Interactions of Low-Frequency, Pulsed Electromagnetic Fields with Living Tissue: Biochemical Responses and Clinical Results

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In recent years many studies have demonstrated stimulatory effects of pulsed electromagnetic fields (PEMF) on biological tissue. However, controversies have also surrounded the research often due to the lack of knowledge of the different physical consequences of static versus pulsed electromagnetic. PEMF is widely used for treating fractures and non-unions as well as for treating diseases of the joints. Furthermore, new research has suggested that the technology can be used for nerve regeneration and wound healing although conclusive clinical trials, besides those for fracture healing, are still lacking. Despite the apparent success of the PEMF technology very little is known regarding the coupling between pulsed electrical fields and biochemical events leading to cellular responses. Insight into this research area is therefore of great importance. In this review we describe the physical properties of PEMF-activated electrical fields and explain the typical set up for coils and pulse patterns. Furthermore, we discuss possible models that can account for mechanisms by which induced electric fields are able to enhance cellular signaling. We have emphasized the currently well-documented effects of PEMF on cell function from tissue culture and animal studies as well as from studies describing clinical effects on bone growth, nerve growth and angiogenesis. We believe this relatively new technology can become relevant for treating a variety of physiological conditions demanding enhanced cellular activity.

Key words: PEMF, tyrosine kinase, Src, Lyn, bone healing

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INTRODUCTION

Since the mid-1950s it has been known that bone becomes electrically polarized when deformed as in a fracture, and this led to the discovery that the application of weak electric fields increases the formation and growth of bones (osteogenesis). However, the invasive technique of implanting current inducing electrodes on the broken bone in order to increase the bone growth severely restricted the use of this new method. Since the mid-1970s the non-invasive technique of inducing electric currents in bones by use of PEMF has been widely used to speed up fraction repair (Basset et al, 1977; Basset et al, 1981) and this method has been approved by the Food and Drug Administration (FDA) in USA. In the late 1970s it was found that application of PEMF resulted in a significant and reproducible enhancement in the regeneration of nerve fibers exposed to a lesion and, since then, the PEMF technique has been successfully applied to stimulate growth and regeneration of various types of nerve cells both *in vitro* and *in vivo* (Sisken et al, 1989; Longo et al, 1999; Macias et al, 2000). More recently evidence has been found, showing that application of PEMF has therapeutic benefits in the treatment of painful inflammation of bone joints (osteoarthritis) as well as in wound healing (Trock et al, 1994; Patino et al, 1996). The latest findings show, that low frequency, PEMF is capable of inducing cell proliferation in several cell culture models, of which especially cultures of cartilage cells (chondrocytes) has been tested vigorously, in the hope of obtaining a treatment for rheumatism and other cartilage-based diseases (Pezetti et al.,1999; De Mattei et al, 2001). From the first discovery of its effects, the clinical spectrum for treatment of various afflictions with PEMF has been ever-broadening and the recent findings suggest that new uses for clinical PEMF-therapy may well be under way.

The articles cited above are only a small extract of the large amount of research articles available, documenting the effects of PEMF. Common to them all is the fact that application of PEMF stimulates or enhances growth-related responses of cells. A connection between the pulsed electromagnetic fields and the events responsible for cell growth must therefore exist, and in this review we outline the most attractive models accounting for this interaction.

THE PRINCIPLE BEHIND PEMF STIMULATION

The method of magnetic stimulation takes advantage of the fact that pulsed electromagnetic fields applied to tissue will induce an electric field, giving rise to a stimulating current in the tissue and a driving force on charged particles, ie. on proteins. The main advantage of using PEMF to create these effects, instead of electrical stimulation by use of electrodes, is that the magnetic field penetrates unaffected through electrically insulating regions, such as the skin or bones of a human being. In this way a high density of stimulating current at these surfaces is avoided, thus preventing a pain sensation in the test subject.

Inducing the Electrical Fields

In the 19th century Michael Faraday found that when he placed a wire loop in a changing magnetic field, an electrical current started running in the loop. His result can be interpreted in the following way: a magnetic field consists of so-called magnetic flux lines. A change in the number of flux lines intersecting the wire loop produces an electrical current in the loop. The resulting current running in the wire is said to be induced by the changing magnetic field.

Faraday formulated his discovery in the following equation:

$$E = -\frac{d\Phi(t)}{dt}$$

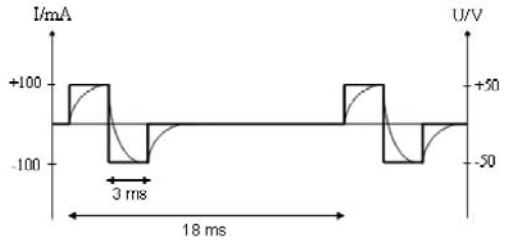

Fig. 1 Output from the generator system. A series of pulses in which +50V is imposed for 3 mINSs then followed by -50V for 3 mINSs is shown as is the current change in a coil.

The above equation, known as Faraday's law, states that an electromotive force, E, is induced by the time-varying magnetic flux $\Phi(t)$. The electromotive force will affect charged particles situated in the time-varying magnetic field and will hereby give rise to the observed electrical current. The fact that a time-varying magnetic field applied to a conductive material like tissue or a cell culture will induce a driving force on charged particles resulting in an induced current if the resistance is low in the material is the basic principle behind electromagnetic stimulation known as PEMF (Pulsed ElectroMagnetic Fields).

Coils Used for Stimulation

A time-varying magnetic field is commonly produced by sending a pulsed current through coils consisting of many turns of cobber wire. Since a single wire loop carrying a relative large amount of current will only produce a weak magnetic field, many turns of wire are needed to produce even modest sized fields, a fact taken into account during the construction of current carrying coils. In these the magnetic field resulting from a single turn of wire is multiplied by the total number of turns of the wire in the coil.

No conventions exist on the size and shape of the coils used for stimulation purposes, resulting in the fact that most research groups use their own set of coil specifications. An example could be coils having 2200 windings of 0.15mm Cu-wire, with a diameter of 2.5cm and a length of 1cm. The resistance and inductance of a single such coil can be measured to 90 Ω and 32 mH respectively. The coils are connected to an external pulse generator system, which generates bipolar square pulse pairs. Many different pulse shapes are currently in use. The goal is to obtain as fast a current rise in the coils as possible in order to induce a larger electrical field in the tissue. In the above example pulses are produced every 18 mins with pulse duration of three mins. The bipolar square pulses cause a rapid change in the currents in the coils that in turn gives rise to a rapidly changing magnetic field. The output signal is illustrated in Fig 1.

The current running through each coil is ie. 100mA. The current shape in the coil during the positive pulse from the pulse generator is given by

$$I(t) = I_{coil}\left(1 - e^{-\frac{t}{\tau}}\right)$$

where $I(t)$ is the time-dependent current in the coil, I_{coil} is the maximum current running in the coil (100 mA) and $\tau = \frac{L}{R}$, where L is the inductance and R is the resistance of the coil, is called the rise time of the current and reflects the fact that the inductance of the coil will

act by resisting the change in induced current when the voltage from the pulse generator is turned on. The exponential current shape in the coil is illustrated in Fig 1.

Recalling that it is the change in magnetic field that induces an electrical current, it is observed that a small τ will give rise to a steeper current slope and thereby a greater change in magnetic field resulting in a larger induced electric field. Decreasing the value of L relative to R reduces the value of τ, a fact that was taken into account during coil construction when evaluating number of windings and geometry.

Frequency and Power Spectrum

It is informative to calculate the actual power spectrum of the pulse pattern in order to understand the type of electrical field the tissue is exposed to. In the above mentioned example, a pulse configuration such as that provided by the pulse generator gives rise to a relative broad band of frequencies that reside in the extremely low frequency (ELF) range (<1000Hz). The highest frequencies obtained are due to the three mins interval between the bipolar pulses. The frequency here is

$$\nu_{max} \approx 333 \text{ Hz}$$

The time between the bipolar pulses and the negative square pulses, where maximal current is induced by the coils is 18 ms, resulting in a frequency of

$$\nu \approx 55 \text{ Hz}$$

A range of lower frequencies is also produced due to the continuous generation of bipolar pulses from the pulse generator.

By use of the power spectral density theory, the frequency composition of a function can be found by Fourier transforming the function of interest. In this case it is the square wave function resulting from the bipolar pulses from the pulse generator that needs to be Fourier transformed. The power spectral density, $|f_n^2|$ for coils with the above specifications is shown in Fig 2. As seen from this, the frequency composition of the square wave function is located primarily below 333 Hz, in the extremely low frequency range and is therefore comparable with the time scales of biologically relevant frequencies (<100 Hz) where biological action occurs (Tenforde, 1996).

Size of the Induced Electrical Fields

In the following section we describe an expression that can be used to calculate the magnitude of the induced electric field in tissue from the rise time of the current in the coil, the overall magnetic field induced as well as the coil characteristics.

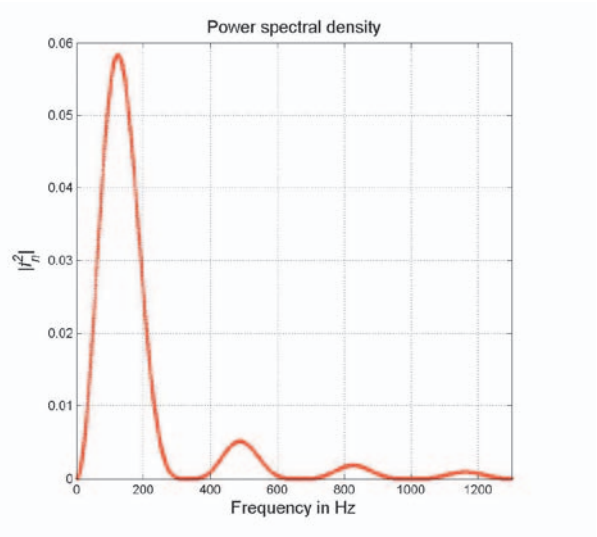

Fig. 2 The power spectral density. Fourier transforming the square wave function seen in Fig 1 reveals the frequency composition of the pulsed signal.

When applying a pulsed electromagnetic field to tissue, Faraday's law states that the induced electromotive force on a charged particle in the tissue can be found by calculating the time derivative of the magnetic flux Φ. However, to calculate the spatial distribution of the electrical field is not as straightforward as it appears. To ease the calculation of the induced current in the cells, a physical quantity known as the vector potential, $\mathbf{A}$ must first be calculated. The vector potential is directly related to the magnetic field and thereby to the magnetic flux. The vector potential at a point P located in the immediate vicinity of a coil carrying a current is given by

$$\bar{A} = \frac{\mu_r \mu_0}{4\pi} \int_V \frac{\bar{J}(\bar{r})}{|r_P|} dV$$

where $\mu 0 = 4\pi \cdot 10^{-7}$ N/A^2 is the vacuum permeability and $|r_P|$ is the distance from the conductor element in the coil to the arbitrary point P, at which we wish to estimate the size of the induced field. dV is a small volume in the coil with current density $\bar{J}(\bar{r})$. In order to find the total vector potential we must integrate across the entire volume V of the coil, since all the small volumes in the coil containing a current density contributes to the vector potential in P. The dimensionless constant μ_r takes account for the material surrounding P. For biological tissue $\mu_r \approx 1$ and can therefore be omitted in the following formulas.

Due to the symmetric geometry of the coil, the normally three-dimensional vector potential reduces to only a single component, a fact that eases the following calculations. When an expression for the vector potential has been obtained, the magnetic flux can be found and

by insertion into Faraday's law the following equation for the induced electric field results

$$E = \frac{\mu_0 N}{2\pi r_2}\sqrt{h^2 + (r_1 + r_2)^2}\left(\left(1 - \frac{k^2}{2}\right)K(k) - E(k)\right)\frac{dI}{dt}$$

where

$$k = \frac{4r_1 r_2}{h^2 + (r_1 + r_2)^2}$$

In the above equations N is the number of windings of the coil, r_1 is the radius of the coil, r_2 is the distance of P from the axis through the center of the coil and h is the height of P above the coil. $K(k)$ and $E(k)$ are the elliptic integrals of the first and second kind respectively and they can be found by reference to a mathematical compendium. The parameters of interest in the coil setup are illustrated in Fig 3.

An expression for $\frac{dI}{dt}$ can be obtained by differentiating the equation for $I(t)$ in the previous section.

To verify the expression obtained for the induced electric field, the physical units are examined.

$$[E] = \frac{\mathrm{N}}{\mathrm{A}^2 \cdot \mathrm{m}} \cdot \mathrm{m} \cdot \frac{\mathrm{A}}{\mathrm{s}} = \frac{\mathrm{N}}{\mathrm{A} \cdot \mathrm{m}} = \frac{\mathrm{V} \cdot \mathrm{s}}{\mathrm{m} \cdot \mathrm{s}} = \frac{\mathrm{V}}{\mathrm{m}}$$

From the above it is seen that the correct unit of the electric field, volts per meter, is obtained.

By inserting the specifications of the coils in the above equation, the induced electric field can be found as the following example will show.

Example: Calculation of the Induced E-Field from a Coil

When using coils to stimulate tissue or cell cultures, the coils are frequently placed together to create a larger area of induced currents. Fig 4 shows an example of such a coil structure.

If more coils are placed in close proximity, the induced electric field from a single coil will also add up with the induced fields created by the coils situated around it, thereby creating a larger induced electric field.

When calculating the induced field from a single coil, the resistance of any such parallel or serial connections to surrounding coils must also be taken into account. Assuming a resistance of 90Ω for a single coil, the total resistance of the coilbed shown in Fig 4 can be found as

$$\frac{1}{R_{coilbed}} = 3\left(\frac{1}{6 \cdot 90\Omega}\right) + 2\left(\frac{1}{5 \cdot 90\Omega}\right) \leftrightarrow R_{coilbed} = 100\Omega$$

With a voltage pulse height of U = 50V produced by the pulse generator (see Fig 2) the current running through the system is given by

$$I_{total} = \frac{U}{R_{coilbed}} = \frac{50\mathrm{V}}{100\Omega} = 0.500\mathrm{A}$$

The current running through each coil is given by

$$I_{coil} = \frac{I_{total}}{5} = 100\mathrm{mA}$$

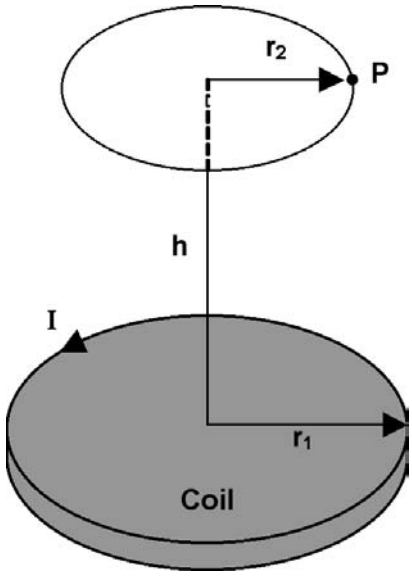

Fig. 3 Geometric parameters. Illustration of the geometry of the coil and the arbitrary point P, at which we wish to estimate the induced electric field.

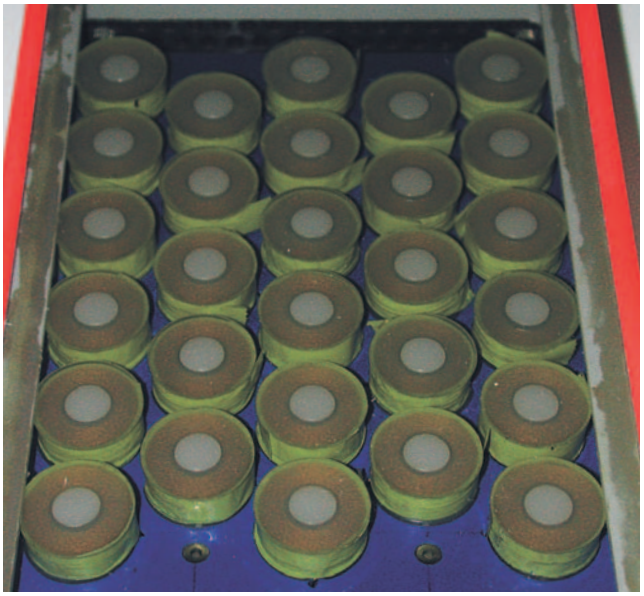

Fig. 4 Coilbed. Close up of the structure of the coils used for stimulation.

The maximum current slope $\frac{dI}{dt}\Big|_{max}$ is found by differentiation:

$$\frac{dI(t)}{dt} = -\frac{U}{R}\frac{R}{L}e^{-\frac{tR}{L}} \longleftrightarrow \frac{dI(t)}{dt}\Big|_{max} = \frac{dI(t)}{dt}\Big|_{t=0} = -\frac{U}{L}$$

The sign of the current can be neglected, since the decisive factor is its numerical size. By insertion of the values specific for these coils, i.e. U = 50 V, L = 32 mH, it is found that

$$\frac{dI(t)}{dt}\Big|_{max} = 1563\frac{A}{s}$$

This value is obtained immediately after the voltage pulse is turned on and it represents the maximum attainable current change.

The characteristics of a single coil have previously been described as having 2200 windings and r_1=1.25cm. The induced electric field can now be calculated at an arbitrary point P. If P is chosen at h = 2cm and r_2=1 cm k is found as

$$k = \frac{4 \cdot 1.25\text{cm} \cdot 1\text{cm}}{(2\text{cm})^2 + (1.25\text{cm} + 1\text{cm})^2} = 0.552$$

By use of a mathematical handbook, the values of the elliptic integrals of the first and second kind can be found as

$$K\ (0.552) = 1.716$$
$$E\ (0.552) = 1.443$$

The maximum induced electric field in this point, due to the pulsed current running in a single coil, can now be found upon insertion into the above equation for the E-field. The E-field is found to be of the magnitude

$$E = 0.025\frac{V}{m} = 0.250\frac{mV}{cm}$$

This is the field a charged particle situated exactly at point P is exposed to when applying electric stimulation to tissue through the use of coils with the above specifications. Most studies within the last decades focusing on fracture healing have applied a rise time of current on the order of several hundreds of microseconds yielding E-fields of 10-100 mV/cm with pulses generally above 15Hz (McLeod and Rubin, 1990).

The induced electromotoric force is found to reach a maximum almost directly above the periphery of the coil and vanishes on the axis through the center of the coil,

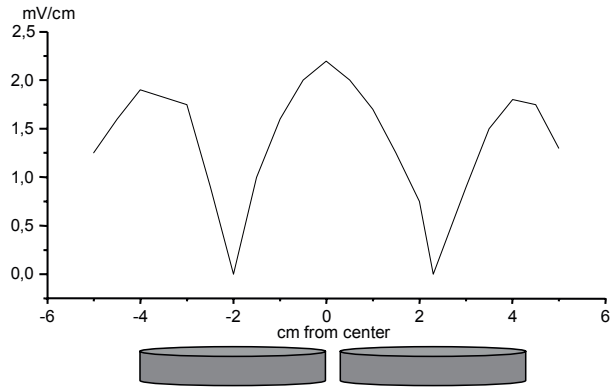

Fig. 5 Induced electromotoric force. The electromotoric force induced on charged particles above the coils. The electric field was measured at a distance of 0.5cm above the coils by use of a differential amplifier. Notice the additive properties of the fields from the two coils when they are in close proximity. No force is induced directly above the center of the coils.

as Fig 5 showing the directly measured electromotoric force above two coils reveals.

Across the 5nm-wide plasma membrane of a cell, the electrical potential is approximately 70 -mV. Compared to the E-field across a cell membrane, the field induced by coils such as the ones used above is completely unable to induce action potentials in excitable cells, since the fields required for this are of the order 15 MV/m. The observed effects of pulsed electromagnetic fields must have a source other than the direct electrical stimulation of excitable cells. The next section will focus on some of the proposed mechanisms of action underlying the well documented effects of electrical stimulation with fields in this magnitude range (ie. mV/cm).

BIOCHEMICAL SENSORS

Since the 1970s it has been known that application of PEMF enhances the growth-related responses of cells. However, the actual mechanisms, governing the conversion of the induced electric field into the cellular signaling events responsible for the observed clinical effects, are still not fully characterized. In the following some of the generally accepted models are discussed.

Tyrosine Kinase Activity

The phosphorylation of amino acid residues of intracellular proteins is responsible for regulating and controlling a wide range of intracellular signaling mechanisms. Several studies have revealed that pulsed electromagnetic fields applied to cells have a stimulatory effect on intracellular tyrosine kinase activity (Uckun et al, 1995; Dibidirk et al, 1998; Kristupaitis et al, 1998), specifically the members of a class of tyrosine kinase proteins known as the Src family.

Tyrosine kinases are able to phosphorylate tyrosine residues on other proteins, leading to a modification of their state of activity, a change in their subcellular localization and in their protein binding properties. Through the tyrosine phosphorylation-induced changes the protein mediates its effects on other intracellular proteins in its signaling cascade ultimately leading to cellular responses.

Important Tyrosine Kinase-Binding Domains

Without the ability of enzymes to recognize the specific motifs contained in their substrates, the complex intracellular signaling pathways would not be able to transmit information at all. It turns out, that many of the proteins involved in intracellular signaling contain similar regions of amino acids called domains, of which many have been shown to be involved in protein-protein binding. These binding domains are highly conserved in different species and appear to be a major constituent in regulating protein-protein interactions, protein activity and cellular localization of proteins. One such binding domain involved during tyrosine kinase activity is the Src homology 2 (SH2) domain. The SH2 domain is a conserved region of approximately 100 residues, which was first discovered in the Src protein as its name implies. Phosphorylation of tyrosine residues in proteins often act as on/off switches for activity and the SH2 domain recognizes and binds the phosphorylated tyrosine residues with high affinity (Yaffe, 2002). The human genome codes for 87 proteins containing SH2 domains. Interactions between phosphotyrosine residues and SH2 domains are involved in the formation of protein complexes, as is the case in the Src protein association with the VEGFR-2/KDR receptor (Chou et al, 2002), as well as protein interactions with its own residues as seen for the inactivated Src protein (Abram and Courtneidge, 2000).

Another region in the Src protein, consisting of about 60 residues, was found to be conserved in other enzymes. This domain was named the Src homology 3 (SH3) domain. The SH3 domain binds the proline-rich sequences contained in various proteins, thereby inducing formation of protein complexes (Cowburn et al, 1998). The SH3 domain has also been shown to interact with residues located on the same protein, hereby contributing to the tertiary structure of the protein (Abram and Courtneidge, 2000).

The Src-Family

The ubiquitously expressed Src protein is involved in the regulation of several signaling pathways including the phospholipase C gamma (PLCγ) pathway producing inositol (1,4,5)-triphosphate (IP_3) resulting in intracellular Ca^{2+} signaling and the Akt pathway involved in cellular activation and survival (Thodeti et al, 2000; Jiang and Qiu, 2003). The Src protein is furthermore a key enzyme in controlling the MAPK signaling pathway leading to the formation of transcription factors and cell proliferation (Abram and Courtneidge, 2000). A few of the multiple roles of the Src kinase are illustrated in Fig 6.

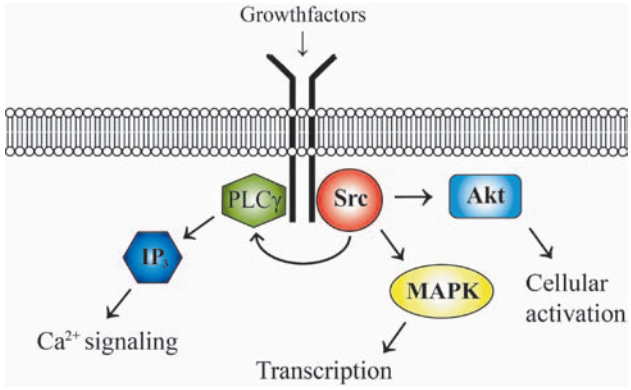

Fig. 6 Src interacts with important signaling pathways. Src has been shown to mediate the effects of growth factor stimulation on various intracellular signaling cascades like the PLCγ, Akt and MAPK pathways.

A whole family of tyrosine kinases with structural and functional similarities to Src has been found, collectively known as the Src-family. Members of this family have furthermore been shown to be involved in PEMF-induced cell signaling (Uckun et al, 1995; Dibidirk et al, 1998; Kristupaitis et al, 1998). Its major constituents are the Src, Fyn and Yes all ubiquitously expressed in mammalian cells, while other of its members such as Lyn, Hck and Lck are more restrictively expressed (Abram and Courtneidge, 2000). Protein kinases of the Src-family each contain a unique domain, which is involved in mediating interactions with receptors or proteins, specific for the Src-family member (Thomas et al, 1997), thereby governing its substrate specificity and controlling its biochemical role. Following the unique domain are two binding domains, the first of which is a SH3 domain, involved in proline motif recognition and binding. The SH3 domain is connected with a SH2 domain which is linked to the kinase domain of the enzyme (Xu et al, 1997).

Due to its many different subcellular locations and its versatile protein-binding properties, the Src family of tyrosine kinases is able to regulate a wide range of cellular processes from mitogenesis to cytoskeletal organization and cell trafficking (Thomas et al, 1997). New substrates and protein interactions of the Src family are continuously found, ever increasing the importance of the Src family enzymes in controlling intracellular signaling events. The structure of a human Src family kinase is shown in Fig 7.

The phosphorylation state of two tyrosine residues on the Src family members has been found to be primarily responsible for regulating the activity of the kinases.

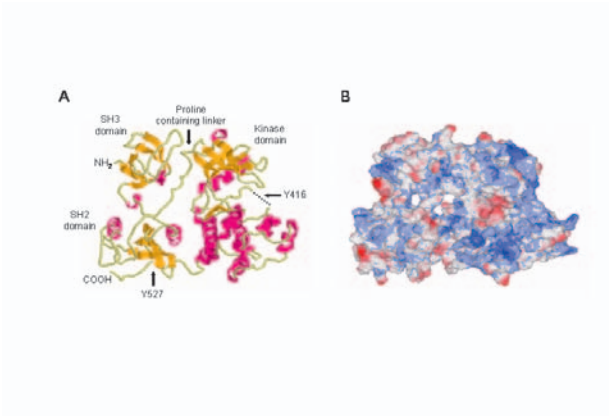

Fig. 7 Src family structure. A, Structure of the inactive conformation of the human Src kinase. α-helices colored red, β-sheets colored yellow. The different domains and key regulatory tyrosine phosphorylation sites are marked. B, Surface map of the Src protein illustrating the charged areas. Acidic (anion) regions colored red, basic (cation) regions colored blue. Protein Data Bank id: 1fmk. Images created using MDL Chime via Protein Explorer (http://proteinexplorer.org).

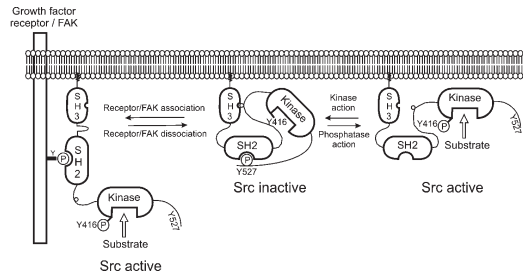

Fig. 8 Activation of membrane-bound Src. Receptor or FAK association as well as phosphatase (PTP) action causes an unfolding of Src allowing substrates to bind the kinase domain. Mutations in the Src protein truncating the tail region containing the Y527 residue prevent phosphorylated Y527 - SH2 domain association, keeping the kinase in a constitutively activated state.

The numbering of the tyrosine residues is based on those appearing on the chicken Src protein. Located in the activation loop of the kinase domain, the Y416 residue occupies the substrate binding pocket of the protein. Upon phosphorylation it is displaced from this position, allowing the kinase domain of the protein access to substrates. In this way phosphorylation of Y416 functions as a positive regulator of Src kinase activity. The phosphorylation state of Y416 is controlled by intramolecular autophosphorylation, by other tyrosine kinases and by various phosphatases (Ma et al, 2002).

In the C-terminal tail of the Src kinases, the Y527 residue is located. When Y527 is phosphorylated an intramolecular contact with the SH2 phosphotyrosine binding domain is established, keeping the protein in a closed conformation, repressing the kinase activity. Upon dephosphorylation the intramolecular contact is broken, displacing the tail from the SH2 domain. This leads to an unfolding of the protein to a conformation where Y416 is either autophosphorylated or exposed to kinases capable of phosphorylating it, resulting in activation of the Src kinase (Abram and Courtneidge, 2000). Phosphorylation of Y527 acts as a negative regulator, repressing the kinase activity of the protein. The phosphorylation state of Y527 is controlled by protein tyrosine phosphatases such as PTPα and PTPλ and the tyrosine kinase C-terminal Src kinase (Csk) (Bjorge et al, 2000). The finding that a mutated form of Src in which the Y527 tail has been truncated, exists in colon cancer cells can explain the observed increase in Src kinase activity, which is due to the inability of phosphorylated Y527 to bind the SH2 domain (Irby et al, 2000). In Fig 8 the activation of Src by association to a growth factor receptor or a focal adhesion kinase (FAK) or by phosphatase action on Y527 is illustrated.

The Lyn Kinase

Human Lyn is a tyrosine kinase containing 512 amino acids with a total molecular weight of 58 kDa. Lyn is found primarily in hematopoietic cells and in neurons although this kinase may function in additional cell types (Thomas et al, 1997).

Several studies on the intracellular effects of applied pulsed electromagnetic fields implicate the Src family kinase member Lyn as a pivotal player in initiating the intracellular signaling events. Exposure of a DT40 lymphoma B-cell line to a 60 Hz pulsed electromagnetic field resulted in the activation of the Lyn kinase (Uckun et al, 1995). An increase in the phosphorylation level of the membrane associated phospholipase PLCÁ following stimulation by PEMF has furthermore been shown to be dependent on the Lyn kinase (Dibirdik et al, 1998; Kristupaitis et al, 1998). Following phosphorylation and activation of the PLCγ enzyme, it associates with the polar head groups of small membrane-bound phospholipids. By hydrolyzing a chemical bond in the phospholipids, it generates the two important second messengers, inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG), capable of releasing Ca^{2+} from the intracellular stores and activating protein kinase C (PKC), hereby influencing a broad spectrum of intracellular processes, leading to several diverse cellular responses such as gene transcription, regulation of glycogen metabolism and cell growth (Carpenter et al, 1999; Uckun et al, 1995).

The studies by Dibidirk et al (1998) and Kristupaitis et al (1998) have also shown a PEMF-induced increase in the phosphorylation levels of two of the intracellular

targets of Lyn, the spleen tyrosine kinase (SYK) and Bruton's tyrosine kinase (BTK) both of which are able to affect intracellular signaling processes such as cell development, growth and apoptosis (Cheng et al, 1995; Qiu et al, 2000). In both these studies the Src-family kinase Lyn appears to play an integral part in transducing the intracellular effects of PEMF. However, a study trying to reproduce the findings of Uckun et al (1995) using the same experimental setup and stimulation method, detected no significant effect of PEMF on the protein tyrosine phosphorylation level or on the activities of Lyn and Syk protein kinases (Woods et al, 2000). We investigated whether another member of the Src family, the Src protein itself, is activated by the application of pulsed electromagnetic fields, possibly resolving the ongoing debate arising from these conflicting results.

The Src Kinase

Human Src is a tyrosine kinase containing 536 amino acids with a total molecular weight of 60 kDa. As stated earlier the numbering of the amino acids have been based on the chicken Src (533 amino acids), since this appears conventional in the majority of the literature. The Src enzyme can be associated with the plasma membrane, the nuclear envelope and the membranes of organelles through a myristoylated N-terminal (Bjorge et al, 2000). The cytosolic non-myristoylated form of Src is sometimes referred to as c-Src to distinguish it from the membrane bound form.

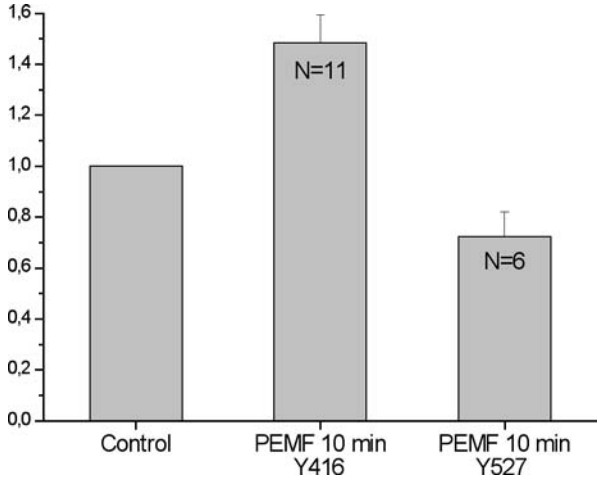

Fig. 9 Activation of the Src kinase by PEMF. Pig aortic endothelial (PAE) cells stably transfected with a KDR-receptor cDNA ligated expression vector, were incubated in a serum free medium for 60 min (Control) before stimulation with pulsed electromagnetic fields generated by coils with the previously described characteristics. Phosphorylation of Src before and after stimulation with PEMF was measured by western blot analysis using polyclonal antibodies raised against Y416 and Y527 (Eriksson et al., 2003). The degree of phosphorylation was normalized with respect to the control experiments and expressed ± S.E. At the p=0.02 level a significant difference of the two means compared with the control was found to exist.

Factors that alter the delicate balance between phosphorylation and dephosphorylation of Y527 are able to cause changes in the Src activity and it has therefore been suggested that pulsed electromagnetic fields are able to activate the Src family kinases through small changes in the electrochemical environment of their charged residues. We have measured the phosphorylation levels of the two key phosphorylation sites of the Src kinase in order to investigate whether stimulation by pulsed electromagnetic fields activates the kinase. The results are shown in Fig 9.

Fig 9 shows an increase in the phosphorylation level of the Y416 residue of Src as well as a decrease in the phosphorylation level of the Y527 residue following endothelial cell stimulation by pulsed electromagnetic fields. Both phosphorylation events are consistent with activation of the kinase. This shows that stimulation by PEMF is able to activate the Src kinase. Our results are consistent with the findings on the Lyn kinase of Uckun et al (1995), Dibirdik et al (1998) and Kristupaitis et al (1998) and it therefore seems likely that the entire family of Src tyrosine kinases is involved in mediating the effects of PEMF.

The next section will focus on the origin of the observed intracellular effects following PEMF stimulation.

SITES OF INITIATION OF THE SIGNALLING EVENTS

From the study of elasmobranchs (ie. fish like sharks and rays), it has been found that they hunt for small fish buried in the seabed by sensing the electrical signals originating from the neural tissue of their prey (Petracchi et al, 1998; Montgomery et al, 1999). They have specialized cells, which are able to intercept and recognize these electrical signals coming from the surroundings, while at the same time being able to differentiate them from the electrical "noise" produced by their own neural tissue, which is greater by many orders of magnitude. Behavioral responses were found in rays when exposed to fields in water as low as 1-2 nV/cm (Kalmijn, 1997).

Even though the explanation for this signal recognition mechanism remains unknown, this example shows that biological mechanisms of action are able to sense and react to the presence of very small electric fields, compared to what they are normally exposed to. In this section a few of the proposed models for PEMF-induced cellular effects are discussed.

Several studies have shown the effects of PEMF to depend on the activation of members of the Src-family of tyrosine kinases. Activation of the Src kinases influences a broad range of signal pathways controlling cellular functions such as cell growth, metabolism and survival. The fact that many of the Src-family kinases are

ubiquitously expressed throughout the interior of cells leads to a broad range of PEMF affected targets. From fig. 7B the surface charge distribution of the Src protein is observed to be close to homogenous, showing that no net dipole moment exists for the enzyme. However, the charge distribution is not entirely uniform either, suggesting that small sub-domains positioned in close vicinity could have a different net charge, giving rise to localized dipole moments. The effect of PEMF could therefore be envisaged to affect the transition between the active (open) and inactive (closed) conformations of the kinases, through subtle changes in the energy landscape of the sub-domains governing the transition process. A transition to the active state of a Src-family kinase could be favored, through the accumulative effects of PEMF on the electrochemical potential environment of the charged residues located in the Src kinase and the multiple charged particles in the surrounding medium.

Another factor that could explain the observed increase in Src-family kinase activity could be if the frequency of the applied pulsed electromagnetic field coincided with the frequency of the transitions of the Src enzymes. A resonance frequency like this would result in increased energy transfer from the pulsed electromagnetic field to the Src-family kinases possibly leading to a modulation of the Src transitions. However, until the resonance frequencies of the Src-family enzymes have been determined the resonance model is purely speculative.

Whether the observed increase in Src kinase activity and the dependence of cellular effects on Lyn activation following stimulation by pulsed electromagnetic fields is due to a direct effect on the Src family members by the induced electric field is currently unknown. It is possible that the effect on the Src family kinases is initiated at some other cellular site, resulting in a subsequent activation and dependence on the Src kinases. One such proposed site is the cellular membrane.

It has been suggested that the PEMF-induced intracellular effects originates at the cell membrane, since membrane constituent should be much better detectors of an applied electric field than isolated molecules in solution (Astumian et al, 1990; NIEHS Working Group Report, 1998). The induced E-field and corresponding currents in the extracellular medium could alter ion binding to macromolecules situated in the membrane, influence ligand-receptor association and modify the general membrane transport processes taking place (Tenforde, 1996). These effects could result in changes in the cell environment capable of initiating intracellular signaling pathways leading to the observed responses such as cell growth and proliferation. Supporting this hypothesis is the finding that stimulation by PEMF is able to alter the association and dissociation kinetic parameters for agonist binding to the adenosine receptor A_{2A} in human neutrophil cells, leading to an increase in receptor-ligand affinity thereby resulting in increased intracellular effects of agonist stimulation (Varani et al, 2002).

The minimum signal strength required to exceed the thermally generated electrical noise in the membrane was estimated to be approximately 1 mV/cm, dependent on the form and shape of the cell of interest (Astumian et al, 1990; Tenforde, 1996). Many of the induced electric fields used for stimulatory purposes are above this threshold by an order of magnitude, indicating that the sensory event, which converts the PEMF-induced electric field into an initiation of intracellular signal transduction, could take place at the extracellular surface of the plasma membrane. However, so far only circumstantial evidence supporting this hypothesis is available, giving rise to the need for further experiments to investigate the effects of PEMF on the cell membrane.

As seen above, several theoretical models have been proposed seeking to explain the interaction mechanisms between PEMF and biological systems. It is often difficult to evaluate the relevance of these models, due to the lack of reliable experimental data with which to test the predictions made by the models. Nevertheless the theoretical models presented here are useful, since they indicate which data are needed and suggest new experimental procedures or modify existing ones, hereby generating new 'reliable' information, which will lead to the eventual discovery of the cellular mechanisms underlying the effects of PEMF.

CLINICAL EFFECTS OF PEMF

Currently PEMF are most widely used for the promotion of bone growth. The first studies were initiated as a result of previous observed effects of bone healing from applying direct electrode coupling. PEMF have subsequently been used for conditions of non-union, delayed union, osteotomy and bone fusion and have been approved by the FDA as safe and effective for the treatment of non-unions and failed fusions (Basett et al, 1981). Studies have later shown that the technique is applicable for promoting nerve growth, promoting bone formation around dental implants, initiating growth and differentiation of chondrocytes and promoting angiogenesis.

The first therapeutic use of PEMF was in the treatment of patients suffering from pseudoarthrosis and non-unions (Basett et al, 1977). The overall success rate in this study was estimated to 70%. In a subsequent study treating 125 patients for ununited fractures of the tibial diaphysis a success rate of 87% was observed using an average E-field along the long axes of the tibia

of 1.5mV/cm. (Basett et al, 1981). Successful healing of osteotomies was shown in a study by Borsalino et al. (1988) in a double blind treatment of 32 patients with femoral intertrochanteric osteotemy. The study revealed a significant difference between osteotomy healing in treated patients compared to controls.

To clarify the mechanism by which electrical stimulation promotes osteogenesis in rabbits, bone marrow was stimulated by either direct electrical current through inserted Kirshner wires or by PEMF alone also in the presence of a Kirschner wire. It was found that both processes initiate osteoblast proliferation in close proximity to the wire (Yonemori et al, 1996). It was subsequently shown by Matsumoto et al (2000) that PEMF stimulation promotes bone formation around dental implants inserted into the femur of rabbits using 100Hz pulses applied 4-8 hrs/day. These studies have indicated that an enhanced rate of bone material around dental implants can be attained by use of PEMF at the area in which the implant is inserted.

Recently, a number of animal studies and *in vitro* studies using cell cultures have tested PEMF for treatment of growth of chondrocytes and treatment of osteoarthritis. A general finding has been that in presence of growth factors PEMF cause an enhanced proliferation of chondrocytes in serum (Pezzetti et al, 1999) showing that growth factors are important mediators of PEMF effects. Ciombor et al (2003) have in a guinea pig model in which animals spontaneously develop arthritis, shown that PEMF reduce the severity of osteoarthritis and cause preservation of articular cartilage. The key player in this model is transforming growth factor β (TGF-β), the expression of which was increased by 72% following PEMF exposure (1 hr/day for 6 months). TGF-β upregulates gene expression of both aggrecan and various tissue inhibitors of metalloproteases. Furthermore, it suppresses interleukin-1 activity. An important signaling pathway for TGF-β, involves Src capable of sensing pulsed electrical fields as described above.

Several investigations have now demonstrated that PEMF enhances peripheral nerve regeneration (Sisken et al, 1989, Longo et al, 1999, Macias et al, 2000). The most important growth factors that play a role in nerve regeneration are nerve growth factor (NGF), insulin like growth factors (IGF) and fibroblast growth factors (FGF), and they are all dependent on the activity of Src or members of the Src family. Activation of neural tissue by PEMF has also been seen with regard to enhancing the cAMP concentration – an effect that may have effect on synaptic efficiency and neuronal excitability (Hogan and Wieraszo, 2004). Low frequency pulsed electromagnetic fields have also been used for treatment of depression – for review see Gershon et al (2003). Recently it was shown that PEMF have beneficial effects on refractory depressions and pulses of the duration of 30 minutes per day for two weeks strongly improve the mood of patients suffering from refractory depressions (Martini, 2003). Another recent study has shown that several FGF receptors play a central role in the frontal cortex in the development of mood disorders since low expression of the receptors can be associated with major depressive disorders (Evans et al, 2004). Since Src is a key player in FGF signaling, Src activation by pulsed electrical fields could thus turn out to be a plausible explanation for the observed clinical effects.

Finally, in animal studies it was shown that PEMF imposed on rats 36 minutes twice a day for up to 21 days gave rise to an enhanced rate of wound healing (Patino et al, 1996). Along these lines it was also shown that PEMF causes an enhanced rate of angiogenesis and endothelial release of FGF-2 (Tepper et al, 2004) as well as to a smaller extent epidermal growth factor (EGF) and angiopoietin-2 - all essential factors for promoting growth of new vessels. An enhanced growth of blood vessels in bone treated with PEMF for an extended period of time might thus provide the basis for explaining an accelerated bone healing process.

A general picture emerges from studies on cell cultures and living tissue revealing that PEMF facilitate existing biochemical processes - especially those related to growth factor stimulation. Most of these factors stimulate cytoplasmic tyrosine kinases of the Src family and they lead to many different responses depending on the cell type i.e. proliferation of osteoblasts, endothelial cells, nerve cells and chondrocytes.

New technologies including small interference RNA (siRNA) and microarrays, will undoubtedly yield valuable information regarding the intracellular signal pathways influenced by PEMF as well as the induced cellular responses. In the future this will enable us to obtain a better understanding of the interactions of pulsed electromagnetic fields with biological tissue.

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