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Biochemical effects of low frequency electromagnetic fields on cells

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BIOCHEMICAL EFFECTS OF LOW FREQUENCY ELECTROMAGNETIC FIELDS ON CELLS

by

JAMES THOMAS RYABY

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

BIOCHEMICAL EFFECTS OF LOW FREQUENCY ELECTROMAGNETIC FIELDS ON CELLS

by

JAMES THOMAS RYABY

Advisor: Arthur A. Pilla, Ph.D.

The interaction of electric and magnetic fields with biological systems is an area of growing research interest due to both development of novel therapeutic applications as well as increasing awareness of potential environmental hazards. The question which was addressed in this thesis is what is the role of the cyclic AMP signal transductive pathway in the biological effects of low energy, low frequency electromagnetic fields (PEMF) at the cellular level? The model system employed was the Cloudman melanoma cell line which can be induced to differentiate with the peptide hormone, α-melanocyte stimulating hormone (MSH). The data indicate that both MSH and PEMF affect both adenylate cyclase (AC) activity and cAMP-dependent protein kinase (PKA) activity, and the sensitivity to PEMF and MSH is partially modulated through the GTP-binding protein, Gi. The link to Gi was established by inhibition of Gi activity through use of pertussis toxin catalyzed ADP-

Ribosylation of Gi. Phosphorylation of proteins including the oncogene proteins, c-fos and c-ras, is altered and this may be a reflection of the effects on AC and PKA. In order to target receptor specificity of the PEMF interaction, homologous and heterologous desensitization with the α-adrenergic receptor agonist, epinephrine (EPI) and the ß-adrenergic agonist, isoproterenol (ISO) was performed. Both EPI and ISO pretreatment of melanoma cells decreased the response to PEMF with no effects observed on the MSH response. These results indicate a linkage between adrenergic receptor expression and senstivity to PEMF; and further suggest that PEMF does not work through the MSH receptor pathway. The conclusion from these studies is PEMF can induce differentiation in a melanoma cell line by manifesting certain functional alterations associated with the normal physiological regulator, MSH. PEMF may also be capable of altering the phenotypic expression of the melanoma cell by differential regulation of protein synthesis and phosphorylation. This PEMF-induced differentiation is AC and PKA dependent and the results are consistent with an adrenergic receptor target site for PEMF. The generality of these findings in other model systems remains to be investigated.

ACKNOWLEDGEMENTS

I wish to express my sincerest thanks and gratitude to Dr. Arthur A. Pilla for providing the environment in which these studies were conducted. The members of his lab, in particular Dr. Jonathan J. Kaufman and Ms. Marisa Figueiredo, were instrumental in the progress of this work.

During the course of this work I had the opportunity for many collaborations and developed many close friendships. I thank Dr. Alessandro Chiabrera for more than 14 years of interest on my behalf, and Dr. Robert S. Siffert for supporting our laboratory effort. Special thanks to Drs. David B. Jones and R. Barbara Pedley, who introduced me to the melanoma model system and collaborated very fruitfully on this project in the early stages.

I sincerely appreciate the collective knowledge and input of Drs. Carter Bancroft, Thomas A. Einhorn, Ravi Iyengar, Stave Kohtz, Marko Markov, and Martin J. Walsh; and the excellent assistance of Steven Tannenbaum, Priscilla de Gouvea Duarte-Alves, Gita Bosch, Judy Sy, Patrick F. Dalton, Dr. Evan J. Bachner, and Dr. Michael Mont.

DEDICATION

This work is dedicated to my father, John Peter Ryaby, whose contributions in this area are great but not acknowledged to the degree they deserve; and to my wife, Deborah, and our children, John Peter and Eloise Helle, for their support, love and inspiration.

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LIST OF ABBREVIATIONS

αAR - Alpha Adrenergic Receptor

ßAR - Beta Adrenergic Receptor

Con A - Concanavalin A

EM - Electromagnetic

EPI - Epinephrine

FOR - Forskolin

ICYP - Iodocyanopindolol

ISO - Isoproterenol

kd - Kilodalton

MSH - Melanocyte Stimulating Hormone

PEMF - Pulsing Electromagnetic Fields

PHA - Phytohemagglutinin

2D - Two Dimensional

1D - One Dimensional

INTRODUCTION

The interaction of electric and magnetic fields with biological systems is an area of growing research interest. This is due in part to the clinical application of electric stimuli in orthopaedics, physical therapy, neurology, and oncology (Bassett,1989); and also due to concern regarding the environmental influences of electromagnetic fields (Savitz et al., 1990).

The focus of research at the cellular level has been on membrane mediated signal transduction. Many effects indicate that the receptor-adenylate cyclase second messenger system may be a site of interaction of electromagnetic (EM) fields. Early work by Rodan (1978) demonstrated that EM fields could stimulate cAMP formation in cartilage and correlated this with effects on alkaline phosphatase activity. It was reported that pulsed electromagnetic fields (PEMF) could inhibit the hormonally stimulated adenylate cyclase in osteoblast-like cells (Luben et al.,1982). Other investigators have described suppression of cAMP levels in fibroblast cultures after long term stimulation with PEMF (Farndale and Murray,1986). Changes in cAMP levels as a function of the electrical field strength induced capacitively have been correlated with a change in DNA synthesis (Korenstein et al., 1984). Cell type specific changes in cAMP levels with demonstrated recovery phenomenon after PEMF removal have been described (Jones, 1984). These investigators all consider the adenylate cyclase system as a potential mediator of functional response to electromagnetic field stimulation. The protein kinase C transductive pathway, as well

as calcium signalling pathways, have also been implicated in electric field interactions (Byus et al.,1984; Adey, 1988). Finally, the stimulation of autocrine growth factor production has been postulated as both messenger and regulator in electric field stimulation (Fitzsimmons et al.,1990, Cadossi et al., 1989). Therefore, it seems that many signal transductive pathways may be sensitive to electric field interaction. In this study, we use a clinically effective pulsing electromagnetic field on a well-defined model system to define the biochemical signal transductive mechanism underlying the reported biological effects.

BACKGROUND

INTERACTION OF ELECTROMAGNETIC FIELDS WITH BIOSYSTEMS

THERAPEUTIC APPLICATIONS OF ELECTROMAGNETIC FIELDS

In the orthopaedic field, electric and magnetic fields have been demonstrated to promote healing in both delayed and non-unions of bone fractures. The development of this method of treatment was based on the discovery of the electrical properties of bone tissue in the 1950's and 60's. The first report of the piezoelectric properties of bone originated from Yasuda in Japan (1954) who measured an electric potential upon deformation of dry bone. In the early 1960's, several groups reported the generation of electrical potentials in wet bone upon mechanical deformation (Bassett

and Becker, 1962; Shamos et al., 1963; Friedenberg and Brighton, 1966). These studies resulted in the hypothesis that strain generated electric potentials may be a signal for regulation of cellular processes in bone (Bassett, 1968).

The above body of experimental work culminated in the development of therapeutic devices which employed signals related to those measured in the strain generated potential experiments. The first applications of these devices was in the treatment of bone fracture non-union (Friedenberg et al. 1971; Bassett et al., 1975) and has been subsequently expanded to include fresh fractures as well. Devices are currently under basic and clinical investigation for the treatment of avascular necrosis (Aaron et al., 1990; Steinberg, 1990) as well as osteoporosis (Tabrah, 1990). Beyond the orthopaedic field, these devices are being investigated for application in wound healing (Pollack et al., 1990), peripheral nerve regeneration (Zanakis et al, 1991) , and antibacterial therapy (Spadaro et al., 1990). The above is not exhaustive but encompasses only those areas where sufficient in vivo data has justified human clinical trials.

Recalcitrant fracture repair (delayed and non-union of bone) has had the longest history in PEMF application (Bassett, Brighton). Different methods of electric current induction have been developed, and both surgically invasive or noninvasive technologies are available. The first studies were published in the 1970's and developed from work which originally employed direct current stimulation. Direct current devices use electrodes either surgically implanted directly adjacent to the fracture site (Patterson,1990), or use one internal/one external electrode

combination . Brighton (1981) reported the largest series of patients treated with direct current stimulation. Major problems encountered with dc stimulation are those associated with surgical intervention: infection, inflammation, and initial mobility impairment. The major advantage of this system is the minimal patient compliance requirement. However, the mechanism of dc stimulation may be distinctly different from that of the noninvasive technology (see below) due to: generation of electrodic byproducts (Black, 1987): modification of oxygen tension (Baranowski et al.,1983; Black, 1984): and possible direct realignment and electrophoresis of cells and matrix components (Cooper, 1984; Robinson, 1985; Ross et al., 1988; Onuma, 1988).

Noninvasive devices have been based on two different principles of electric current induction. The most widely employed system is that of electromagnetic induction using external coils (Bassett,1989). This technology provides a pulsed asymmetric electric signal of low frequency and low energy (hence nonthermal) with current values of ≈ 5-10 $\mu A/cm^2$. Over 100,000 patients have been treated worldwide with application to all areas of fracture management. The first large series of patients exhibited a success rate of 80% in bone non-unions (Bassett, 1982) and has been followed recently by randomized, double blind clinical trials which confirm this success rate (Sharrard, 1990). In the past decade, over thirty clinical trials in the literature document the efficacy of PEMF as an alternative to invasive surgical intervention (Bassett, 1989; Hinsenkamp et al, 1990). As with surgical treatment, the success rate of PEMF is determined by the location and severity of the fracture.

Based on this documented success over the past twenty years, new applications

include: avascular necrosis, osteoporosis, spinal fusion, and tendinitis. Avascular necrosis (osteonecrosis) highlights this rapidly progressing area of PEMF application. Avascular necrosis is a condition of bone loss, primarily centered in the proximal femur, which is due to loss of vascularity with increasing incidence in both the elderly and patients with endocrinopathies resulting in mineral metabolism defects. Erosion of the joint with collapse of the femoral head is a common outcome with total hip arthroplasty following (Aaron et al., 1989a; Steinberg et al., 1989). One recent trial compared core decompression (standard surgical technique) with PEMF treatment and patient followup was for a minimum of three years. This study demonstrated a higher success rate for PEMF than for surgery with less degeneration of the femoral head observed in the PEMF group (Aaron et al., 1989a). Osteoporosis, a disease of bone loss observed in the elderly which is due to decreased production of the steroids 17-beta estradiol and 5-α dihydrotestosterone, respectively in women and men, was the subject of a recent clinical trial in osteoporosis-prone women. The investigators measured bone mineral density changes during 12 weeks of daily exposure, and for 9 months subsequent to PEMF treatment. Increased bone density during treatment in the PEMF treated limbs was reported, with a return to contralateral control values after treatment termination (Tabrah, 1990). Double blind clinical trials have also demonstrated therapeutic efficacy of PEMF for treatment of spinal fusions (Mooney, 1990), tendinitis (Binder, 1984), femoral osteotomies (Borsalino et al., 1988), and venous ulcers (Ieran, 1990). The above trials clearly demonstrate the ability of PEMF to stimulate bone repair and remodeling as well as

general tissue repair, and are indicative of the promising future applications of this technology.

Capacitively coupled devices have been developed more recently and their clinical application has been limited at present to nonunion treatment (Brighton and Pollack, 1985). This device employs a low energy 60 kHz sinusoidal signal and the current induced in the tissue is on the same level as that of the inductively coupled device, on the order of $\approx 10\mu A/cm^2$. Clinical trials are currently under way in the treatment of avascular necrosis (Steinberg et al., 1990) and osteoporosis (Brighton et al., 1991).

THEORETICAL BASIS AND ANALYTICAL MODELS

The physical mechanism(s) of interaction of electric and magnetic fields on biological tissues as well as the biological transductive mechanism(s) remain to be elucidated. An important question concerning these interactions is the following: Is there a unifying mechanism which can explain the wide range of results and provide for predictive ability of electromagnetically induced biological effects?

It was proposed in the early 1970's that electromagnetic fields may affect ion adsorption/binding and therefore trigger a cascade of biological processes (Pilla 1974,1987). This electrochemical information transfer hypothesis postulated that the cell membrane would be the site of interaction of low level electromagnetic fields by altering the rate of binding of i.e. calcium ion to enzyme and/or receptor sites.

Empirical data based on impedance measurements at cell membranes provided information on time constants due to specific adsorption/interaction (ion binding) pathways at the cell surface. Models were employed which used equivalent circuit models of cell membranes and tested these models against real time impedance measurements. The first model system studied was the toad urinary bladder membrane whose transport properties are well understood. The biological advantage to using this single cell thickness epithelial layer is the tight junctional electrical contact between cells, which minimize electrical leak pathways around cells, thereby affording high resolution impedance values (Pilla and Margules, 1977). Isolated cell impedance studies followed which created artificial epithelial layers by deforming red blood cells under hydrostatic pressure into Nucleopore membranes (Schmukler et al, 1985). This technique was further applied to other mammalian cells such as melanoma, fibroblasts, and osteoblasts. The result was prediction of optimization parameters for electromagnetic signal components (Pilla et al, 1987).

Theoretical studies initiated in the late 1970's focused on the potential of electromagnetic fields to alter binding rates of specific ions and ligands to receptor types, or to affect the ion or charged particles (ligands) motion in solution (Chiabrera et al, 1986). The implication of the latter would be to increase the kinetic availability of these ions in ionic dependent processes, such as ion selective channels, etc. Theoretical modeling of ligand binding to cells employed phytohemagglutinin (PHA) as the mitogenic ligand and lymphocytes as the target cell (Chiabrera, 1984). A microelectrophoretic effect was calculated which decreased the

mean lifetime of ligand-receptor complexes. The net effect would be to reduce the mitogenic efficiency of the ligand, PHA. Reduction of mitogenic stimulation of lymphocytes by PEMF (Conti, 1983) may be explained by this model. These experimental studies, in conjunction with previous and subsequent work on PEMF influence on calcium efflux from brain tissue (Bawin and Adey, 1976; Blackman, 1982,1985), demonstrated the appearance of windows in PEMF effects. These "windows" are combinations of amplitude and frequency within which there is an observed response, and once outside this range the response is nonexistent. Wei et al (1990) describe succinctly a "window effect" in their report on transcriptional changes in HL60 cells. These results demonstrated up to a four fold increase in transcripts of c-myc and histone H2B with the peak effect being at 45 Hz. This frequency response provides the first evidence for regulation at the nuclear level.

Three complementary theories evolved from this "windows" concept, and provide a framework for mechanistic modeling. These theories are: 1) Ion resonance theory, 2) Lorentz/Langevin theory, and 3) quantum parametric resonance theory. Further, these physical models are presently being tested in biochemical systems. Liboff (distinct from below, 1966) demonstrated effects of crossed electric and magnetic fields on Brownian motion of charged particles, although not in biological systems. Without reference to the above work, these three complementary theories all involve the combined effects of DC and AC magnetic fields in biological interaction mechanisms.

Ion cyclotron resonance, proposed by Liboff (1985) and advanced by Liboff and

McLeod (1987), described frequency specific combinations of DC and AC magnetic fields which couple directly to calcium dependent processes, by increasing ion mobility near receptor and/or ion channel sites. Experimental verification of these models, although still in early stages, has provided encouraging data to support the notion of direct coupling to ion dependent processes (Liboff and Mcleod, 1988). McLeod et al.(1987) reported that combinations of DC and AC magnetic fields calculated to couple to a resonant frequency for Ca^{2+} stimulated diatom motility while detuning to a resonant frequency for K^{+} led to loss of this effect. These studies were extended to mitogenic stimulation of lymphocytes (which is calcium dependent), and it was again observed that tuning to calcium frequencies led to enhancement of mitogenic stimulation (Liboff, 1987). Of particular interest in the latter study is that nifedipine (a dihydopyridine calcium channel blocker) inhibited the AC/DC effect, indicating a role for calcium channels. Lyle et al (1991) and Reese et al (1991) have recently reported qualitatively similar results in lymphocytes and diatoms, respectively.

The Lorentz force equation was used by Chiabrera (1987) to relate individual influences of both AC and DC electromagnetic (EMF) fields to ligand receptor binding and motions of ions (or other charged molecules). This work was further expanded to include thermal noise effects on the ion binding kinetics with a Langevin-Lorentz model (Chiabrera et al., 1988; Kaufman et al., 1990). This stochastic analysis modeled the motion of the charged ligand as a random walk, i.e., Brownian motion with drift (Chiabrera et al., 1989). EMF signals designed with the

Lorentz theory were used in an attempt to affect the calcium dependent motility of paramecium, and resonance effects were again observed which correlated with combined AC/DC effects on calcium ion motion (Chiabrera et al., 1989). Lednev (1991) recently reported on a paramagnetic resonance quantum formulation which modeled the calcium ion inside a calcium binding protein (i.e calmodulin) as a charged oscillator. These calculations suggest that resonance occurs when the alternating AC field frequency is equal to the calcium ion cyclotron frequency. Experimental evidence for this effect utilized the calcium/calmodulin dependent myosin light chain kinase reaction in a cell-free system (Lednev 1990; Markov et al., 1991). The results indicate a frequency specific decrease in phosphorylation, which correlates with effects on calcium binding. These results again demonstrate the ability to tune to resonance frequencies for calcium with appropriate combinations of AC and DC magnetic fields.

Weaver and Astumian (1990) have discussed the lower limit of cell sensitivity to external electric fields based on considerations of thermal noise. They postulate a maximal sensitivity of between 10^{-3} and 10^{-6} volt/cm by allowing for a time averaging capability of the biochemical sensor, and accounting for the spatial amplification produced by the cell/cell membrane geometry. These field strengths are below the levels used in the research described in this work. An example of a time averaging mechanism is provided by Tsong et al. (1987, 1990) and Westerhoff (1989), although these studies employed fields on the order of 100 volt/cm. Membrane ATPases were employed in this work as transducers capable of absorbing energy from electric fields

of defined frequency and using this to influence chemical reaction rates. The experimental results demonstrate stimulation effects of these electric fields on Rb^+ uptake by electric fields which is ouabain inhibitable, directly implicating the Na/K ATPase.

CELLULAR STUDIES

Cellular studies on skeletal systems illustrate effects concordant with those described above for therapeutic applications. Stimulation of collagen synthesis in fibroblasts has been observed by numerous investigators including results which indicate a frequency dependence of the applied electric field (Farndale and Murray, 1986; McLeod et al.,1987). Additional studies have demonstrated modulation of lysosomal enzyme activity, associated with an alteration of catabolic activity in response to electric fields (Murray and Fitton-Jackson, 1988). Chondrocytes exposed to electric fields in vitro exhibited increased proteoglycan biosynthesis and sensitivity to proteolytic digestion (Sah et al.,1989). These results are consistent with an earlier onset of matrix biosynthesis reported in vivo. In addition, stimulation of cellular proliferation in chondrocytes reported recently has shown a dependence on magnetic field orientation and growth conditions (Smith et al.,1988). The above in vitro results are additionally supported by many other studies linking electric field exposure to a stimulation of cellular events relevant to the repair processes observed both in vivo

and clinically (see Table 1 below).

However, the response of cells to electric and magnetic fields is not limited to skeletally related tissues. A prominent model system used by many investigators has been human peripheral blood lymphocytes, and the major effect reported is on mitogen (phytohemagglutinin, PHA; concanavilin A, ConA) stimulation of blastogenesis (Bersani et al., 1985; Cadossi et al., 1986). Conti et al. (1983) demonstrated inhibition of PHA induced mitogenesis over the range of 1-200 Hz. ConA induced mitogenesis was also inhibited, but only at 3 and 50 Hz. These authors explained the different results with the two mitogens as indicating that different lymphocyte subclasses would be sensitive to different EMF frequencies, in agreement with the original proposal (Chiabrera et al., 1985) that calcium mediated signal transduction would be the underlying mechanism. Experiments to confirm the role of calcium in EMF effects have been reported by Cadossi et al (1988), Conti et al. (1985) ,and Liboff et al.(1987). These investigators used calcium channel blockers which modulated EMF sensitivity, confirming the role of calcium and indicating a potential membrane target site. Data correlating calcium dependent processes with EMF sensitivity in other systems support these results; EMF stimulated calcium efflux and insulin release from isolated rabbit pancreatic islet cells was reported by Jolley et al.(1984); calcium efflux and insulin receptor is increased in fibroblasts (Bourguignon, 1989), and cytosolic free Ca^{2+} is increased after exposure to PEMF in HL60 cells (Carson et al., 1990).

Induction of specific mRNA synthesis after PEMF exposure in both Drosophila

and Sciara salivary glands (Goodman et al., 1988; Goodman et al., 1990) have been reported as well as induction of oncogene expression in HL60 cells (Wei et al., 1990). Effects on induction of p53 and histone H3 mRNA have been reported by Cadossi et al. (1989) in spleen tissue. The rate of regeneration of rat liver (Ottani et al., 1983), B cell growth factor synthesis (Cadossi et al., 1986) and induction of angiogenesis in endothelial cells are stimulated by exposure to EM fields (Yen Patton et al., 1988). EM fields have been shown to modulate neurotransmitter release in PC12 cells (Dixey and Rein, 1983), increase transferrin receptor number in colon carcinoma cells (Phillips et al., 1986), and inhibit retinoic acid induced differentiation in F9 teratocarcinoma cells (Okamine et al., 1985). This wide range of results conclusively demonstrates that electric and magnetic fields can affect cellular processes.

A selected list of cellular effects of electric fields is presented in Table 1.

TABLE 1

CELL/TISSUE TYPE	EFFECT REPORTED	REFERENCE
Bone cells	IGF II Synthesis Increase	Fitzsimmon,1988
Bone cells	Actin Polymer. Changes	Laub, 1982
Bone cells	cAMP/DNA Synth.Increase	Korenstein,1984
Bone Cells	PTH Response inhibition	Cain,1987
Bone Cells	PTH Response inhibition	Luben,1982

Chondroblast	Calcium Incorp. increase	Norton,1988
Chondrocytes	Cell Proliferation increase	Brighton,1985
Chondrocytes	Cell Proliferation decrease	Smith,1988
Chondrocytes	DNA Synthesis Increase	Rodan,1978
Chondrocytes	PTH Response increase	Hiraki,1988
Colon Carcinoma	Transferrin Receptor Incr.	Phillips,1986
Colon Carcinoma	Colony Formation Increase	Phillips,1987
CHO V79 Cells	DNA Synthesis Increase	Takahashi,1989
Endothel.Cells	Stim. of Angiogenesis	YenPatton,1988
F9 Teratocarc.	Differentiation Inhib.	Akamine,1985
Fibroblasts	Collagen Synth. Increase	Farndale,1986
Fibroblasts	Collagen Synth. Effect	McLeod,1987
Fibroblasts	Collagen Synth. Increase	Murray,1986
Fibroblasts	PGE_2 Response inhibition	Farndale,1987
HL 60 cells	Myc/H2b RNA increase	Goodman,1990
Human Fibrobl.	DNA Synthesis Increase	Liboff,1984
Human Fibrobl.	Differentiation Increase	Rodeman,1989
Human Lymphoma	ODC increase	Byus,1988
Human Lymph.	Inhib. of Mitogen Stim.	Conti,1983
Human Lymph.	IL-2 Receptor increase	Cossarizza,1989
Human Lymph.	Mitogen Stim. Increase	Cossarizza,1988
Melanoma	Tyrosinase Increase	Jones,1986

Melanoma	Protein Kinase Effects	Ryaby,1988
Osteoblasts	DNA Synthesis increase	Ozawa,1989
PC12 Cells	Noradrenaline Rel.Incr.	Dixey,1983
Physarum	Surface Charge increase	Marron,1990
Salivary Glands	Protein Synthesis change	Goodman,1988
Salivary Glands	mRNA Synth. Increase	Goodman,1983
Sensory Ganglia	Neurite Outgrowth Increase	Sisken,1986
Skin	Protein Synth. Increase	De Loeker,1988
Synov.Fibroblast	Lysosomal Enzyme decrease	Murray,1988
Tendon	DNA Synthesis Increase	Cleary,1987
Tibia	cAMP Metabolism Changes	Jones,1984
U937 Cells	Surface Charge Alteration	Smith,1990
Xenopus	Nerve Elongation Increase	McCaig,1990

IN VIVO STUDIES

In vivo animal models have been successfully employed to assess the effects of PEMF and provide information regarding both time and amplitude dosimetry allowing for maximal therapeutic effectiveness (Carter et al., 1989; Chakkalakal et al.. 1990). Models were designed to mimic clinical situations and provide information on biomechanical indices of bone repair (Pollack et al., 1985). Furthermore they address the cellular process(es) which are sensitive to the physical stimuli. In the

normal bone fracture repair sequence, inflammation, cellular migration, differentiation of mesenchymal cell precursors, and proliferation of bone forming cells and subsequent mineralization of extracellular matrix are required for complete resolution of the fracture (McKibbin, 1978; Bolander et al., 1990). Evidence for the effectiveness of electric fields in stimulating cellular differentiation has been supplied by a bone induction model system which mimics the normal bone formation process (Reddi, 1980). Electric fields can promote early mesenchymal cell differentiation to fibroblasts and chondrocytes active in the matrix biosynthetic phase of endochondral ossification (Ciombor et al., 1989). Using a cartilage growth plate in vivo system, other investigators have demonstrated proliferative effects of applied electric fields on chondrocytes (Iannacone et al.,1988). These studies are complemented by earlier work which used PEMF to stimulate proteoglycan synthesis in articular cartilage (Smith and Nagel, 1983). The effect on mesenchymal cell differentiation was applied to ingrowth into porous ceramic biomaterials, where it was reported that PEMF stimulated bony ingrowth and mineralization (Shimizu et al., 1988). Finally, using mechanical evaluation as an indicator of strength and mineralization, it has recently been demonstrated that electrical stimulation can enhance the rate of fracture healing (Pienkowski et al., 1987).

Osteoporosis models have been employed to assess the therapeutic potential of applied electric fields in prevention or reversal of progressive bone loss. Kenner et al. (1975) and Bassett et al. (1979) reported on the usefullness of electric fields in the prevention of osteoporosis (osteopenia). Brighton et al. (1985) illustrated the

ability of capacitively coupled electric fields to inhibit bone loss in two osteoporosis models (denervation and castration). Rubin et al. (1989) and Skerry et al. (1991) reported on PEMF use to prevent bone loss associated with disuse in the functionally isolated turkey ulnae and dog fibula respectively. These studies demonstrate another unique feature of electric fields; the ability to replace the normal mechanical input mechanism which regulates bone homeostasis under normal conditions.

Mechanical loading, as stated above, regulates bone homeostasis through the formation of stress generated or streaming potentials. These electrokinetic potentials exist both in bone and cartilage, and are due to compressive fluid flow in a confined space or volume (Pollack et al.,1984, Grodzinsky, 1983). Loading also causes strain in the tissue and can be measured with gauges which measure uniaxial deformation (Rubin and Lanyon, 1984). Rubin et al. (1987) developed the functionally isolated turkey ulna model to address the issue of strain magnitude and dosimetry of applied load. Based on the synthesis of results from these related studies, Mcleod et al.(1990) have calculated the induced electric field levels due to mechanical loading, and found them to be in the μV/cm range, which is 2-3 orders of magnitude lower than that used in the inductive and capacitive devices above. Sinusoidal signals in this amplitude and frequency range (<75 Hz) demonstrate cellular effects similar to that reported above (Cellular Effects section), and require significantly less power, on account of better tuning to the endogenous cellular response (McLeod and Rubin, 1990; McLeod et al, 1991).

In other medical disciplines, electrical stimulation has promoted the healing of

soft tissue wounds in rats, rabbits, and pigs (Glassman et al., 1986; Dunn et al., 1988; Pollack et al., 1991), stimulated the healing of tendons in rats (Nessler and Mass, 1987), and decreased cardiac tissue damage after experimental myocardial infarction in dogs (Cadossi et al., 1990). PEMF has been reported to stimulate peripheral nerve regeneration in both rat and cat models (Orgel et al., 1984; Sisken et al., 1990; Zienowicz et al., 1991). Direct current has also been applied successfully in both peripheral and central nervous regeneration (Borgens et al., 1990; Politis et al., 1989). These studies emphasize that basic cellular biochemical control processes are affected by applied electric fields, and manifestations of these cellular effects at the tissue level provide for future clinical indications.

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in dogs (Cadossi et al., 1990). PEMF has been reported to stimulate peripheral nerve regeneration in both rat and cat models (Orgel et al., 1984; Sisken et al., 1990; Zienowicz et al., 1991). Direct current has also been applied successfully in both peripheral and central nervous regeneration (Borgens et al., 1990; Politis et al., 1989). These studies emphasize that basic cellular biochemical control processes are affected by applied electric fields, and manifestations of these cellular effects at the tissue level provide for future clinical indications.

ENVIRONMENTAL EFFECTS OF ELECTROMAGNETIC FIELDS

Recent reports in the scientific and lay press have discussed associations between electromagnetic exposure to ELF (50-60 Hz transmission lines) and incidence of cancer (Pool,1990; Brodeur,1990). Signals from ELF sources (sinusoidal waves, continuous) are quite different than currently employed therapeutic signals. The earliest report in this area is that of Wertheimer and Leeper (1979), which demonstrated an association between incidence of childhood leukemias and electromagnetic field exposure. This finding was reinforced recently by Savitz et al.(1988), correlating magnetic field exposure to an increased rate of cancer, especially of childhood leukemias. Three significant epidemiological studies have been published since these two landmark publications, and these associations hold although the risk factor is low (generally less than a 2 fold increased risk). Included in the above three reports is exposure from household electrical appliances, electric

blankets, power lines (in occupational exposure), and electrical transmission equipment (Savitz et al., 1990; Gamberale et al., 1989; Coleman et al., 1989). It must be emphasized that the above effects are associative, not causal (Savitz et al., 1989). In contrast to the above work, a recent study in Naval personnel (where ELF exposure is well documented) found no association with electromagnetic exposure and cancer risk (Garland et al., 1990). A mechanistic model has been proposed by Wilson et al. (1989), whose studies on ELF induced pineal gland dysfunction links neuroendocrine effects with immune system suppression. Melatonin levels and serotonin metabolism have been shown to be altered by exposure to ELF fields (Wilson et al., 1990; Lerchl et al., 1990).

Cellular responses to environmental electromagnetic signals (ELF, 60 Hz) include calcium metabolism alterations in different model systems. Walleczek and Liburdy (1990) demonstrated that ^{45}calcium uptake could be increased 2.7 fold by a 60Hz ELF field (1mV/cm) in Con A stimulated thymocytes, with no effect observed in non-mitogenically stimulated thymocytes. Calcium efflux from brain tissue is stimulated by ELF and provided the experimental foundation for novel theoretical models of electrical field interaction (Blackman et al, 1982, 1985, 1990)(see Theoretical Models Section, above). Cellular responses to 60Hz at the gene level include a reported by Goodman et al.(1989 ,1990 ,1991) on changes in both c-myc and c-src transcription in HL60 cells. Phillips (1990) also reported increased transcription of oncogene mRNA due to ELF exposure. The connection with cancer promotion is unknown, since oncogene expression is a normal step in both growth

and differentiation pathways (Bishop et al., 1990; Weinberg et al., 1989). Finally, experiments have shown effects on DNA synthesis and proliferation which could correlate with ELF induced in vivo abnormalities. Byus et al. (1986, 1988) used ornithine decarboxylase (ODC) as a marker since ODC is elevated in growing cells and during the process of tumor promotion. Short term (1 hour) exposure to 60Hz ELF increased ODC activity up to 5 fold in human lymphoma cells and elevated ODC levels remained for several hours after ELF exposure. Changes in protein kinase C which correspond well to the above studies on calcium metabolism have been reported (Byus et al., 1984). Adey (1988) proposed a ELF induced tumor promotion model based on the above results, as have Goodman and Henderson (1990). A recent report has failed to find any effect of 60Hz ELF on cell growth, clonogenicity, or cell cycle kinetics (Livingston et al., 1991). These contradictory results with power frequency fields warrant further investigation in order to ascertain a real versus perceived risk(s).

MODEL SYSTEM EMPLOYED IN THESE STUDIES

In the present study we use a well described cellular differentiation system in which a direct correlation between adenylate cyclase activation and a functional change (induction of differentiation) can be elucidated. The Cloudman S91 melanoma is induced to differentiate by the action of alpha melanocyte stimulating hormone (MSH). MSH activates adenylate cyclase and cAMP-dependent protein

kinase (PKA), resulting in induction of the enzyme tyrosinase, the rate limiting enzyme in melanin biosynthesis. Upon induction of tyrosinase, melanin content increases and the cellular proliferation rate decreases (Korner et al., 1979; Pawelek et al., 1984; Fuller et al., 1989). Insulin stimulated tyrosine phosphorylation is linked to regulation of growth control in this cell line, but no effect is observed on tyrosinase (Fleischman et al., 1986). It is a well characterized cell line which can be used as a model system for other cAMP dependent mechanisms, such as the parathyroid hormone (PTH) response in osteoblasts.

OBJECTIVES AND SIGNIFICANCE

The question to be addressed in this thesis is what is the role of the cyclic AMP signal transductive pathway in the biological effects of low energy, low frequency electromagnetic fields at the cellular level?

To this end, the first objective is to characterize the short term response of adenylate cyclase to PEMF, to MSH, and to combinations of the two. Other agonists which activate the cAMP pathway, such as forskolin and isoproterenol, will also be investigated to provide information on the MSH receptor role in the PEMF effect. The second aspect of this portion of the study will be to determine the cAMP dependent protein kinase (PKA) activity and compare it to the induced changes in adenylate cyclase. Changes observed in the cAMP pathway should be manifested in alterations in tyrosinase activity, and this will be assessed under the above conditions.

To quantitatively assess PEMF at the biochemical signal transductive level, the short term alterations in cAMP levels after PEMF exposure will be compared with MSH. We would expect that short term stimulation will show the same rapid rise in cAMP as with MSH. The measurement of the PKA activity ratio is the only accurate assessment of PKA activity due to the complex kinetics of the enzyme activation (Corbin,1983). This assay will provide us with the in situ total percentage of active enzyme - the key element in determining whether a "kinase cascade" (Hunter, 1987; Nishizuka, 1986, 1990) reaction has been triggered, enhanced, or inhibited. Furthermore, we can bypass questions regarding any secondary modification of cAMP levels, such as phosphodiesterase activation. We intend to show whether PEMF or MSH demonstrates an effect at the level of the regulatory step, i.e. kinase. Study of the PEMF effects on other adenylate cyclase activators should help to elucidate the site of interaction of PEMF.

The second objective is to determine whether GTP binding proteins are involved in the PEMF and MSH response. GTP binding proteins function as regulators of the receptor stimulated adenylate cyclase (Simon et al.,1991). When hormone binds to receptor, the receptor associates with a G protein, which then activates the G protein. This activated G protein can now bind GTP (releasing GDP), and undergoes a conformational change which allows the G protein to activate adenylate cyclase. The intrinsic GTPase activity of the G protein then hydrolyses the GTP to GDP, now rendering the G protein inactive and dissociating from adenylate cyclase and receptor. The G protein in its GDP bound state is now capable of stimulation once

again by hormone/receptor complex (Levitski, 1987). Pertussis toxin, which ADP ribosylates the inhibitory G protein (Gi)(Reisine,1991), and removes the tonic inhibition of receptor mediated stimulation of adenylate cyclase, was used. In addition, forskolin, a diterpene activator of adenylate cyclase at high concentrations, can determine PEMF interactions at the level of cyclase, and consequently PKA (Ruiz et al., 1986). This allows us to potentially isolate effects of PEMF mediated through G proteins. It is likely that PEMF is not affecting the cyclase directly if PEMF does not influence the ability of forskolin to activate PKA. The normal regulator of adenylate cyclase activity are the G proteins, Gi and Gs, which are likely targets for PEMF interaction. The final, and most signficant indication of PEMF interaction at the G protein level will be accomplished with studies using pertussis toxin, a specific modifier of Gi. If pre-treatment of cells with pertussis toxin (Bokoch and Gilman, 1984) results in increased sensitivity to PEMF or MSH interaction, then Gi is playing a role in PEMF signal transduction. It is known that both Gi and Gs are required for maximal adenylate cyclase and PKA stimulation (Cerione et al., 1985, Asano et al., 1985).

A schematic diagram of the overall approach and objectives of this proposal is illustrated in figure B1.

FIGURE B1

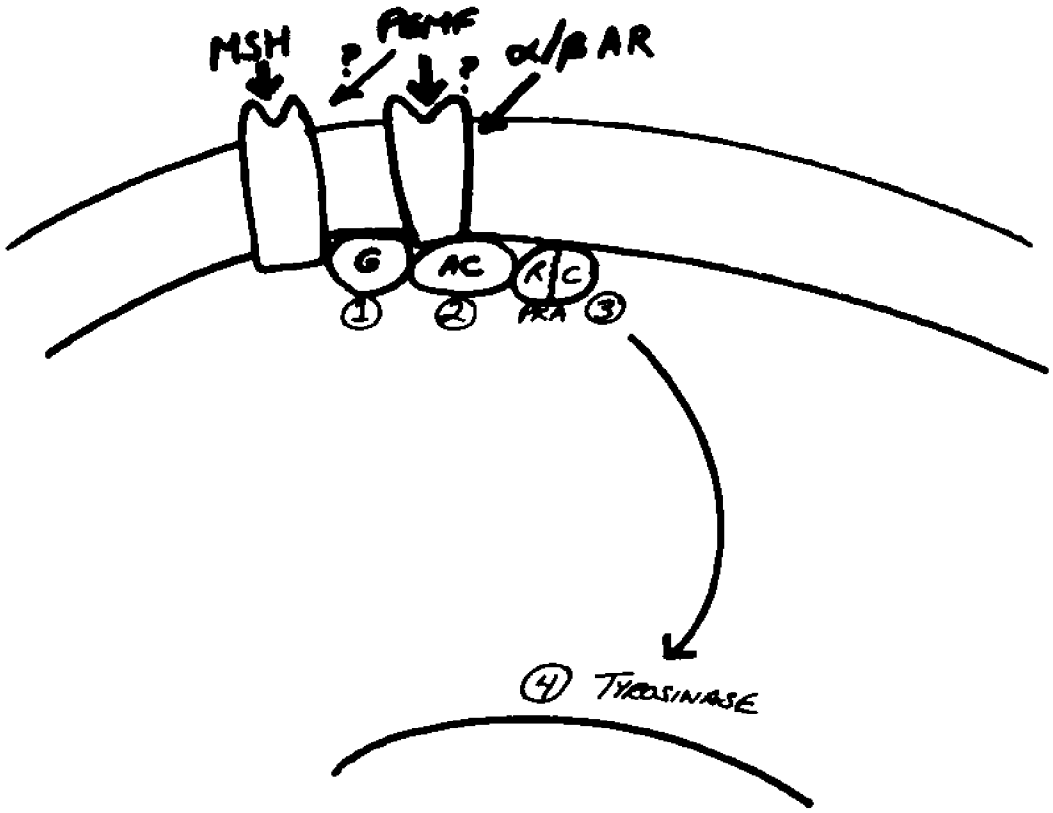

Figure B-1. Schematic diagram of cAMP signal transduction in Cloudman S-91 melanoma. The abbreviations used are 1) G, GTP Binding Proteins; 2) AC, Adenylate Cyclase; 3) PKA, cAMP-Dependent Protein Kinase; R, Regulatory Subunit; C, Catalytic Subunit; 4) Tyrosinase; MSH, α-Melanocyte Stimulating Hormone; PEMF, Pulsing Electromagnetic Field; α/BAR, Alpha/Beta Adrenergic Receptor.

CHAPTER 1

THE EFFECTS OF PULSING ELECTROMAGNETIC FIELDS ON ADENYLATE CYCLASE AND cAMP-DEPENDENT PROTEIN KINASE IN MELANOMA CELLS

INTRODUCTION

The interaction of electric and magnetic fields with biological systems is an area of growing research interest today. This is due in part to the clinical application of electric stimuli in orthopaedics, physical therapy, neurology, and oncology (Bassett, 1990); and also due to concern regarding the environmental influences of electromagnetic fields (Savitz et al.,1990). In fibroblasts, stimulation of collagen synthesis has been observed in numerous studies (Murray and Farndale, 1984; Fitzsimmons et al.,1986), including results which indicate a frequency dependence of the external electric field (Mcleod et al., 1987). Additional studies have demonstrated modulation of lysosomal enzyme activity, suggesting an alteration of catabolic activity in the response to electric fields (Murray et al., 1988). EMF fields have been shown to modulate neurotransmitter release in PC12 cells (Dixey and Rein,1983), increase the transferrin receptor number in colon carcinoma cells (Phillips et al.,1986) , inhibit retinoic acid induced differentiation in F9 teratocarcinoma cells (Akamine et al., 1985), and stimulate transcription of oncogene mRNA levels (Goodman et al., 1989,1990). This wide range of results conclusively demonstrates that electric and magnetic fields can affect cellular processes. The physical mechanism(s) of interaction of electric and magnetic fields as well as the biological transductive mechanism(s) remain to be elucidated.

It was proposed in the early 1970's that electromagnetic fields may affect adsorption/binding and therefore trigger a cascade of biological processes

(Pilla,1974). Theoretical models based on ion-dependent resonance effects (Mcleod and Liboff, 1987; Chiabrera et al., 1985) as well as electroconformational coupling (Tsong, 1989) have been developed to address the observed biological effects. The major locus of agreement in these models is that the membrane is the cellular interaction site of PEMF. This is due to the small field induced intracellularly from the dielectric shielding of the cell membrane and the small current loops induced within the cell. Based upon these physical and analytical considerations, many reported effects indicate that the membrane bound receptor-adenylate cyclase second messenger system may be one site of interaction of electromagnetic fields (Norton et al., 1978; Luben et al., 1982; Jones, 1984; Jones et al, 1986).

In the present study, we use a well characterized cellular differentiation system, in which a direct correlation between adenylate cyclase activation and a functional change (induction of differentiation) can be elucidated. The Cloudman S91 melanoma is induced to differentiate by the action of alpha melanocyte stimulating hormone (MSH, Pawelek et al, 1975). MSH activates adenylate cyclase and cAMP-dependent protein kinase (PKA), which results in induction of the enzyme tyrosinase; the rate limiting enzyme in melanin biosynthesis (Pawelek et al,1977). This induction requires transcriptional activation of the tyrosinase gene and is cAMP dependent (Fuller et al.,1989, Kwon et al.,1988). Melanin content increases and the cellular proliferation rate decreases upon induction of tyrosinase (Moellman et al., 1988). In this chapter, we demonstrate specific effects on signal transduction at the cell surface with PEMF and correlate these alterations with a functional cellular change.

MATERIALS AND METHODS

ELECTROMAGNETIC EXPOSURE APPARATUS

The PEMF waveforms used in these studies were generated with an exposure system consisting of two parallel circular Helmholtz-aiding coils (18 cm or 10 cm diameter) 8 cm apart or 4.5 cm apart, respectively (Figure 1-2). The waveform was monitored with a coil probe amplified in a Tektronix Model 5441 Storage Oscilloscope, as described previously (McLeod et al.,1985). The probe is designed to provide for a 10 fold amplification of the induced voltage when placed parallel to the plane of the coils. The EMF employed is a 5 msec burst consisting of 21 pulses having 200 μsec main and 20 μsec opposite polarity repeating at 15 hz. dB/dt in the main polarity is 0.1 G/μsec corresponding to an induced electric field of 1mV/cm. This corresponds to an average induced current in the culture dish of approximately 5μA/cm^2 (figure 1-1) (McLeod et al., 1984).. All experiments reported herein were performed with the tissue culture dishes or plates oriented parallel to the plane of the coils.

TYROSINASE ASSAY

Cloudman S91 cells (American Type Culture Collection CCL 53.1 clone M-3) are grown to confluency in Ham's F10 medium (Gibco, Grand Island, NY; or

FIGURE 1-1

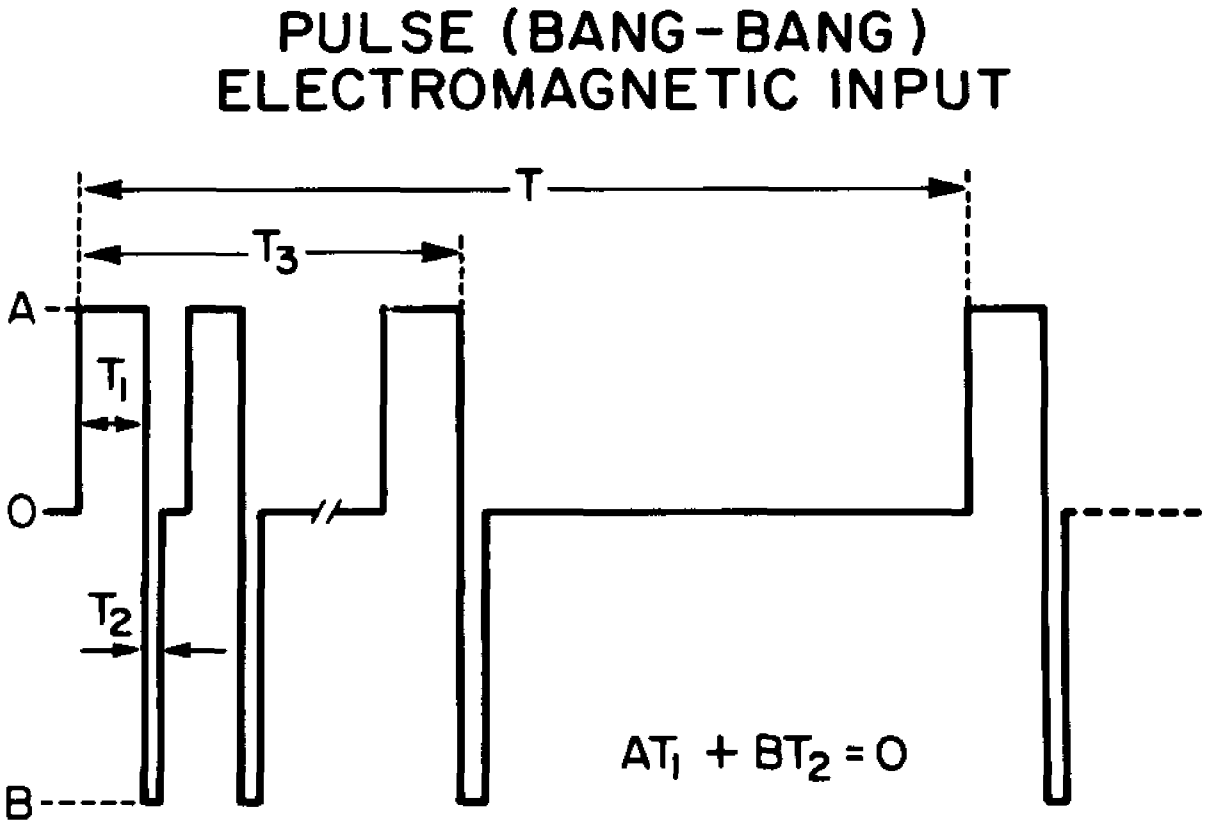

Figure 1-1. Schematic diagram of electric field signal induced by Helmholtz coil exposure system. A, positive amplitude; B, negative amplitude; T, 67 msec (15 Hz); T_1, 200 μsec; T_2, 20 μsec; T_3, 5 msec. The exposure system consisted of two parallel circular Helmholtz-aiding coils (18 cm or 10 cm diameter) 8 cm apart or 4.5 cm apart, respectively (Figure 1-2). The waveform was monitored with a coil probe amplified in a Tektronix Model 5441 Storage Oscilloscope, as described previously (McLeod et al.,1985).

FIGURE 1-2

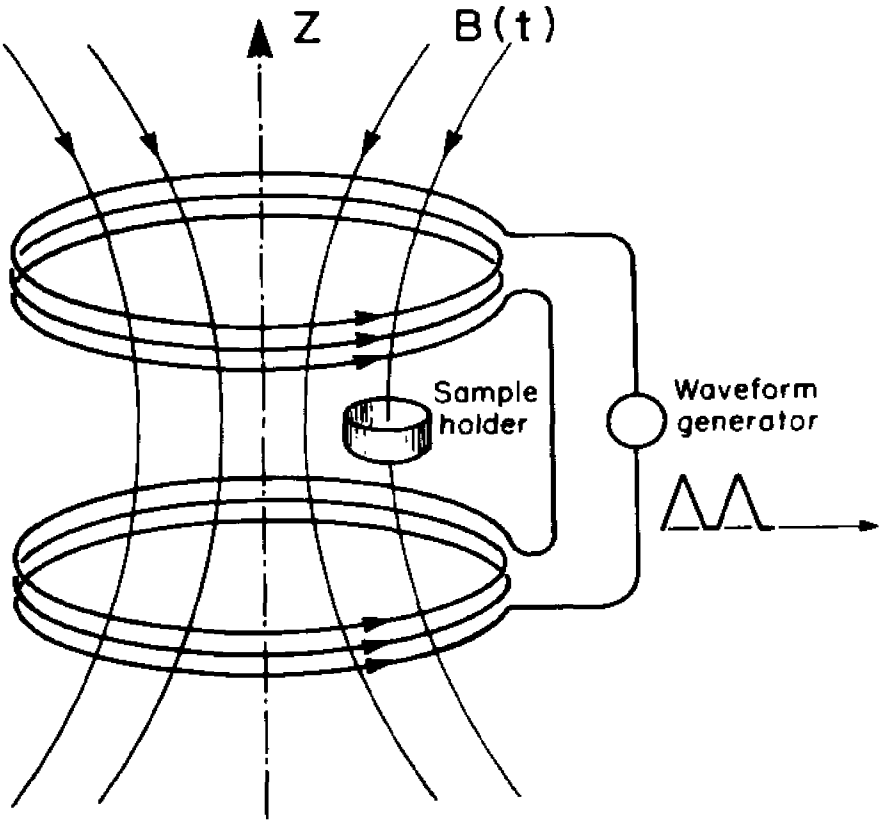

Figure 1-2). Schematic diagram of electromagnetic field exposure system. Z, Vertical Axis; B(t), Time Varying Magnetic Field; Sample holder is representative of culture dish. Note that the magnetic flux lines are perpendicular to the cellular growth surface.

Mediatech, Herndon, VA) supplemented with 15% horse serum / 2.5% fetal calf serum (Hyclone USA, Logan, UT) and 2mM glutamine (Pawelek,1984). No antibiotics are used during either maintenance or experimental culture. Cells were subcultured by incubating briefly with 1mM EDTA in calcium and magnesium-free Hanks solution (Gibco) and diluted 1:5 into 75 or 150 cm flasks according to need. For tyrosinase experiments, cells are seeded at a density of 75,000 (±1%) per 35 mm culture dish (Nunc) in 2 ml of culture medium. Cells are counted in a Coulter ZM cell counter calibrated with 14.8 μm latex particles using a 100 μm aperture and a threshold setting eliminating cells smaller than 8.0μm. Tyrosinase experiments are conducted in glass culture jars (resin reaction vessels), whose ground glass stoppers are sealed with vacuum grease. Cells are grown in an atmosphere of 20% 0_2, 75% N, and 5% C0, at saturated humidity in a warm room at 37°C, or a 10 cubic foot CO_2 incubator under the same conditions were used for the experiments. Control cultures grown under these conditions showed growth and tyrosinase response to MSH and insulin identical to that reported in the literature (Pawelek et al., 1975,1976). Tyrosinase relative activity (monophenol monooxygenase; monophenol, dihydroxy-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) (cpm released per 24 hours from L-[3,5-^{3}H] tyrosine, Amersham, Arlington Hts, IL) was measured by the method of Pomerantz (1969). Cells are grown under control conditions for 24 to 72 hours, then incubated in serum free medium containing 0.1% BSA for an additional 24 hours. At the start of the experiment, cultures are incubated with HF10 growth media containing 0.3μCi/ml [^{3}H] Tyrosine and exposed to PEMF, α-melanocyte

stimulating hormone (MSH, Bachem, Torrance, CA), forskolin (FOR, Sigma, St.Louis, MO), isoproterenol (ISO, Sigma), or under control conditions as described above. At the end of each 24 hour interval, the media is collected and fresh media is added containing the stimulus (as above). Free $^3H_20$ in the culture media is separated from ^{3}H-Tyrosine by activated charcoal/Dowex 50W chromatography and counted in Hydrofluor (National Diagnostics, Manville, NJ) by liquid scintillation counting in a LKB-Wallac LSC. For cellular tyrosine hydroxylase activity, aliquots (prepared as described below in PKA assay method) are incubated in a reaction mixture consisting of 0.1 mM tyrosine, 2μCi/ml of [^{3}H] tyrosine, and 0.1 mM L-DOPA (Sigma) in 0.1 M sodium phosphate buffer, pH 6.8 at 37°C. To terminate the reaction, 1 ml of charcoal (10% w/v, in 0.1 N HCl) was added to each assay tube and the samples centrifuged at 2000 x g for 10 min and processed as above. Data is presented as cpm $^3H_20$ per culture, per well, or per cell. For DNA synthesis experiments, cultures were incubated for 22 hours and then media containing ^{3}H-thymidine (Amersham) at a final concentration of 1μCi/ml was added for 2 hours. Cells were fixed with 200μl of methanol/acetic acid (3:1) for 1 hour, then washed 2X with 200 μl of 80% methanol. Cells were air dried and extracted with 0.5% trypsin for 30 minutes followed by 100 μl 2%SDS. Samples were assayed in triplicate and counted as above (Noda et al., 1989).

ADENYLATE CYCLASE ASSAY

Adenylate cyclase activity was performed according to the method of Salomon (1979). Confluent CS91 melanoma cultures (see above) were subcultured into 35 mm petri dishes(9.6cm^2), or 4 and 24 well plates (16 mm diameter,2cm^2) at a density of 20,000 cells/cm^2 (determined by Coulter Counter as above) and grown under control conditions for a minimum of 24 hours before the start of an experiment. For comparison of serum-free and normal growth conditions, cells were incubated for an additional 24 hours in fresh medium respectively. Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, α-melanocyte stimulating hormone , forskolin, isoproterenol , or under control conditions for the times indicated. The PEMF and MSH costimulated group was achieved by placing cultures first in the PEMF apparatus then immediately adding the MSH stock. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography. For analysis of the ^{3}H-cAMP formation, cultures were thawed at room temperature and transferred to 12 X 75mm glass tubes containing 100 μl of nucleotide carrier solution (5mM [final] of Adenine, Adenosine, AMP, ADP, ATP, and cAMP (Sigma) ; to prevent non-specific binding of ^{3}H-cAMP or ^{14}C-cAMP), 50 μl of ^{14}C-cAMP (Amersham) standard (3000 cpm/tube), and then 50 μl of 4N KOH was added and the mixture

is vortexed. Samples were applied to the column by inversion and 4 ml volumes were collected from the alumina columns in scintillation vials. Fourteen ml of Hydrofluor was added and counted in a LKB-Wallac scintillation counter with internal standard. Data was collected directly onto disk with backup hardcopy printout. The data was edited with Epsilon text editor, and determination of ^{3}H-cAMP performed with PC-Matlab (The Mathworks). Data was expressed as cpm/10^5 cells or cpm/culture.

cAMP DEPENDENT PROTEIN KINASE ASSAY

Confluent CS91 melanoma cultures (see above) were subcultured into 35 mm petri dishes(9.6cm^2), at a density of 20,000 cells/cm^2 (determined by Coulter Counter as above) and grown under control conditions for a minimum of 24 hours before the start of an experiment. For comparison of serum-free and normal growth conditions cells were incubated for an additional 24 hours in fresh medium, respectively. Cultures were then exposed to either PEMF, MSH or other agonists as described. At the end of the timed incubations, cultures are washed 1X in ice cold PBS, then frozen rapidly by immersion into liquid N_2. Cultures were then prepared for analysis by sonication in an ice bath for two 5 second intervals at a power setting of 3 using a microtip (Heat Systems-Ultrasonics Model W385, Farmingdale, NY). cAMP-dependent protein kinase (PKA) was assayed by a combination of the methods of Livesey and Martin (1988) and Levin et al (1988). Kemptide (Bachem) was used as the phosphoacceptor substrate and gamma ^{32}P-ATP (Amersham) as the phosphate

donor. The assay buffer contained 150 μm kemptide (Bachem), 1mM EDTA, 10mM MgAcetate, 15 μM BSA (fraction V), 125 μM ATP, and 0.25 - 1.0 μCi gamma ^{32}P-ATP. The incubations were performed in 40μl total volume in assay plates (Falcon, polystyrene), containing either plus or minus cAMP (6.25 μm) for 15 minutes at 30°C. The assay plates were floated in the water bath with a styrofoam frame. The reaction was stopped by the addition of 10 μl of a 1mM EDTA solution (Mg2+ is a required cofactor for kinase activity). 10 μl samples were then spotted onto Whatman P81 phosphocellulose paper, air dried, then washed with 75mM phosphoric acid 4X for 5 minutes, followed by one 5 minute wash with 95% EtOH, and dried with a infrared lamp. Samples were subsequently counted in 15 ml of H_2O in a LKB Wallac scintillation counter . Soluble protein was measured by the Coomassie blue method of Bradford (61).

STATISTICAL ANALYSIS

In the experiments reported here a minimum of three experiments were performed with a minimum of four culture wells/point/experiment (5 culture wells/point/experiment when using 35 mm dishes). The mean and standard deviation were evaluated for each sample group. Paired two-tailed Student's t test was employed to test the percentage differences for statistical significance. Significance was accepted at $P \leq 0.05$.

RESULTS

The first series of experiments were performed with cultures grown under normal growth conditions (serum containing media) and at early passage numbers (passage 20-32, defined as in methods). Adenylate cyclase activity in control cultures maintained a stable basal level of ≈ 1000 cpm/10^5 cells (Figure 1-3). PEMF inhibited adenylate cyclase slightly at 3,5,and 15 minutes with little change at 30 minutes. In contrast, MSH stimulated adenylate cyclase up to 4 fold (peak at 10 minutes) at these corresponding time points. PEMF and MSH costimulation did not modulate the MSH stimulation of adenylate cyclase at any of the four time points. Isoproterenol (ISO), to our surprise, had little stimulatory effect on adenylate cyclase activity (an average of ≈ 35% above control).

To further address activation, the PKA activity ratio was determined under the above conditions. The results overall paralleled those observed in the adenylate cyclase series. As can be seen in Figure 1-4, the PKA activity ratio (the percentage of active catalytic subunit) remains constant between 10 and 20% in the control group over the range of 3 to 15 minutes (30 minute time points were not performed an adequate number of times). MSH demonstrated a rapid rise to 40% activation at 3 minutes, and this level rose to a significant PKA stimulation at all time points, ranging from 50% to 70% of maximal activity. PEMF did not exhibit any significant effect versus control with only 10 to 20% free catalytic subunit present.

Costimulation with MSH and PEMF demonstrated no change from MSH alone. As in the cyclase experiments above, little effect was observed with ISO.

The question of cell cycle was addressed by serum starvation to achieve partial synchronization. This was verified in experiments by ^{3}H-Thymidine incorporation (data not shown). Partial synchronization was achieved (60-70%) after incubation for 24 hours in serum free medium. Identical adenylate cyclase and PKA experiments were performed under these conditions. Figure 1-5 exhibits stable basal adenylate cyclase levels in the control group in the range of 350 cpm/10^5 cells. (Note that this basal cyclase level is one third that in actively proliferating (cycling) cultures). PEMF treated cultures (Figure 1-5A) demonstrated statistically significant cyclase increases at 1,3,5,15, and 30 minutes. No effect was observed at 10 minutes, although there is a stimulatory trend (large standard deviation). MSH demonstrated a subtle stimulation (Figure 1-5), slighty higher than PEMF for the first five minutes. This stimulation then increased dramatically to peak values at 6 fold over control at 10 minutes, and maintained at least a 3 fold elevation out to 30 minutes. The ISO group demonstrated a 10 fold stimulation at one minute, followed by multifold stimulation at 3,5, and 10 minutes, returning close to basal at 30 minutes.

The PKA assay results followed the same trend as above. PEMF stimulated PKA and raised the activity ratio almost 2 fold over control at 3 and 5 minutes, and 15 minutes (Figure 1-6A). The 10 minute time point did not exhibit a statistically significant increase above control. MSH showed a significant increase versus the control with ratio means of 70% (Figure 1-6) over the 5, 10, and 15 minute time

Figure 1-3

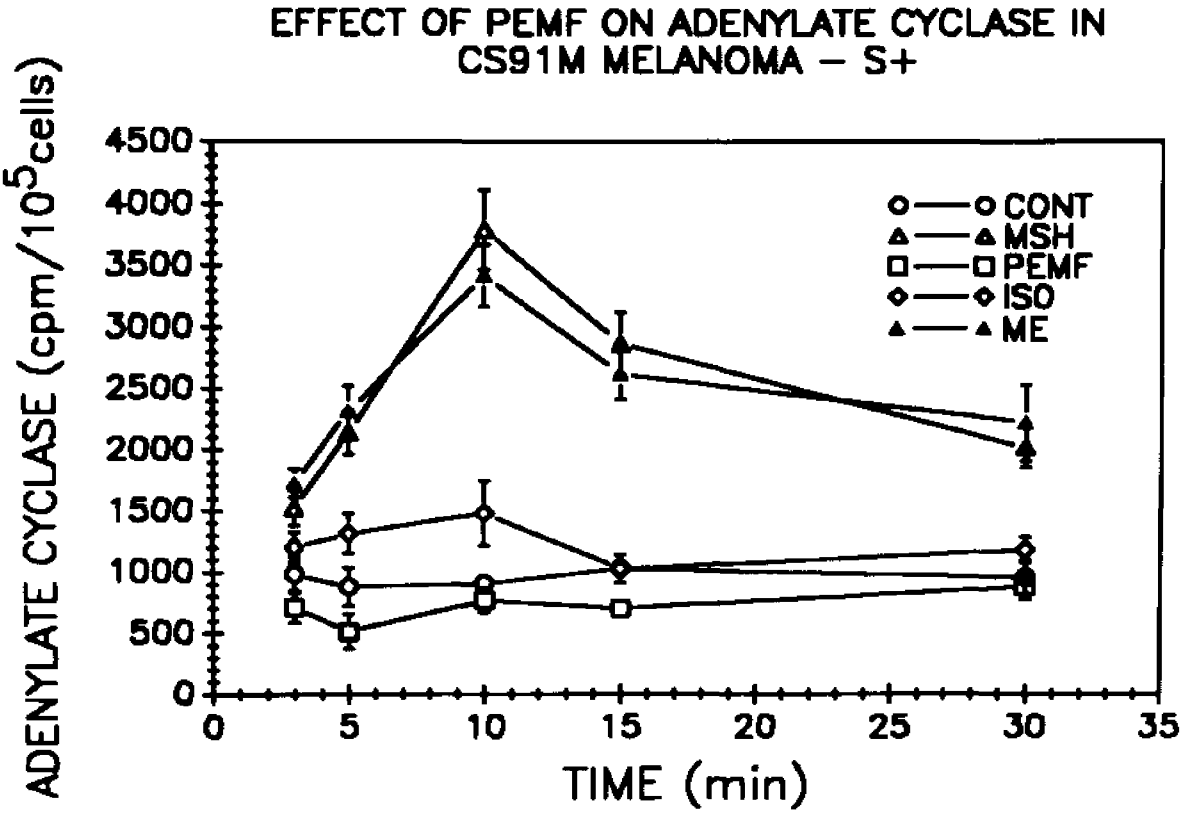

Figure 1-3. Regulation of adenylate cyclase activity in response to MSH, PEMF, and ISO. Melanoma cells were exposed to stimuli for the indicated times. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods); ISO, isoproterenol, 1 μM; ME, MSH and PEMF costimulation. Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, α-melanocyte stimulating hormone , isoproterenol , or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon,1979).

Figure 1-4

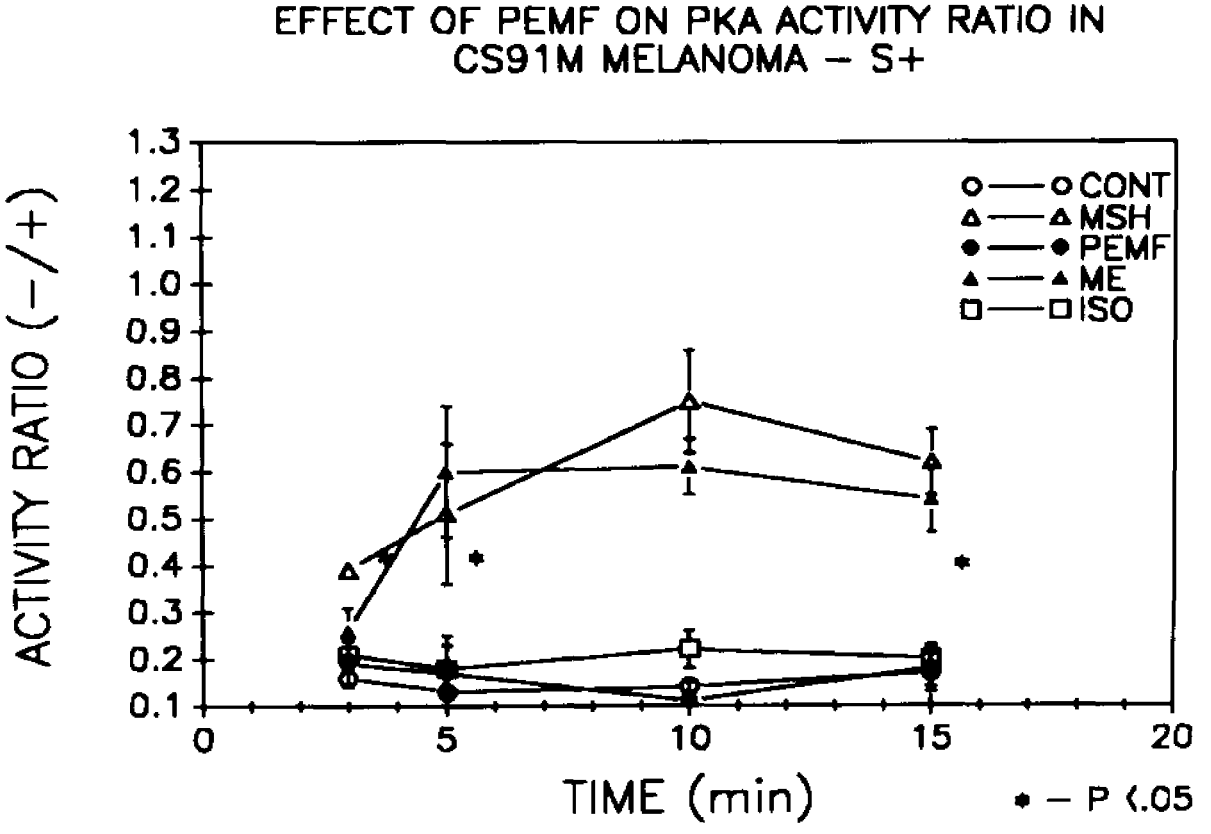

Figure 1-4. Regulation of cAMP-dependent protein kinase activity ratio in response to MSH, PEMF, and ISO. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods); ISO, isoproterenol, 1 μM; ME, MSH and PEMF costimulation. Melanoma cells were exposed to stimuli for the indicated times, washed 1X in ice cold PBS, frozen in liquid N_2, and sonicated in an ice bath 2 X 5 sec. cAMP-dependent protein kinase (PKA) was assayed using Kemptide substrate; the assay buffer contained 150 μm kemptide (Bachem), 1mM EDTA, 10mM MgAcetate, 15 μM BSA (fraction V), 125 μM ATP, and 0.25 - 1.0 μCi gamma ^{32}P-ATP in 40μl total volume plus or minus cAMP (6.25 μm) for 15 minutes at 30°C. 10 μl samples were separated by Whatman P81 phosphocellulose chromatography and counted by Cerenkov emission as described in Methods (Livesey and Martin,1988; Levin et al.,1988).

periods. Costimulation with MSH and PEMF exhibited enhanced activity over MSH alone at 5 minutes, but with obvious overlap of the standard deviations, hence this remains unclear whether this was significant. ISO, as in the adenylate cyclase assay, showed a supramaximal stimulation, achieving over 100% activation. This greater than 100% activation was misleading since this data was pooled from several experiments.

MSH stimulates tyrosinase (Figure 1-7) by over 2 fold over control at the 24,48, and 72 hour time points. PEMF showed significant stimulation only at 24 hours, and returns to control values during the next 48 hours. Forskolin, added as a positive control, stimulated tyrosinase to a greater degree than even MSH (≈ 2.8 fold versus 2.1 fold). ISO increased tyrosinase by 70% and 30% at 24 and 48 hours respectively, with no effect at 72 hours. Cellular tyrosinase activity corresponded qualitatively with the above results at 72 hours, with lower activity.

FIGURE 1-5

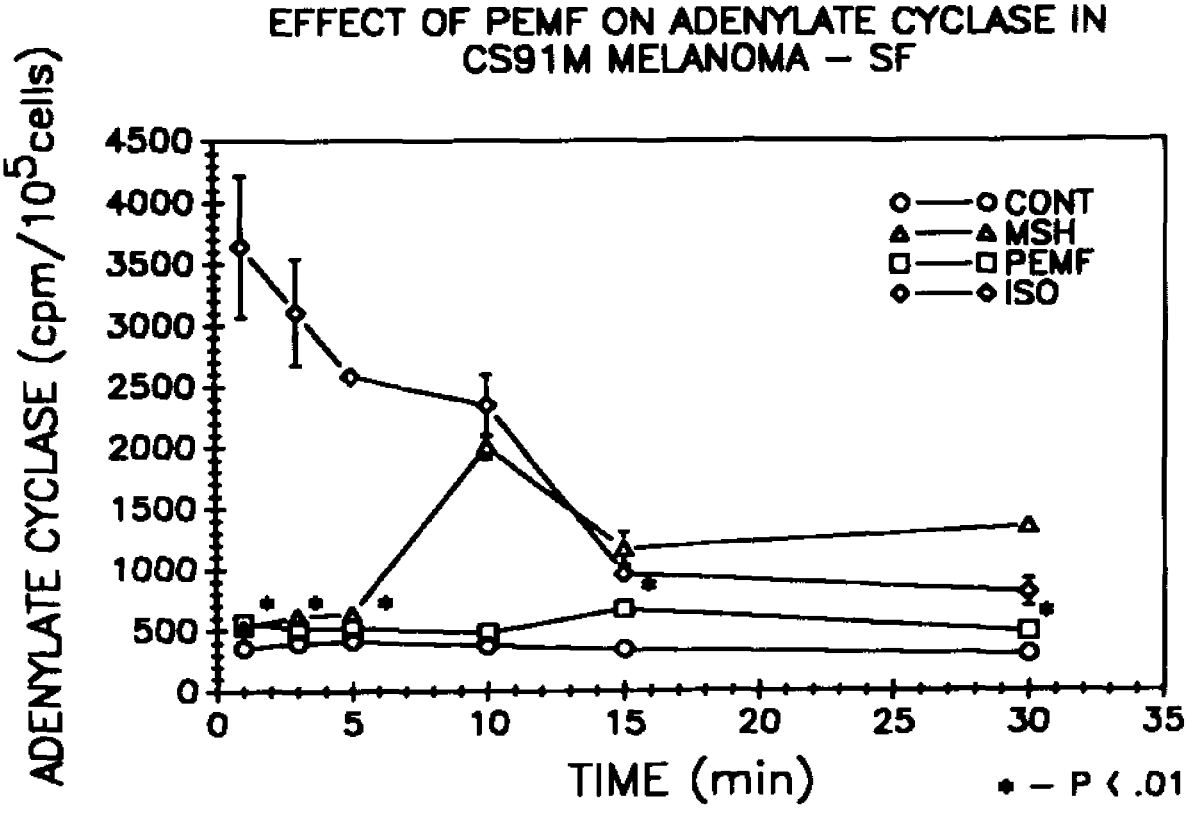

Figure 1-5. Regulation of adenylate cyclase activity in response to MSH, PEMF, and ISO in partially synchronized cultures. Melanoma cells were exposed to stimuli for the indicated times. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods); ISO, isoproterenol, 1 μM; ME, MSH and PEMF costimulation. Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, MSH, ISO, or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

FIGURE 1-5A

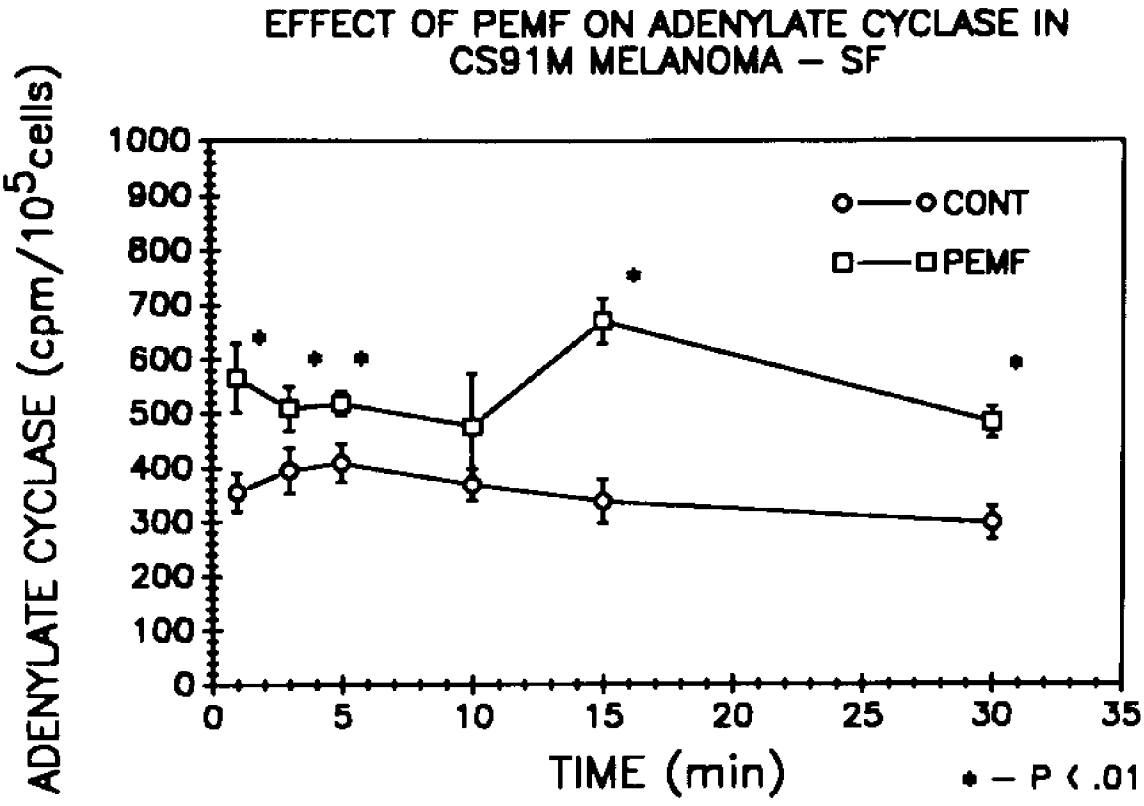

Figure 1-5A. Regulation of adenylate cyclase activity in response to PEMF, higher resolution. Same data from figure 1-6 presented in expanded scale. CONT, Control; PEMF, as above.

FIGURE 1-6

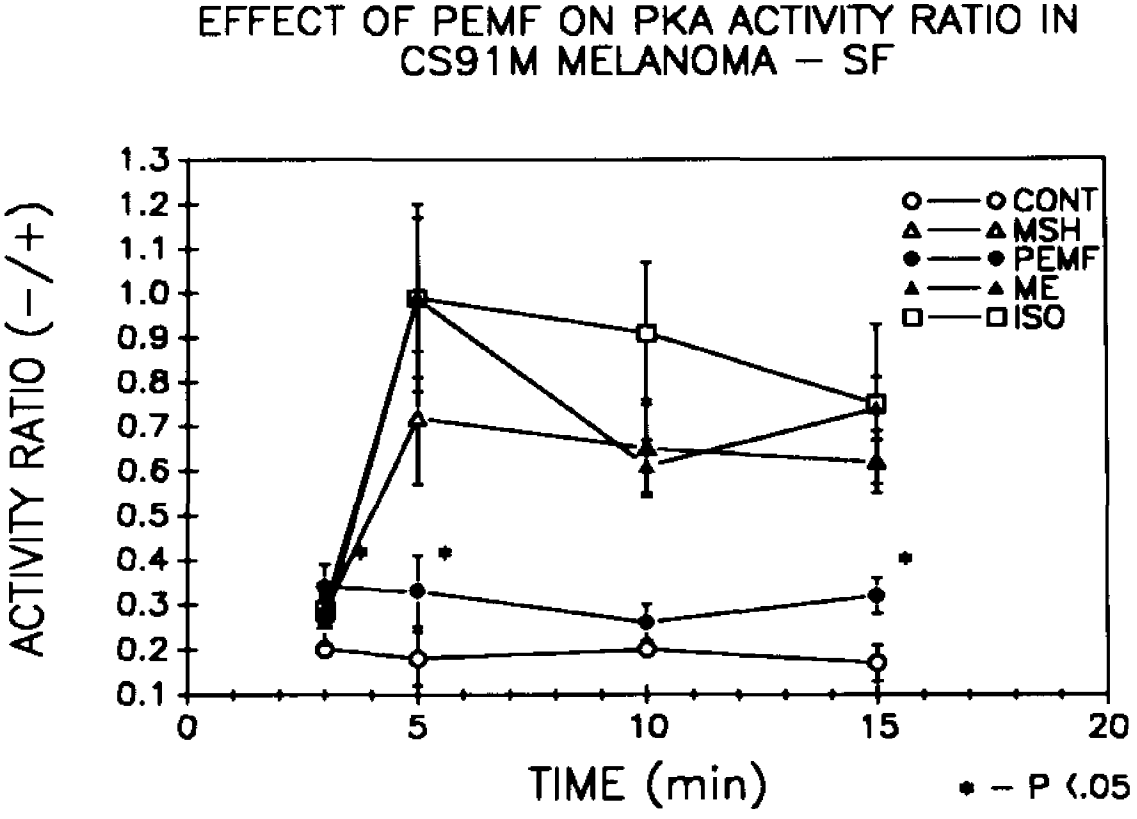

Figure 1-6. Regulation of cAMP-dependent protein kinase activity ratio in partially synchronized cultures in response to MSH, PEMF, and ISO. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods); ISO, isoproterenol, 1 μM; ME, MSH and PEMF costimulation. Melanoma cells were exposed to stimuli for the indicated times, washed 1X in ice cold PBS, frozen in liquid N_2, and sonicated in an ice bath 2 X 5 sec. cAMP-dependent protein kinase (PKA) was assayed using Kemptide substrate; the assay buffer contained 150 μm kemptide (Bachem), 1mM EDTA, 10mM MgAcetate, 15 μM BSA (fraction V), 125 μM ATP, and 0.25 - 1.0 μCi gamma ^{32}P-ATP in 40μl total volume plus or minus cAMP (6.25 μm) for 15 minutes at 30°C. 10 μl samples were separated by Whatman P81 phosphocellulose chromatography and counted by Cerenkov emission as described in Methods (Livesey and Martin,1988; Levin et al.,1988).

FIGURE 1-6A

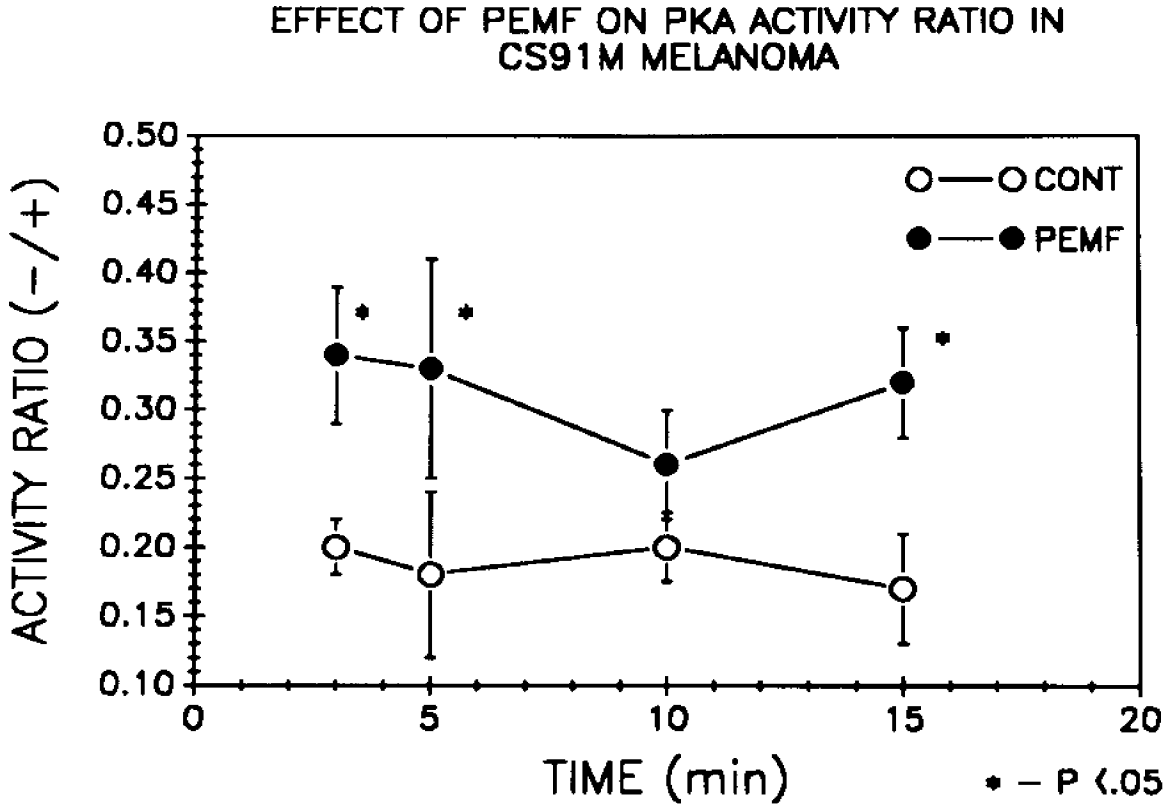

Figure 1-6A. Activity ratio of PKA in response to PEMF, higher resolution. Same data from figure 1-7 presented in expanded scale. CONT, Control; PEMF, as above.

DISCUSSION AND CONCLUSION

Previous studies have demonstrated the ability of PEMF to increase tyrosinase through a cAMP mechanism (Jones et al.,1986; Jones and Ryaby,1987). The depressed PKA levels after PEMF exposure resulted in a recovery phenomenon, which after PEMF removal was suggested to activate tyrosinase. We characterize in the present work the conditions which allow maximal direct activation of adenylate cyclase and PKA by PEMF. The direct stimulatory effect of both PEMF and ISO is observed only after preincubation in serum free media. Two explanations can be proposed for these results: 1) both PEMF and ISO act only when the cultures are quiescent and partially synchronized, perhaps due to expression of a common receptor pathway at this Go/G1 juncture, and 2) growth factors or other compounds in the serum interfere with the ability of these stimuli to elicit their effects. The two above explanations are not mutually exclusive, as they perhaps suggest that PEMF and ISO might function through the same receptor pathway. The rationale underlying the ISO response desensitization may be the presence of compounds with ß-agonist properties capable of downregulating the ß-adrenergic receptor (ßAR). Epinephrine is an example, due to low affinity ßAR binding activity, or PGE_2 which itself stimulates cAMP formation. Investigations of the crosstalk between αAR and ßAR receptor kinase pathways (Sibley et al., 1987) and coordinate regulation through G proteins (Hausdorf et al., 1989) support this speculation. Desensitization may also

FIGURE 1-7

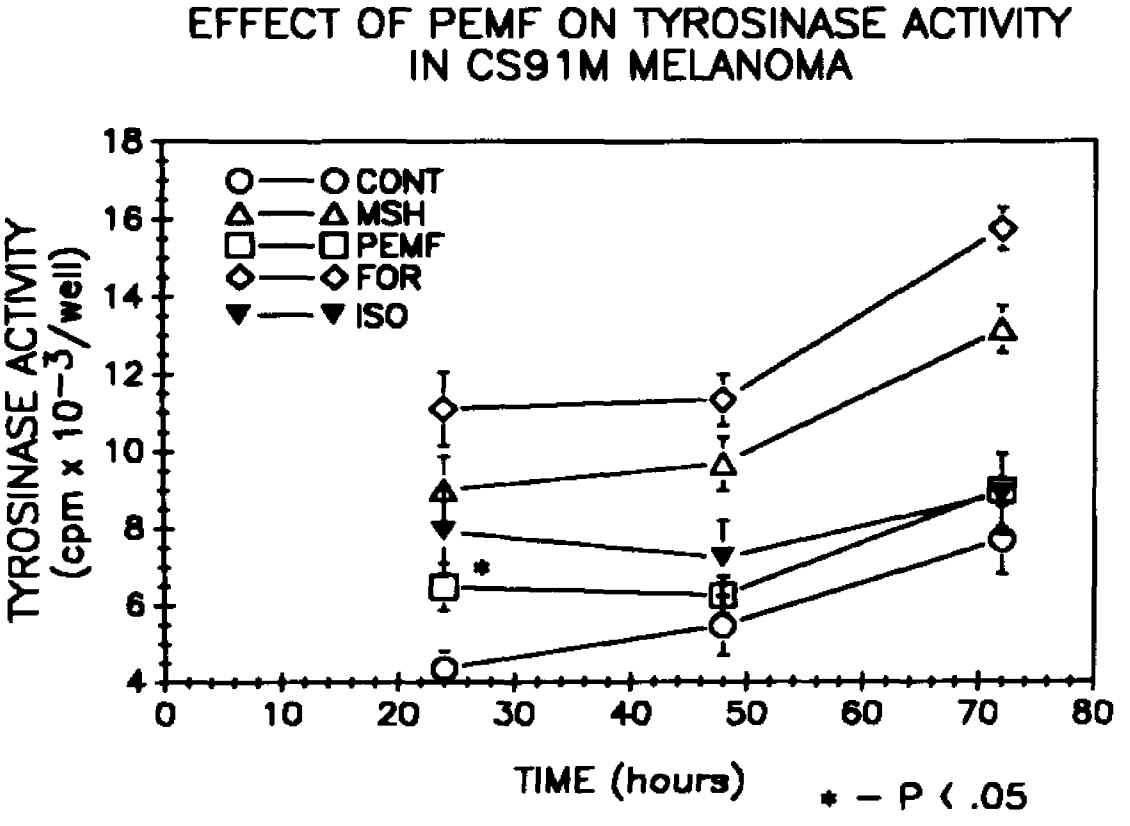

Figure 1-7. Tyrosinase activity in response to MSH, PEMF, FOR, and ISO. Melanoma cells were exposed to stimuli for the indicated times. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, pulsing electromagnetic field, as above; FOR, forskolin, 1 μM; ISO, isoproterenol, 1 μM. Melanoma cultures are incubated with media containing 0.3μCi/ml [^{3}H] and exposed to PEMF, MSH, FOR, ISO, or under control conditions as described above. At the end of each 24 hour interval, the media is collected and fresh media is added containing the stimulus (as above). Free $^3H_20$ in the culture media is separated from ^{3}H-Tyrosine by activated charcoal/Dowex 50W chromatography quantified by LSC.

be mediated by the PKA pathway; this downregulation of ISO activity may be secondary (Hausdorf et al., 1989) to other hormonal stimuli. These results cannot clearly delineate receptor specificities for the PEMF response, yet other potential mechanisms remain to be discussed. Receptor levels for polypeptide hormones are known to fluctuate during the cell cycle (Boynton et al., 1982; Leffert and Koch, 1982). In particular, MSH receptors are maximally expressed during the G_2 phase of the cell cycle (Abdel Malek et al.,1989). There is no information regarding the percentage of cells which respond positively to the hormone at a given time in the results above with MSH activation. Our assay only provides the summated response over the entire cellular population. In the case of PEMF, serum free conditions may enhance the percentage of cells capable of responding to this stimuli (providing a better signal to noise ratio). The PEMF response may not work through either the MSH or ISO receptor pathway, but through a calcium dependent pathway, as has been demonstrated in electric field effects in bone cells and lymphocytes (Conti et al.,1985; Cadossi et al,1988; Ozawa et al., 1990; McLeod et al.,1991).

The time lag for PKA activation merits discussion. MSH, ISO, and PEMF all demonstrate little PKA activation at 3 minutes with maximal activation at 5 minutes. The reason lies in activation of phosphodiesterase (PDE), which concomitant with adenylate cyclase activation is capable of attenuating the cAMP signal (Shacter et al., 1988). Pilot experiments were performed to look at PKA activation in the presence of IBMX. Unfortunately, under these conditions, even basal PKA activity ratios were at or close to 100%. This should have been predictable, as the stoichiometry ratio

for cAMP versus regulatory subunit is strongly in favor of cAMP (Swillens, 1988). Our interpretation states that the initial stimulus must be perceived for a sufficient time for cAMP levels to overcome the PDE attenuation, therefore activating PKA to measurable activity levels.

The tyrosinase response reported with PEMF is not as large as that reported previously, but makes sense in light of the adenylate cyclase and PKA data. After 24 hours under serum free conditions, PEMF will stimulate the cAMP pathway, initiating the transcription of tyrosinase mRNA and synthesis of active enzyme (Fuller at al.,1990). The cells in the tyrosinase assay are grown in serum so the desensitization will manifest itself throughout the first few hours, but not rapidly enough to suppress the initial cAMP 'trigger'. This leads to PEMF having an effect during the first 24 hours, but none thereafter, due to the inability of the cAMP/PKA pathway to respond.

In this chapter we characterized conditions which allow for reproducible adenylate cyclase and PKA activation by PEMF and ISO. MSH, on the other hand, induces adenylate cyclase and PKA under both serum containing and serum-free conditions. These results suggest that PEMF and ISO stimulation may be related and act through similar pathways.

CHAPTER 2

THE BIOLOGICAL MECHANISM OF LOW ENERGY ELECTROMAGNETIC FIELD EFFECTS IN MELANOMA CELLS. DIFFERENTIAL RESPONSE ON PROTEIN PHOSPHORYLATION AND SYNTHESIS.

INTRODUCTION

In the previous chapter, we presented evidence that the cAMP signal transductive mechanism is a target of PEMF stimulation. In this chapter, we compare the effects of the hormone MSH and PEMF on protein phosphorylation and synthesis to: (1) identify relevant substrates which may act as mediators; and (2) examine the complexity of the PEMF response. The PEMF and MSH effects on the phosphorylation of two oncogene proteins (c-fos and c-ras) is compared, in accordance with reports on fos induction being cAMP-dependent and phosphorylatable by PKA (Curran et al.,1986). Goodman and Henderson (1988) demonstrate PEMF signal specific alterations in polypeptide synthetic patterns, thus indicating induction of phenotypic response. Sisken et al.(1989) reported on alterations in synthesis of neurofilament proteins in PEMF stimulated nerve regeneration. Rodeman et al.(1989) described alterations in polypeptide synthetic patterns in response to PEMF stimulation, which correlated with virally-induced differentiation. The data indicate that both MSH and PEMF affect phosphorylation of proteins with similarities at early time points and differences over the long term induction of differentiation. This suggests that a correlation can be drawn between the short term stimulation of PKA (chapter 1) and the alterations observed in protein phosphorylation.

MATERIALS AND METHODS

ELECTROMAGNETIC EXPOSURE APPARATUS

The PEMF waveforms used in these studies were generated with an exposure system consisting of two parallel circular Helmholtz-aiding coils (18 cm or 10 cm diameter) 8 cm apart or 4.5 cm apart, respectively (Figure 1-2). The waveform was monitored with a coil probe amplified in a Tektronix Model 5441 Storage Oscilloscope, as described previously (McLeod et al.,1985). The probe was designed to provide for a 10 fold amplification of the induced voltage when placed parallel to the plane of the coils. The EMF employed was a 5 msec burst consisting of 21 pulses having 200 μsec main and 20 μsec opposite polarity repeating at 15 hz. dB/dt in the main polarity is 0.1 G/μsec corresponding to an induced electric field of 1mV/cm. This corresponded to an average induced current in the culture dish of approximately 5μA/cm^2 (figure 1-1) (McLeod et al., 1984).. All experiments reported herein were performed with the tissue culture dishes or plates oriented parallel to the plane of the coils.

PROTEIN PHOSPHORYLATION

Confluent CS91 melanoma cultures were subcultured into 35 mm petri dishes(9.6cm^2) at a density of 20,000 cells/cm^2. Cells were counted in a Coulter ZM

cell counter calibrated with 14.8 μm latex particles with a 100 μm aperture and a threshold setting, eliminating cells smaller than 8.0μm. All cultures were grown under control conditions for a minimum of 24 hours, and then incubated for an additional 24 hours in serum free media containing 0.1% BSA before an experiment commences. Melanoma cells were labeled for protein analysis with [^{32}P] orthophosphoric acid ($^{32}PO_4$) or ^{35}S-methionine and processed as described by Cooper (1983). Briefly, 35 mm Petri dishes were washed two times in PO_4 or methionine-free HF10 and then incubated with $^{32}PO_4$ (0.1-1.0 mCilml) or [$_3^5$S] methionine (200 μci/ml) for the indicated times. Cultures were then exposed to either PEMF, MSH or other agonists (as described in figure legends and in chapter 1). At the end of the timed incubations, cultures were washed 1X in ice cold PBS, solubilized in Laemmli sample buffer, and analyzed by one dimensional gels (Laemmli, 1970) using 25 ma constant current. For two-dimensional electrophoresis cultures were washed 2X in ice cold PBS, treated with nuclease solution (DNase and RNase, New England Biolabs, Beverly, MA), and solubilized in NP40 2D gel buffer according to Garrels (1979). Autoradiography was performed with Kodak XAR5 film and Dupont Lightning Plus intensifying screens at -70°C. Dilutions of the cell lysates were analyzed for protein content by the BCA method (Pierce, Rockford, IL). In all analyses, the same quantity of protein was loaded per well to allow for direct comparison of phosphorylation or synthesis changes.

IMMUNOPRECIPITATION PROCEDURE

Cell cultures were labeled with ^{32}Pi and ^{35}S-Methionine as described above. Cell lysates were prepared by lysing cells in buffer containing 50mM Tris (pH8.0), 0.5% SDS, 1mM DTT, and 100μM PMSF. Lysates were diluted 1:1 in RIPA buffer (1% NP40, 1% sodium deoxycholate, 150mM NaCl, 10mM Tris, pH7.5) and precleared with nonimmune serum. Lysates were incubated with 1:1000 dilution of antibody for 1 hour at 4° C and then an additional 30 minutes with 25 μl of Pansorbin (Calbiochem, La Jolla, CA) for c-fos, or with 25 μl of Protein A-Sepharose (BRL, Gaithersburg, MD) for c-ras (Curran et al., 1985; Clark et al.,1985). Precipitates were analyzed on 9% (c-fos) or 12.5% (c-ras) gels, dried and autoradiographed as above. Antibodies to c-fos (M peptide) was a gift from Dr. Tom Curran, Roche Institute of Molecular Biology, Nutley, NJ. Antibody to C-ras was from Oncogene Science, Manhasset, NY.

RESULTS

The initial question asked was whether PEMF or MSH could affect the phosphorylation state of specific proteins during short term stimulation (5-30 minutes). Figure 2-1 illustrates that both MSH and PEMF decrease the phosphorylation of proteins with molecular weights of 60 and 75 kilodaltons (kd), while increasing the phosphorylation of a 52 kd species. At 15 minutes, only the phosphorylation of the 52 kd protein was observed to increase in both MSH and PEMF stimulated cells. No changes were detected at either 30 or 60 minutes. These results indicated that similar substrates were potential targets for both MSH and PEMF-induced differentiation.

Experiments were also performed on cellular lysates at 15 and 30 minutes. In all cases no effect on phosphorylation was observed with either MSH and PEMF. Activators of tyrosine kinases, such as epidermal growth factor, and of adenylate cyclase, such as IBMX an cholera toxin, were examined in this system. No quantifiable differences were observed at 15 or 30 minutes with these agents. This suggests a requirement for the intact cell to observe changes in phosphorylation.

The differentiation process in these melanoma cells requires at least 24 hours of induction before a tyrosinase change was observed. We performed accordingly, phosphorylation studies at 24, 48, and 72 hours. Following one day (24 hours) of stimulation, no changes were observed, although an overall increase in the uptake

FIGURE 2-1

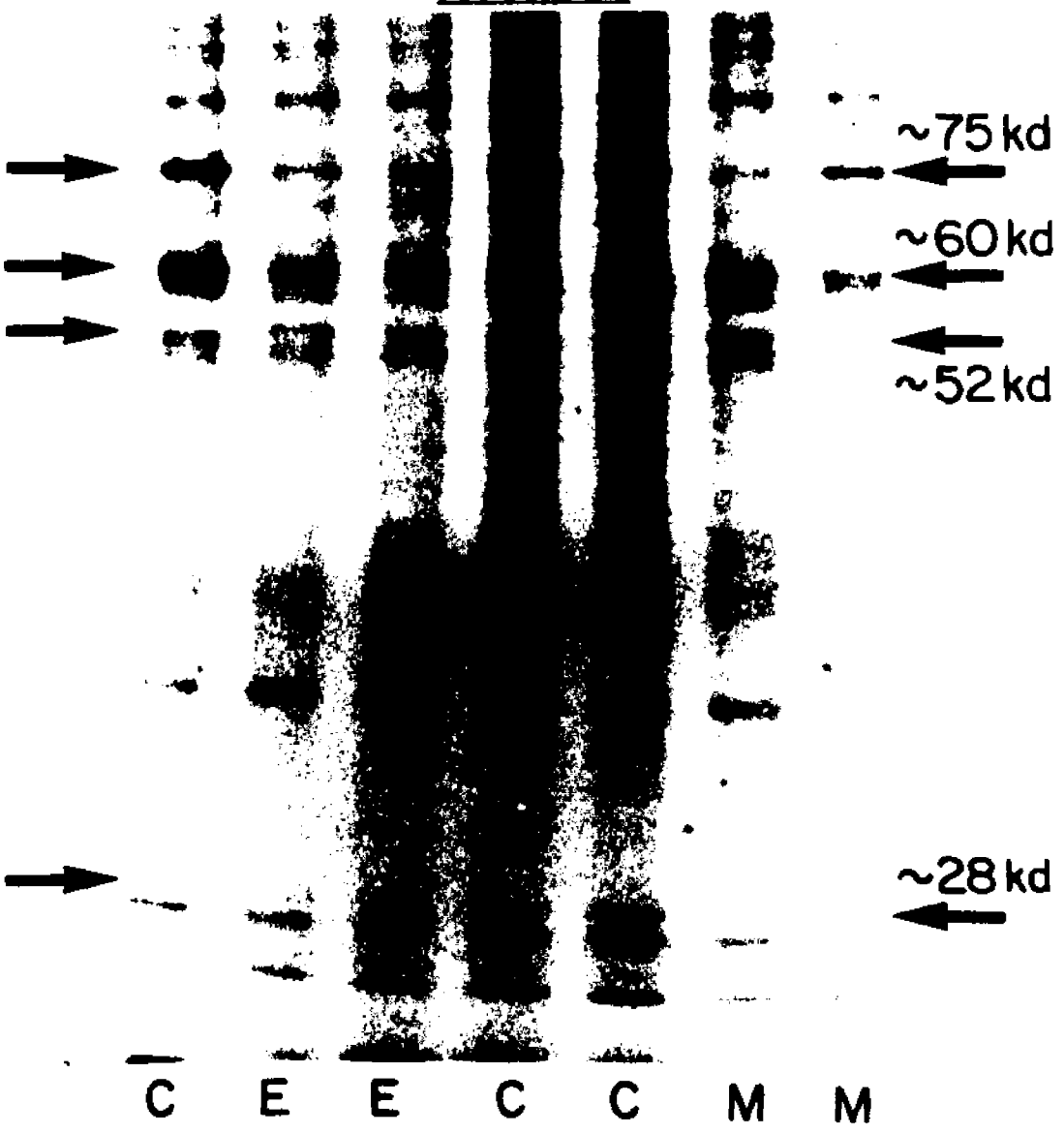

Figure 2-1. One dimensional SDS-PAGE gel of $^{32}PO_4$ -phosphoproteins after 5 minutes of MSH or PEMF exposure. C, Control; M, α-melanocyte stimulating hormone, MSH (10 nM); E, pulsing electromagnetic field, (PEMF; as described in methods). Melanoma cells are washed for 2 hours in PO_4 -free HF10,then incubated with $^{32}PO_4$ (500μCi/ml) and exposed to PEMF or MSH for 5 min. Cultures are then washed 1X in ice cold PBS, solubilized in Laemmli sample buffer, and analyzed by 1D gels (10%, Laemmli, 1970). Autoradiography was performed with Kodak XAR5 film and Dupont Lightning Plus intensifying screens at -70°C. 1:10 dilutions of the cell lysates are analyzed for protein content by the BCA method (Pierce, Rockford, IL). In all analyses, the same quantity of protein is loaded per well to allow for direct comparison of phosphorylation or synthesis changes.

FIGURE 2-2

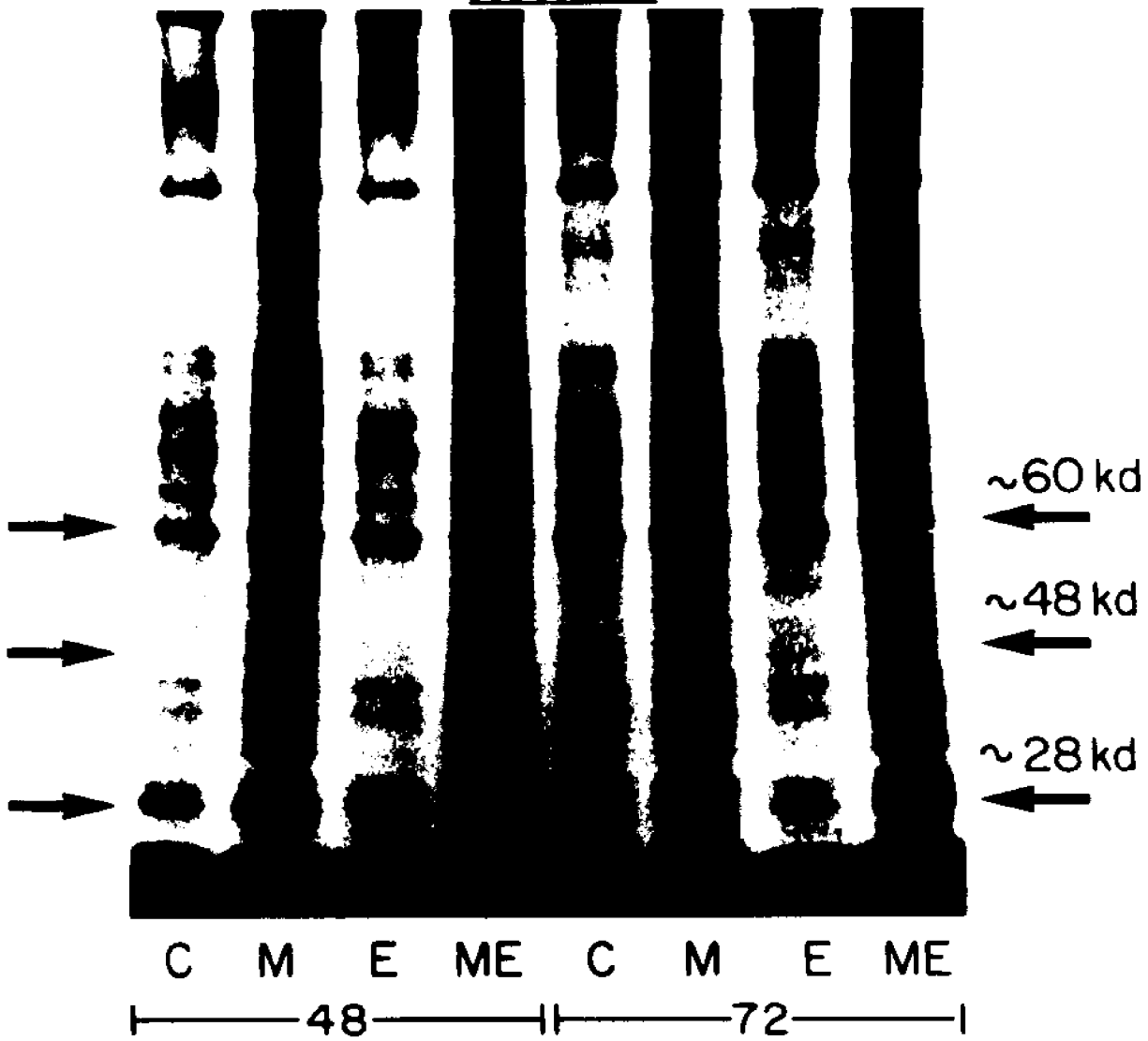

Figure 2-2. One dimensional SDS-PAGE gel of $^{32}PO_4$ -phosphoproteins after 48 and 72 hours of continuous MSH or PEMF exposure. C, Control; M, α-melanocyte stimulating hormone, MSH (10 nM); E, pulsing electromagnetic field, (PEMF; as described in methods). Melanoma cells are washed for 2 hours in PO_4 -free HF10,then incubated with $^{32}PO_4$ (50μCi/ml) and exposed to PEMF or MSH for 48 or 72 hours. Cultures are then washed 1X in ice cold PBS, solubilized in Laemmli sample buffer, and analyzed by 1D gels (10%, Laemmli, 1970). Autoradiography was performed with Kodak XAR5 film and Dupont Lightning Plus intensifying screens at -70°C. 1:10 dilutions of the cell lysates are analyzed for protein content by the BCA method (Pierce, Rockford, IL). In all analyses, the same quantity of protein is loaded per well to allow for direct comparison of phosphorylation or synthesis changes.

FIGURE 2-3

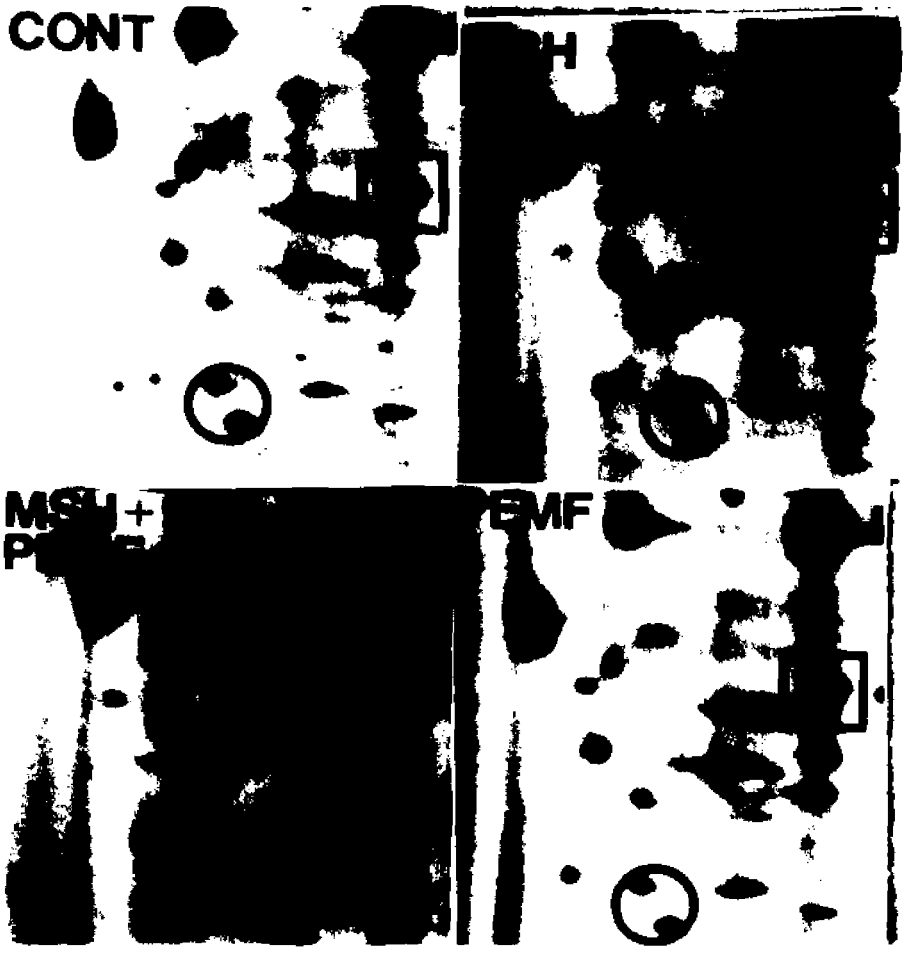

Figure 2-3. Two dimensional SDS-PAGE gel of $^{32}PO_4$ -phosphoproteins after 72 hours of continuous MSH or PEMF exposure. C, Control; M, α-melanocyte stimulating hormone, MSH (10 nM); E, pulsing electromagnetic field, (PEMF; as described in methods); ME, MSH and PEMF costimulation. Melanoma cells are washed for 2 hours in PO_4 -free HF10,then incubated with $^{32}PO_4$ (50μCi/ml) and exposed to PEMF or MSH for 72 hours. For two-dimensional electrophoresis cultures are washed 2X in ice cold PBS, treated with nuclease solution (DNase,RNase), and solubilized in NP40 2D gel buffer according to Garrels (1979). Autoradiography was performed with Kodak XAR5 film and Dupont Lightning Plus intensifying screens at -70°C. Dilutions of the cell lysates are analyzed for protein content by the BCA method (Pierce, Rockford, IL). In all analyses, the same quantity of protein is loaded per tube to allow for direct comparison of phosphorylation or synthesis changes. Note: 66 and 90 kDa molecular weight estimation on right margin.

of $^{32}PO_4$ was noted in the MSH induced cultures. The results at 48 hours (figure 2-2) demonstrated increased phosphorylation of proteins of 28, 48, and 60kd in the MSH induced cultures, with the 60kd species maintained up to 72 hours. No alterations were observed in the PEMF treated cultures at these time points. PEMF and MSH costimulation showed an overall stimulation of $^{32}PO_4$ incorporation at both 48 and 72 hours. These results illustrated that MSH-induced alterations in phosphorylation may be maintained for long periods of time. Further, this demonstrated that PEMF does not mimic the activity of the hormone MSH at long time intervals in terms of phosphorylation.

To provide higher resolution of the long term effects of MSH and PEMF, two dimensional electrophoresis (2D) was employed. Both PEMF and MSH at 72 hours induced phosphorylation of a isoelectric point (pI) 5.6, 90kd protein (Figure 4). Addition of both PEMF and MSH illustrated an additive phosphorylation increase of this protein. In contrast, a second class of proteins, pI 5.3, 60-70kd, were stimulated by MSH but not PEMF. As shown before, PEMF does not display the identical response to that of the hormone MSH.

Another question addressed in this study was whether biosynthetic alterations are triggered during the MSH or PEMF mediated induction of differentiation. MSH induction for 72 hours displayed no noticeable alterations in protein biosynthesis as assessed by 2D gel analysis. However, the PEMF stimulation (Figure 2-4) increased synthesis of a p.I 6.6, 70kd class of proteins, and decreased synthesis of a p.I 6.3, 65-75kd class of proteins. Further differential regulation was observed in the pI 6.6,

60kd protein. The implication here was PEMF can alter the biosynthesis of specific proteins without necessarily affecting the overall biosynthetic activity of the cell.

The final question was whether phosphorylation or synthesis changes can be detected in oncogene proteins related to early events in signal transduction. As can be seen in figure 2-5, alterations in c-fos phosphorylation were seen with PEMF exposure at both 30 and 60 minutes; in fact, more extensive phosphorylation was observed in both the 39 and 55kd proteins than with MSH. Forskolin displays the most phosphorylation at 60 minutes, with MSH and PEMF costimulation demonstrating the largest effect at 30 minutes. ISO did not show any definitive alteration, although a slight decrease in phosphorylation have been observed. The data with c-ras (figure 2-6) is less clear, but preliminary results demonstrated a strong activation of phosphorylation with MSH and PEMF costimulation, while PEMF alone also increased versus control. No effects were observed on synthesis of c-fos or c-ras.

FIGURE 2-4

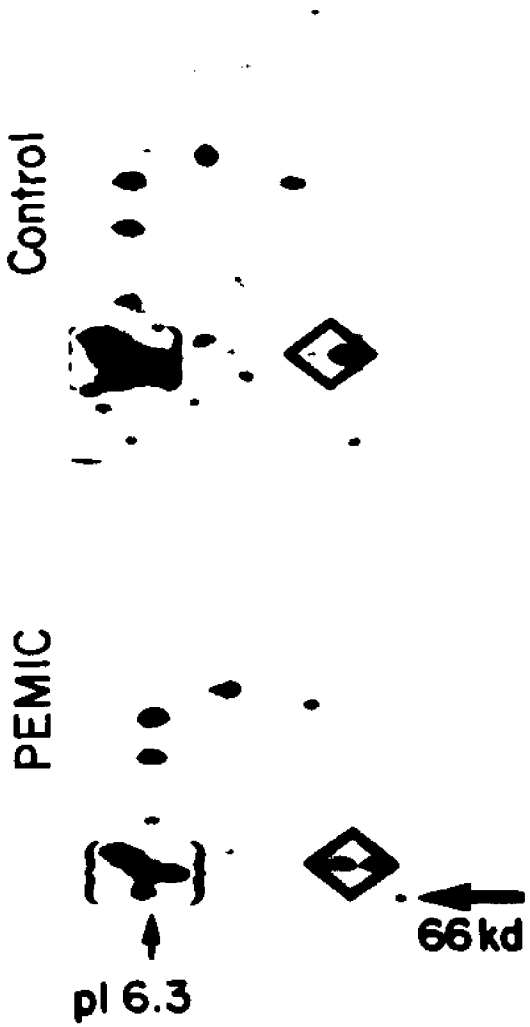

Figure 2-4. Two dimensional SDS-PAGE gel of ^{35}S-Methionine labelled proteins after 72 hours of continuous PEMF exposure. C, Control; PEMIC, pulsing electromagnetic field, (PEMF; as described in methods). Melanoma cells are washed for 2 hours in methionine-free HF10, then incubated with $^{35}SO_4$-Methionine (200μCi/ml), and exposed to PEMF for 72 hours. For two-dimensional electrophoresis cultures are washed 2X in ice cold PBS, treated with nuclease solution (DNase,RNase), and solubilized in NP40 2D gel buffer according to Garrels (1979). Autoradiography was performed with Kodak XAR5 film and Dupont Lightning Plus intensifying screens at -70°C. Dilutions of the cell lysates are analyzed for protein content by the BCA method (Pierce, Rockford, IL). In all analyses, the same quantity of protein is loaded per tube to allow for direct comparison of phosphorylation or synthesis changes. Note: 66 and 90 kDa molecular weight estimation on right margin.

FIGURE 2-5

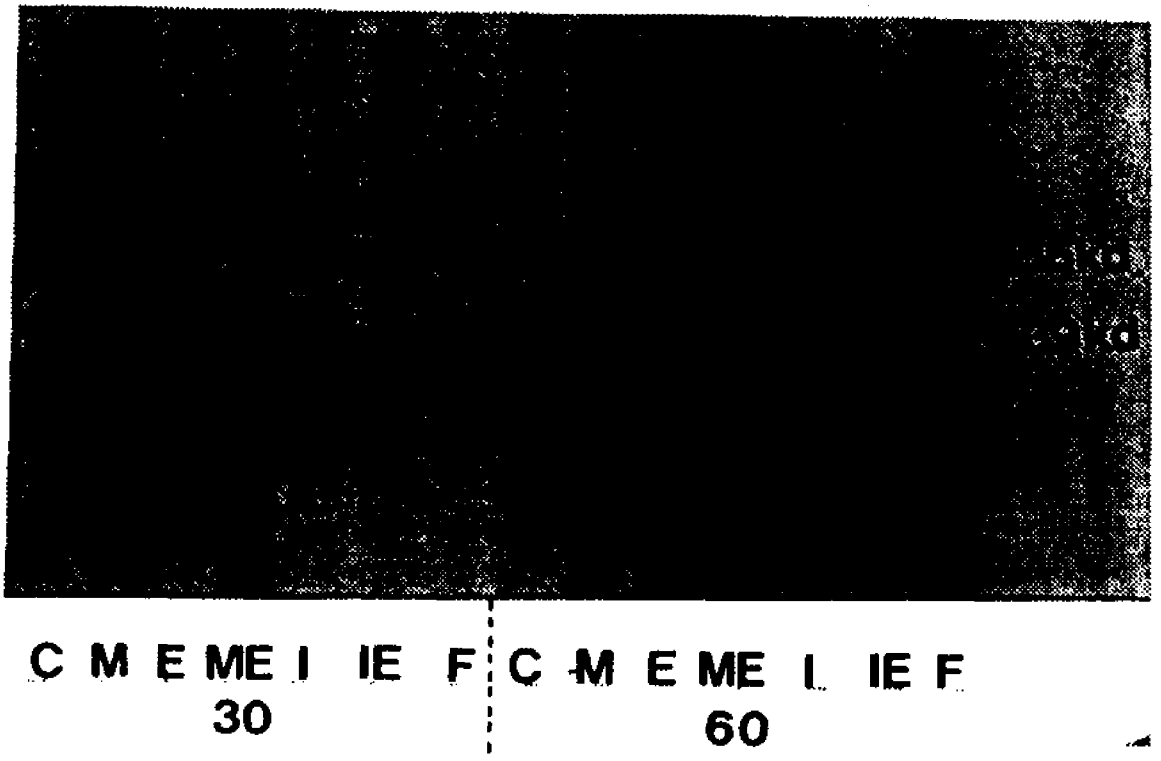

Figure 2-5. Immunoprecipitation analysis of ^{32}-PO_4 labeled phosphoproteins with antibody to c-fos. Melanoma cells were exposed to stimuli for the indicated times. C, Control; M, α-melanocyte stimulating hormone, MSH (10 nM); E, Pulsing electromagnetic field (as above); ME, MSH and PEMF costimulation; I, Isoproterenol, ISO (1 μM); IE, ISO and PEMF costimulation; F, Forskolin, FOR (1 μM). Cell cultures were labeled with ^{32}Pi as described in methods. Cell lysates were prepared by lysing cells in buffer containing 50mM Tris (pH8.0), 0.5% SDS, 1mM DTT, and 100μM PMSF. Lysates were diluted 1:1 in RIPA buffer (1% NP40, 1% sodium deoxycholate, 150mM NaCl, 10mM Tris, pH7.5) and precleared with nonimmune serum. Lysates were incubated with 1:1000 dilution of antibody for 1 hour at 4° C and then an additional 30 minutes with 25 μl of Pansorbin (Calbiochem, La Jolla, CA) for c-fos (Curran et al., 1985). Precipitates were analyzed on 9% gels, dried and autoradiographed as in methods.

FIGURE 2-6

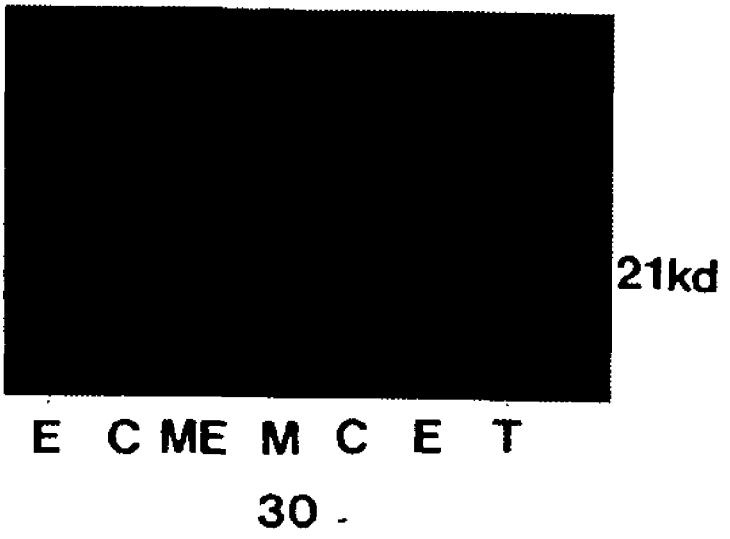

Figure 2-6. Immunoprecipitation analysis of ^{32}-PO_4 labeled phosphoproteins with antibody to c-ras. Melanoma cells were exposed to stimuli for the indicated times. C, Control; M, α-melanocyte stimulating hormone, MSH (10 nM); E, Pulsing electromagnetic field (as above); ME, MSH and PEMF costimulation; T, TPA (10 μM). Cell cultures were labeled with ^{32}Pi as described in methods. Cell lysates were prepared by lysing cells in buffer containing 50mM Tris (pH8.0), 0.5% SDS, 1mM DTT, and 100μM PMSF. Lysates were diluted 1:1 in RIPA buffer (1% NP40, 1% sodium deoxycholate, 150mM NaCl, 10mM Tris, pH7.5) and precleared with nonimmune serum. Lysates were incubated with 1:1000 dilution of antibody for 1 hour at 4° C and then an additional 30 minutes with 25 μl of Pansorbin (Calbiochem, La Jolla, CA) for c-fos (Clark et al., 1985). Precipitates were analyzed on 12.5% gels, dried and autoradiographed as in methods.

DISCUSSION AND CONCLUSION

Previous studies demonstrated that PEMF could induce differentiation, as monitored by tyrosinase activity in melanoma cells in the same temporal period as the hormone, MSH (Jones et al, 1986). The experiments reported herein distinguish MSH induced changes in phosphorylation from those of PEMF at the protein level. Our results showed similarities in the short term phosphorylation pattern (5-60 minutes). This implies that PEMF and MSH induced phosphorylation alterations may be mediated through PKA in the short term stimulation reported here. Other kinase mediated signal transductive mechanisms exist in melanoma cells, such as tyrosine and calmodulin kinase, and these may be responsible for the observed short term changes (Fleischman et al, 1988). Protein kinase C does not seem to be the candidate responsible for the short term changes. The long term phosphorylation alterations demonstrate that PEMF does not display all hormonal response manifestations. As reported in the previous chapter, the direct stimulation of adenylate cyclase and PKA may only occur at a specific phase of the cell cycle, or when appropriate receptors (e.g., adrenergic) were being expressed.

The differentiation process, whether induced by PEMF or MSH, is accompanied by an inhibition of growth (lengthening of the cell cycle time) (Jones and Ryaby, 1987; Pawelek, 1973). The growth regulation of melanoma cells involves the insulin receptor and associated tyrosine specific protein kinase (Fleishmann et al., 1985). Numerous reports document the phosphorylation of both the insulin and epidermal

growth factor receptors by both PKA and PKC (Cochet et al., 1984; Takayama et al., 1984). Accordingly, the effect of both PEMF and MSH on growth inhibition in melanoma cells should involve the modulation of insulin and other growth factor receptors by phosphorylation-dependent mechanisms. Consistent with the role of insulin are the results presented herein on increased phosphorylation of the p.I 5.6, 90kd protein in both MSH, insulin and PEMF treated cells. This protein has tentatively been identified as the peptide mediating insulin growth regulation of growth (Fleischmann et al., 1985). These findings lead us to propose that PKA may co-regulate the phosphorylation state of this peptide and consequently regulate cell cycle transit time.

Previous studies have reported effects of PEMF on specific protein synthesis in Sciara salivary glands (Goodman and Henderson, 1988). This work illustrated protein synthesis alterations by 2D gel electrophoresis which indicated that synthesis of specific proteins could either be augmented or inhibited by different PEMF signals. The authors also established that these responses were different from those induced by heat shock. In this study, we provide some preliminary results on protein synthesis in a mammalian cellular system. Our results indicate that differential regulation is exemplified by the effect on the pI 6.6, 66kd protein. The putative expression of both isoform variants in PEMF treated cultures is supported by parallel experiments, where no phosphorylation change was detected which could account for the isoelectric point shift (Johnson et al.,1988). Further characterization of these proteins will be required before any functional conclusion can be drawn from these

results.

The stimulation of c-fos and c-ras phosphorylation with MSH and PEMF confirms definitively the activation of a signal transductive mechanism, although one cannot conclusively state that these results are cAMP/PKA mediated. These results are interesting because both c-fos itself and the fos related antigen (Curran et al.,1985) are affected, thus indicating similarity between MSH and PEMF induction; and further suggesting a link to a calcium dependent pathway (Morgan and Curran, 1986). The inhibition observed with ISO is puzzling because one would assume that activation of cAMP would result in this response regardless of the agonist used to elicit the cellular response. Perhaps 30 and 60 minutes are too distant temporally from the initial stimulus and any effect of ISO is already back to basal levels. It is well known that the cAMP phosphoprotein phosphatases attenuate these signals rapidly (Leiser et al., 1986) which may explain these findings. The effect seen with c-ras is suggestive at best and indicates that low molecular weight G proteins may be involved in the crosstalk between different signalling pathways (Simon et al., 1990).

The conclusion from these studies is PEMF can induce differentiation in a melanoma cell line by manifesting certain functional alterations associated with the normal physiological regulator, MSH. PEMF may also be capable of altering the phenotypic expression of the melanoma cell by differential regulation of protein synthesis and phosphorylation. The generality of these findings in other model systems remains to be investigated.

CHAPTER 3

MODULATION OF PEMF STIMULATED ADENYLATE CYCLASE AND cAMP DEPENDENT PROTEIN KINASE ACTIVITY BY THE INHIBITORY G PROTEIN, Gi.

INTRODUCTION

The focus of electromagnetic field (PEMF) research at the cellular level has been on membrane mediated signal transduction processes. Many effects indicate that the receptor-adenylate cyclase second messenger system may be a site of interaction of PEMF. Early work by Norton et al.(1977) demonstrated effects on cAMP metabolism and correlated this with effects on DNA synthesis (Rodan et al.,1978). Other investigators have reported changes in cAMP levels as a function of the electrical field strength induced capacitively and correlated this with a change in DNA synthesis (Korenstein et al., 1984). Cell type specific changes in cAMP levels with demonstrated recovery phenomenon after PEMF removal have been described (Jones, 1984), as have changes in cAMP induced differentiation in F9 teratocarcinoma cells (Akamine et al., 1985). These investigators all consider the adenylate cyclase system to be a mediator of a functional response to the electromagnetic field stimulation. The protein kinase C transductive pathway, as well as calcium signalling pathways, have also been implicated in electric field interaction (Byus et al.,1984; Adey, 1988; McLeod et al.,1990). Finally, the stimulation of autocrine growth factor production has been postulated as both messenger and regulator in electric field stimulation (Fitzsimmons et al.,1990, Cadossi et al., 1989). Therefore, many signal transductive pathways may be sensitive to electric field interaction.

The ability to modulate hormonal responses has been a central theme in PEMF

effects on signal transduction. Luben et al. (1982) were the first to demonstrate inhibition of parathyroid hormone stimulated adenylate cyclase activity in bone cells by long term PEMF exposure. Cain et al.(1987) extended this work for short term PEMF stimulation and suggested no role for G protein mediation. Brighton et al.(1988) showed maximal inhibition of PTH response using a capacitively coupled electric field system. In contrast, Hiraki et al.(1987) reported a PTH response increase in differentiating chondrocytes after PEMF exposure.

The remaining question regarding the mechanism of PEMF inhibition of hormonal response is whether it is mediated at receptor, G protein coupling, or cyclase level itself? Furthermore, what is the role of G proteins in the direct stimulation of adenylate cyclase by PEMF? In other systems, G proteins attenuate hormonal response in K^+ and Ca^{2+} ion channel regulation (Brown et al., 1989; Yatani et al., 1989), phospholipase C activity (Fain et al., 1988), as well as in adenylate cyclase/cAMP dependent protein kinase regulation (Gilman, 1987).

In this study, we use a clinically effective pulsing electromagnetic field on a well-defined model system to define the biochemical signal transductive mechanism underlying the reported biological effects.

MATERIALS AND METHODS

ELECTROMAGNETIC EXPOSURE APPARATUS

The PEMF waveforms used in these studies were generated with an exposure system consisting of two parallel circular Helmholtz-aiding coils (18 cm or 10 cm diameter) 8 cm apart or 4.5 cm apart, respectively (Figure 1-2). The waveform was monitored with a coil probe amplified in a Tektronix Model 5441 Storage Oscilloscope, as described previously (McLeod et al.,1985). The probe was designed to provide for a 10 fold amplification of the induced voltage when placed parallel to the plane of the coils. The EMF employed was a 5 msec burst consisting of 21 pulses having 200 μsec main and 20 μsec opposite polarity repeating at 15 hz. dB/dt in the main polarity was 0.1 G/μsec corresponding to an induced electric field of 1mV/cm. This corresponds to an average induced current in the culture dish of approximately 5μA/cm^2 (figure 1-1) (McLeod et al., 1984).. All experiments reported herein were performed with the tissue culture dishes or plates oriented parallel to the plane of the coils.

TYROSINASE ASSAY

Cloudman S91 cells (American Type Culture Collection CCL 53.1 clone M-3) were grown to confluency in Ham's F10 medium (Gibco, Grand Island, NY; or Mediatech, Herndon, VA) supplemented with 15% horse serum / 2.5% fetal calf serum (Hyclone USA, Logan, UT) and glutamine (2mM [final]) (56). No antibiotics

were used during either maintenance or experimental culture. Cells were subcultured by incubating briefly with 1mM EDTA in calcium and magnesium-free Hanks solution (Gibco) and diluted 1:5 into 75 or 150 cm flasks according to need. For tyrosinase experiments, cells were seeded at a density of 75,000 (±1%) per 35 mm culture dish (Nunc) in 2 ml of culture medium. Cells were counted in a Coulter ZM cell counter calibrated with 14.8 μm latex particles using a 100 μm aperture and a threshold setting eliminating cells smaller than 8.0μm. Tyrosinase experiments were conducted in glass culture jars (resin reaction vessels) whose ground glass stoppers were sealed with vacuum grease. Cells were grown in an atmosphere of 20% O_2, 75% N_2 and 5% CO_2 at saturated humidity in a warm room at 37°C or a 10 cubic foot CO_2 incubator under the same conditions were used. Control cultures grown under these conditions showed growth and tyrosinase response to MSH and insulin identical to that reported in the literature (Pawelek et al.,1975,1976). Tyrosinase relative activity (monophenol monooxygenase; monophenol, dihydroxy-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) (cpm released per 24 hours from L-[3,5-^{3}H] tyrosine, Amersham, Arlington Hts, IL) was measured by the method of Pomerantz (1969). Cells were grown under control conditions for 24 to 72 hours, then incubated in serum free medium containing 0.1% BSA for an additional 24 hours. Pertussis toxin (List Biological Laboratories, Campbell, CA) was reconstituted according to the manufacturer. Activation of PT was accomplished according to the methods of Moss et al. (1983). Briefly, PT (100 μg/ml) was incubated in 50mM HEPES, pH 8.0, 1 mg/ml BSA, 20 mM DTT, and 0.125% SDS for 30 minutes at 30°C. At the start of

the experiment, cultures were preincubated with or without PT in fresh serum free media for 4 hours at 37°C. Media is aspirated, cultures were washed 1X with fresh media, refed with fresh media and exposed to either PEMF, α-melanocyte stimulating hormone (MSH, Bachem, Torrance, CA), forskolin (FOR, Sigma, St.Louis, MO), or under control conditions as described above. At the end of each 24 hour interval, the media was collected and fresh media containing PT was added for 4 hours containing the stimulus, after which HF10 growth media containing 0.3μCi/ml [^{3}H]-Tyrosine was added (with appropriate stimulus). Free $^3H_20$ in the culture media was separated from ^{3}H-Tyrosine by activated charcoal/Dowex 50W chromatography and counted in Hydrofluor (National Diagnostics, Manville, NJ) by liquid scintillation counting in a LKB-Wallac LSC. Data was presented as cpm $^3H_20$ per well.

ADENYLATE CYCLASE ASSAY

Adenylate cyclase activity was performed according to the method of Salomon (1979). Confluent CS91 melanoma cultures (see above) were subcultured into 35 mm petri dishes(9.6cm^2), or 4 and 24 well plates (16 mm diameter,2cm^2) at a density of 20,000 cells/cm^2 (determined by Coulter Counter as above) and grown under control conditions for a minimum of 24 hours before the start of an experiment. For comparison of serum-free and normal growth conditions, cells were incubated for an additional 24 hours in fresh medium respectively. Cells were incubated in serum free

HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, α-melanocyte stimulating hormone , forskolin, isoproterenol , or under control conditions for the times indicated. The PEMF and MSH costimulated group was achieved by placing cultures first in the PEMF apparatus then immediately adding the MSH stock. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography. For analysis of the ^{3}H-cAMP formation, cultures were thawed at room temperature and transferred to 12 X 75mm glass tubes containing 100 μl of nucleotide carrier solution (5mM [final] of Adenine, Adenosine, AMP, ADP, ATP, and cAMP (Sigma) ; to prevent non-specific binding of ^{3}H-cAMP or ^{14}C-cAMP), 50 μl of ^{14}C-cAMP (Amersham) standard (3000 cpm/tube), and then 50 μl of 4N KOH was added and the mixture is vortexed. Samples were applied to the column by inversion and 4 ml volumes were collected from the alumina columns in scintillation vials. Fourteen ml of Hydrofluor was added and counted in a LKB-Wallac scintillation counter with internal standard. Data was collected directly onto disk with backup hardcopy printout. The data was edited with Epsilon text editor, and determination of ^{3}H-cAMP performed with PC-Matlab (The Mathworks). Data was expressed as cpm/10^{5} cells or cpm/culture.

cAMP DEPENDENT PROTEIN KINASE ASSAY

Confluent CS91 melanoma cultures (see above) were subcultured into 35 mm petri dishes(9.6cm^2), at a density of 20,000 cells/cm^2 (determined by Coulter Counter as above) and grown under control conditions for a minimum of 24 hours before the start of an experiment. For comparison of serum-free and normal growth conditions cells were incubated for an additional 24 hours in fresh medium, respectively. Cultures were then exposed to either PEMF, MSH or other agonists as described. At the end of the timed incubations, cultures are washed 1X in ice cold PBS, then frozen rapidly by immersion into liquid N_2. Cultures were then prepared for analysis by sonication in an ice bath for two 5 second intervals at a power setting of 3 using a microtip (Heat Systems-Ultrasonics Model W385, Farmingdale, NY). cAMP-dependent protein kinase (PKA) was assayed by a combination of the methods of Livesey and Martin (1988) and Levin et al (1988). Kemptide (Bachem) was used as the phosphoacceptor substrate and gamma ^{32}P-ATP (Amersham) as the phosphate donor. The assay buffer contained 150 μm kemptide (Bachem), 1mM EDTA, 10mM MgAcetate, 15 μM BSA (fraction V), 125 μM ATP, and 0.25 - 1.0 μCi gamma ^{32}P-ATP. The incubations were performed in 40μl total volume in assay plates (Falcon, polystyrene), containing either plus or minus cAMP (6.25 μm) for 15 minutes at 30°C. The assay plates were floated in the water bath with a styrofoam frame. The reaction was stopped by the addition of 10 μl of a 1mM EDTA solution (Mg2+ is a required cofactor for kinase activity). 10 μl samples were then spotted onto

Whatman P81 phosphocellulose paper, air dried, then washed with 75mM phosphoric acid 4X for 5 minutes, followed by one 5 minute wash with 95% EtOH, and dried with a infrared lamp. Samples were subsequently counted in 15 ml of H_2O in a LKB Wallac scintillation counter . Soluble protein was measured by the Coomassie blue method of Bradford (61).

ADP-DEPENDENT RIBOSYLATION OF Gi AND Gs

Confluent CS91 melanoma cultures were subcultured into 35 mm petri dishes(9.6cm^2) at a density of 50,000 cells/cm^2. Cells were counted in a Coulter ZM cell counter as mentioned above. All cultures were grown under control conditions for a minimum of 24 hours, and then incubated for an additional 24 hours in serum free media containing 0.1% BSA before the start of an experiment. Cultures were then exposed to either PEMF, MSH or FOR as described above. At the end of the timed incubations, cultures were washed 1X in ice cold PBS, then scraped into buffer containing 10mM HEPES, pH 8.0, 10mM Thymidine, 1mM EDTA, 5mM DTT, and 0.2 mg/ml BSA. Cell cultures were sonicated as above (PKA assay). Aliquots containing 50 μg protein were then incubated in the above buffer containing 1 μM ^{32}P-NAD (40 Ci/mmol, New England Nuclear, Boston, MA) and 100ng/ml pertussis toxin (List Biological Laboratories, Campbell, CA). The samples (50 μl total volume) were mixed 1:1 with 2X Laemmli sample buffer, boiled for 1 minute, and analyzed by one dimensional 12.5% gels (Laemmli, 1970). Autoradiography was

performed with Kodak XAR5 film and Dupont Lightning Plus intensifying screens at -70°C. Dilution of the cell lysates were analyzed for protein content by the BCA method (Pierce, Rockford ,IL). In all analyses, the same quantity of protein (25 μg) was loaded per well to allow for direct comparison of phosphorylation changes.

STATISTICAL ANALYSIS

In the experiments reported here a minimum of three experiments were performed with a minimum of four culture wells/point/experiment. The mean and standard deviation were evaluated for each sample group. An paired Student's t test was employed to test statistical significance. Significance was accepted at $P \leq 0.05$.

RESULTS

The G protein involvement in the stimulation of adenylate cyclase by PEMF and MSH was examined by use of pertussis toxin. The effects of pertussis toxin pretreatment on the modulation of PEMF response was seen in Figure 3-1. As reported in chapter 1, under serum free conditions PEMF was capable of adenylate cyclase stimulation. In this series of experiments, PEMF increases adenylate cyclase by about 40-50% versus control at 3, 5, and 15 minutes. Pertussis toxin (PT) pretreatment does not affect the basal cyclase activity in the control cells, but in the PEMF treated cultures cyclase stimulation was increased 2 fold versus PEMF alone at all time points. MSH activation of cyclase was also augmented by PT pretreatment, although not as much as in the PEMF group. The increase in cyclase observed with MSH alone is 2-2.5 fold above control, and PT pretreatment increases this by approximately 30-35 %. These experiments were repeated to confirm activation of cAMP-dependent protein kinase (PKA) activity. Figure 3-2 illustrates PKA activity stimulated by both MSH and PEMF at 3, 5, and 15 minutes. PT pretreatment did not affect basal cyclase activity except at 3 minutes, with no effect seen at 5 or 15 minutes. MSH and PEMF response was affected by PT pretreatment as MSH activation increased by 30% at all time points, and PEMF stimulation increased by 60% in the PT group.

The comparison of serum free and serum containing medium was performed to

FIGURE 3-1

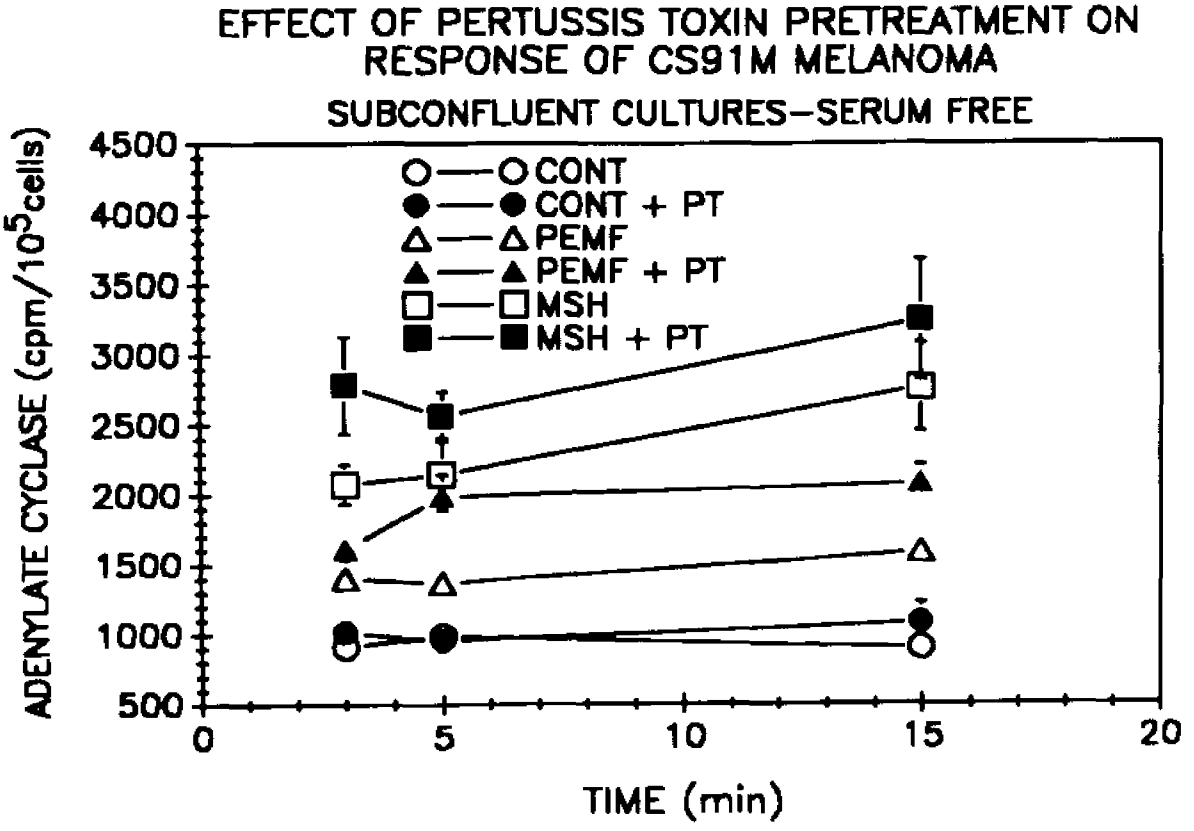

Figure 3-1. Pertussis toxin (PT) pretreatment effect on regulation of adenylate cyclase activity in response to MSH and PEMF in partially synchronized cultures. Melanoma cells were exposed to stimuli for the indicated times post PT pretreatment. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods). Cultures were preincubated with or without PT in fresh serum free media for 4 hours at 37°C.Cells were incubated in serum free HF10 containing 1 µCi/ml 8-3H-Adenine for 2 hours, then the media was replaced with media containing 200 µM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, MSH, or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

determine whether any effect would be observed using PT. In chapter 1, no PEMF effect on cyclase or PKA was observed due to presence of serum factors which perhaps desensitized the cells to PEMF stimulation. In Figure 3-3, the effect of pertussis toxin pretreatment on the PEMF and MSH response in serum free medium was observed. PEMF does not stimulate adenylate cyclase at the 5 and 15 minute time points. MSH, in contrast, increases cyclase 1.5 fold at both 5 and 15 minutes. After PT pretreatment, both the MSH and PEMF responses increase at both 5 and 15 minutes (by 70% and 46% respectively). Forskolin, a positive control, also increased cyclase after PT pretreatment.

The final question is: does the increased response seen with PT pretreatment manifest itself as an increase in tyrosinase activity? The results in figure 3-4 demonstrate that MSH and FOR at 24 hours were able to stimulate tyrosinase activity, unlike PEMF. PT pretreatment does not have any effect on any groups at 24 hours. The effects at 48 hours indicate that PEMF, MSH, and FOR all induce tyrosinase. PT pretreatment did increase the PEMF response by 60%, with no additional effect of PT seen in the MSH or FOR group. The same data presented in bar graph form (for easier comparison), in figure 3-5.

The effect of PEMF, MSH and FOR on ADP-dependent ribosylation of Gi and Gs in cell lysate preparations was studied by in vitro phosphorylation and SDS-PAGE analysis. PEMF, MSH, and FOR groups were stimulated for 15 minutes prior to cell lysate preparation. Figure 3-6 shows PT catalyzed phosphorylation of Gi was

FIGURE 3-2

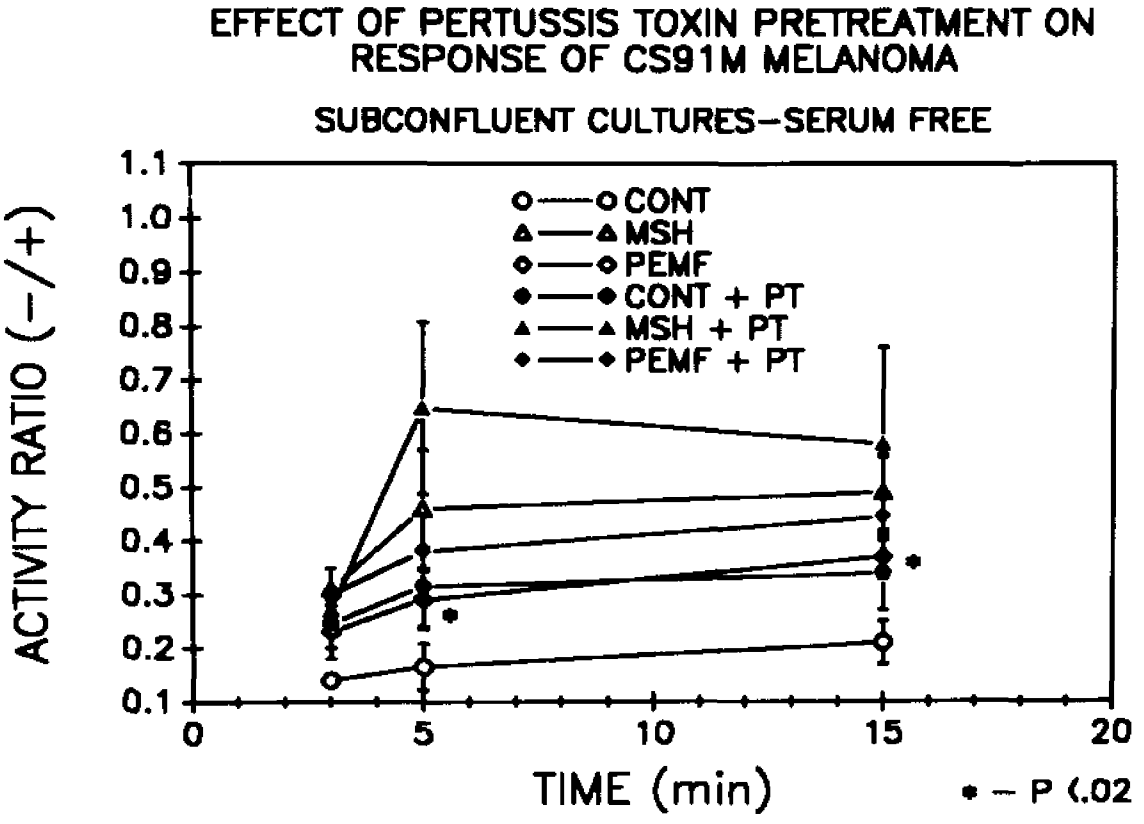

Figure 3-2. Pertussis toxin (PT) pretreatment effect on activity ratio of PKA in response to MSH and PEMF in partially synchronized cultures. Melanoma cells were exposed to stimuli for the indicated times post PT pretreatment.CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods). Cultures were preincubated with or without PT in fresh serum free media for 4 hours at 37°C. Melanoma cells were exposed to stimuli for the indicated times, washed 1X in ice cold PBS, frozen in liquid N_2, and sonicated in an ice bath 2 X 5 sec. cAMP-dependent protein kinase (PKA) was assayed using Kemptide substrate; the assay buffer contained 150 µm kemptide (Bachem), 1mM EDTA, 10mM MgAcetate, 15 µM BSA (fraction V), 125 µM ATP, and 0.25 - 1.0 µCi gamma ^{32}P-ATP in 40µl total volume plus or minus cAMP (6.25 µm) for 15 minutes at 30°C. 10 µl samples were separated by Whatman P81 phosphocellulose chromatography and counted by Cerenkov emission as described in Methods (Livesey and Martin,1988; Levin et al.,1988).

FIGURE 3-3

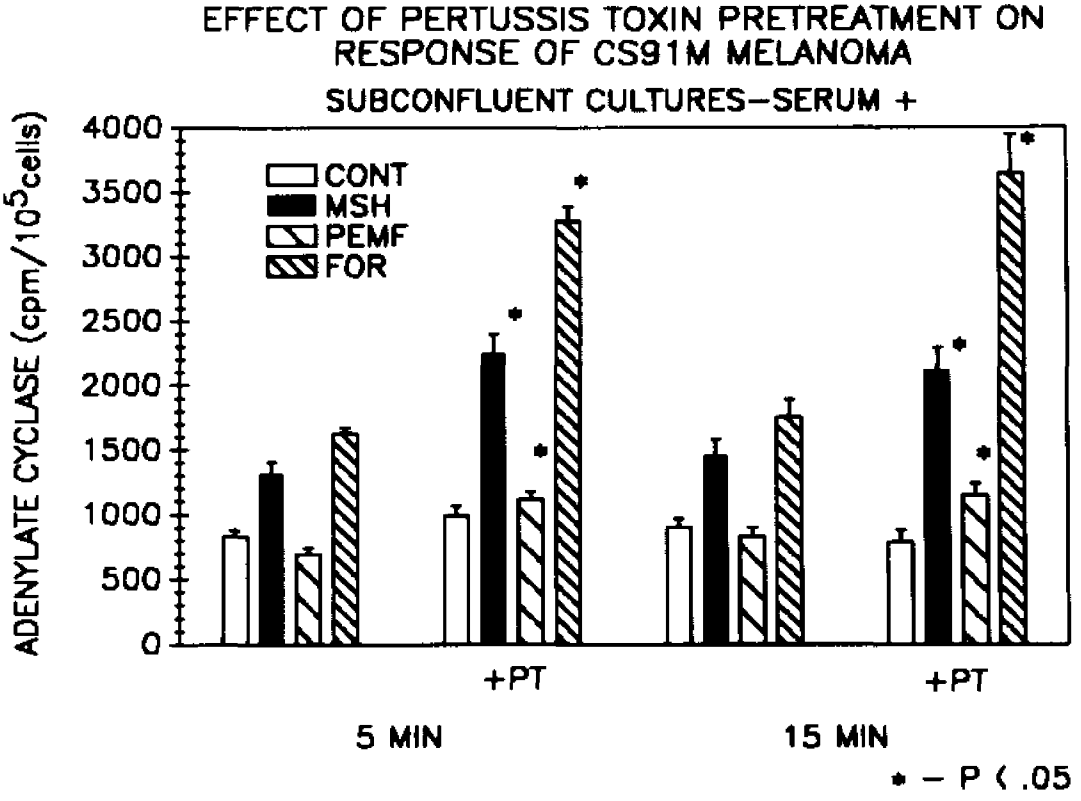

Figure 3-3. Pertussis toxin (PT) pretreatment effect on regulation of adenylate cyclase activity in response to MSH and PEMF. Melanoma cells were exposed to stimuli for the indicated times post PT pretreatment. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods); FOR, forskolin, 1 μM. Cultures were preincubated with or without PT in fresh serum free media for 4 hours at 37°C. Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, α-melanocyte stimulating hormone , forskolin, isoproterenol , or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

FIGURE 3-4

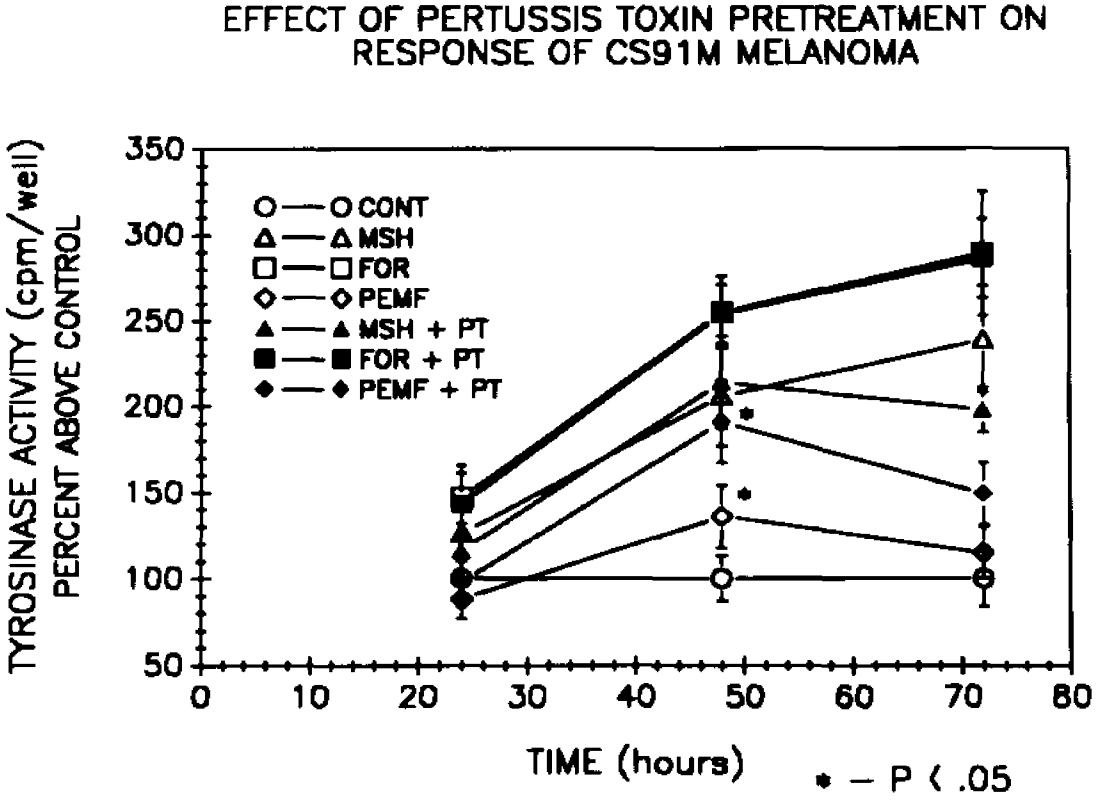

Figure 3-4. Pertussis toxin (PT) pretreatment effect on tyrosinase activity in response to MSH, PEMF, and FOR. Melanoma cells were exposed to stimuli for the indicated times post PT pretreatment. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods); FOR, forskolin, 1 μM. Cultures were preincubated with or without PT in fresh serum free media for 4 hours at 37°C. Cell cultures were incubated with HF10 growth media containing 0.3μCi/ml [^{3}H] Tyrosine; and media was collected after each 24 hour interval. Free $^3H_20$ in the culture media was separated from ^{3}H-Tyrosine by activated charcoal/Dowex 50W chromatography and quantified by liquid scintillation counting (Pomerantz, 1969).

inhibited in the MSH and PEMF samples, with no effect observed in the FOR samples (perhaps a slight increase in phosphorylation). Cholera toxin (figure 3-7), used as a positive control, was not modified under these conditions, except for a slight decrease in phosphorylation in the MSH group. The results in figure 3-8 demonstrate the same results as in figure 3-6 except the exposure time was now 5 minutes. In addition, costimulation with MSH and PEMF, and FOR and PEMF were performed. Again, MSH and PEMF inhibited Gi phosphorylation, as did MSH and PEMF costimulation. ISO also decreased Gi phosphorylation. Both the FOR and FOR plus PEMF group illustrated no effect compared to control. Suboptimal concentrations of MSH (10^{-9}M) had no effect. Pretreatment with PT demonstrated the maximal phosphorylation in this experiment.

FIGURE 3-5

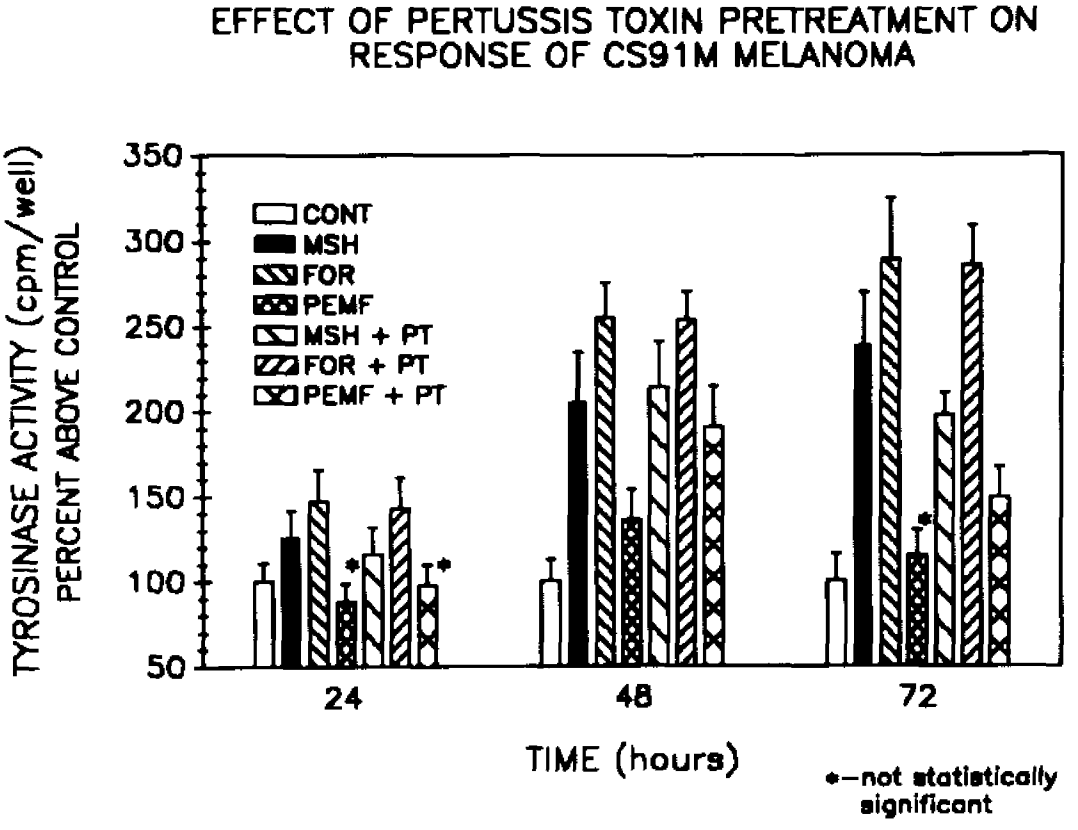

Figure 3-5. Pertussis toxin (PT) pretreatment effect on tyrosinase activity in response to MSH, PEMF, and FOR. Melanoma cells were exposed to stimuli for the indicated times post PT pretreatment. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, Pulsing electromagnetic field (as described in methods); FOR, forskolin, 1 μM. Cultures were preincubated with or without PT in fresh serum free media for 4 hours at 37°C. Cell cultures were incubated with HF10 growth media containing 0.3μCi/ml [^{3}H] Tyrosine; and media was collected after each 24 hour interval. Free $^3H_20$ in the culture media was separated from ^{3}H-Tyrosine by activated charcoal/Dowex 50W chromatography and quantified by liquid scintillation counting (Pomerantz,1969). Data is same as in figure 3-4 only presented as bar graph for direct comparison.

FIGURE 3-6

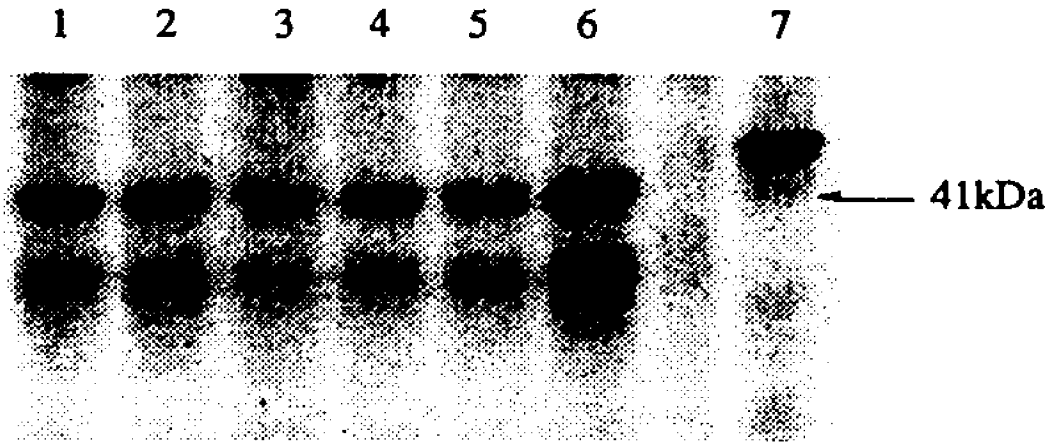

Figure 3-6. Modification of pertussis toxin catalyzed ADP-ribosylation in response to MSH, PEMF, and FOR. Lane 1, Control; Lane 2, Control; Lane 3, α-melanocyte stimulating hormone, MSH (10 nM); Lane 4, pulsing electromagnetic field, PEMF (as above); Lane 5, forskolin, FOR (1 μM) and PEMF costimulation; Lane 6, forskolin, FOR (1 μM); Lane 7, ^{14}C-Ovalbumin (Amersham). Cultures were exposed to either PEMF, MSH or FOR as described above. At the end of the timed incubations, cultures were washed 1X in ice cold PBS, scraped into buffer containing 10mM HEPES, pH 8.0, 10mM Thymidine, 1mM EDTA, 5mM DTT, and 0.2 mg/ml BSA. Cell cultures were sonicated as above (PKA assay); and aliquots containing 50 μg protein were then incubated in the above buffer containing 1 μM ^{32}P-NAD (40 Ci/mmol, New England Nuclear, Boston, MA) and 100ng/ml activated pertussis toxin (Moss et al.,1983). The samples were mixed 1:1 with 2X Laemmli sample buffer, and analyzed by SDS-PAGE on 12.5% gels (Laemmli, 1970). Autoradiography was performed with Kodak XAR5 film and Dupont Lightning Plus intensifying screens at -70°C. Dilutions of the cell lysates were analyzed for protein content by the BCA method (Pierce, Rockford ,IL). In all analyses, the same quantity of protein is loaded per well (25 μl) to allow for direct comparison of phosphorylation changes.

FIGURE 3-7

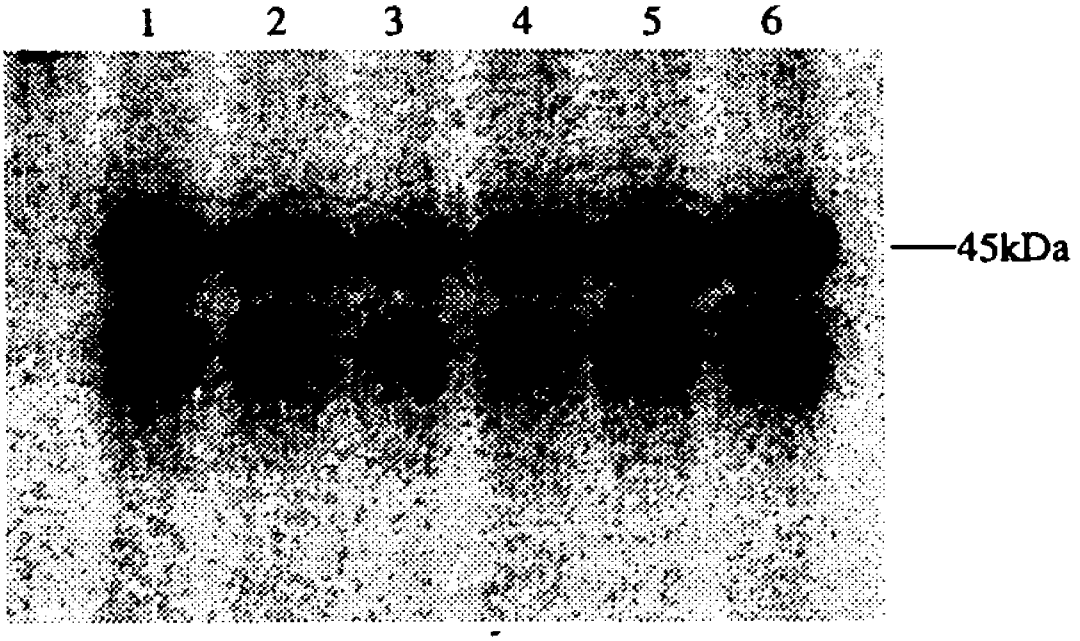

Figure 3-7. Modification of cholera toxin catalyzed ADP-ribosylation in response to MSH, PEMF, and FOR. Melanoma cells were exposed to stimuli for 15 minutes, then analysis was performed as described in figure 3-6 and methods. Lane 1, Control; Lane 2, Control; Lane 3, α-melanocyte stimulating hormone, MSH (10 nM); Lane 4, pulsing electromagnetic field, PEMF (as above); Lane 5, forskolin, FOR (1 μM) and PEMF costimulation; Lane 6, forskolin, FOR (1 μM).

FIGURE 3-8

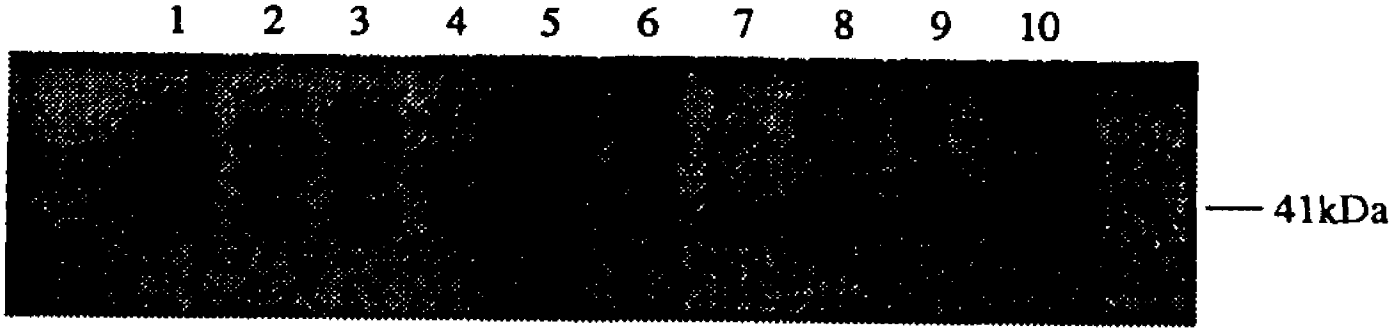

Figure 3-8. Modification of pertussis toxin catalyzed ADP-ribosylation in response to MSH, PEMF, and FOR. Melanoma cells were exposed to stimuli for 5 minutes, then analysis was performed as described in figure 3-6 and in Methods. Lane 1, Control; Lane 2, α-melanocyte stimulating hormone, MSH (10 nM); Lane 3, pulsing electromagnetic field, PEMF; Lane 4, MSH and PEMF costimulation; Lane 5, Control; Lane 6, isoproterenol, ISO (1 μM); Lane 7, forskolin, FOR (1 μM); Lane 8, FOR and PEMF costimulation; Lane 9, MSH (1 nM); Lane 10, Pertussis toxin, PT (100 μg/ml).

DISCUSSION AND CONCLUSIONS

The involvement of the inhibitory G protein, Gi, in the stimulation of melanoma cells by PEMF and MSH has been characterized in this study. The results indicate that Gi did attenuate the effects of PEMF and MSH. The preincubation time with PT was originally tested at 2, 4, and 6 hours, and the maximal response was observed at 4 hours (data not shown). This time was then adopted for the experiments in this chapter. Permeabilization was used to insure cellular incorporation in other cell types, we confirmed the uptake of PT by performing ^{32}P-NAD labeling.

In the first chapter, we demonstrated that the PEMF effect was similar to MSH under serum free conditions, as was isoproterenol (ISO) stimulation. In this study, a comparison between PT pretreated and control cultures was made with activation as previously with MSH or PEMF. Removal of the Gi tonic inhibition with PT results in increased hormone responsiveness in several cell types. Katada et al. (1979) reported on the increase in adenylate cyclase stimulation by glucagon after PT pretreatment. Extension of this work by Katada et al.(1982) illustrated this same phenomenon with isoproterenol, a ßAR agonist, and Wilson et al.(1986) reported on inhibition of αAR induced desensitization with PT pretreatment. Cerione et al.(1985) indicated a role for Gi in a reconstitution assay system, providing evidence that not only does Gi inhibition increase response to ßAR agonists but Gi is also required for maximal stimulation of cyclase (due to suppression of basal activity). Our results

demonstrated a similar response to those above. The interaction of PEMF stimulated adenylate cyclase with PT is increased 2 fold versus PEMF alone, indicating a strong interaction between Gi activity and the ability of PEMF to elicit a response. The same result was seen at the level of cAMP-dependent protein kinase (PKA), although at a lower level (≈60% increased response). The MSH response after PT pretreatment was also increased versus hormone alone, although only by 30%. This data at the signal level was partially mirrored by the results on tyrosinase activity. It was again observed that PT pretreatment increased sensitivity to PEMF exposure. However, one must exercise caution in this series, as the long term effects of PT exposure on cells is not well documented (Reisine, 1990).

In the case of serum containing cultures, previous work in chapter 1 and herein demonstrate no adenylate cyclase response in PEMF treated cultures compared to basal activity. It is quite interesting that following PT pretreatment, the cultures become sensitive once more to PEMF and cyclase is stimulated. Two interpretations can be proposed for this result. The first is the association between ISO and PEMF stimulation is coincidental. Removal of the Gi inhibition should not cause ISO stimulation to reappear. Preliminary results demonstrated this to be the case: there was no ISO response in melanoma cells in serum containing cultures and this lack of response was not affected by PT pretreatment. Therefore, if there was no functional ß-receptor present on these cells, it would be reasonable to assume that the PEMF stimulation after PT pretreatment was either directly on the G protein, Gs, or involves another receptor type, e.g. the MSH receptor. The second

explanation could be other pathways are involved in the PEMF stimulation of adenylate cyclase. PT pretreatment of neuronal cells (N X G hybrids) removes the inhibition of calcium currents (Hescheler et al., 1987), and this increase of calcium can regulate adenylate cyclase secondarily (Ross et al., 1989). Evidence for a role for calcium in electric field stimulation has been presented by Ozawa et al. (1989), but cAMP dependence was not demonstrated. More work is required on the role of signal pathway crosstalk in PEMF stimulation.

Results reported previously by Cain et al.(1987), and Brighton and Townsend (1988) link the PTH receptor to the PEMF effect. Their results showed inhibition of PTH receptor stimulated adenylate cyclase and cAMP formation respectively, and this effect was observed after both short term and long term exposure. Brighton reported direct stimulation of cAMP by 60% in comparison to control (the magnitude of this response was similar to that reported here and in chapter 1). Cain et al. (1987) went further to assert no role of G proteins in their system, based on the finding that the response to forskolin was not affected. Forskolin, at the concentration (1 - 100 μM) used in their studies, activates cyclase directly by promoting interaction with Gs, the stimulatory G protein (Seamon and Daly,1987). Since PEMF did not affect the forskolin response, it can be interpreted that cyclase was not directly affected by PEMF, or assumed that Gs was not involved, yet no provision of data on Gi was presented. In this respect, this study demonstrated a role for Gi and leaves the question of a target for PEMF stimulation still unclear.

CHAPTER 4

SENSITIVITY TO ELECTROMAGNETIC FIELDS IS RELATED TO CELLULAR ADRENERGIC RESPONSIVENESS

INTRODUCTION

Pulsing electromagnetic field (PEMF) signals induce differentiation in the murine melanoma cell line Cloudman S91. Previous work suggests that the cAMP second messenger system is involved in transduction of the PEMF stimulus. Recently, adrenergic receptors have also been implicated in PEMF sensitivity in cell systems such as melanoma (Ryaby et al.,1989), osteoblasts (Luben,1989), and the pineal gland (Rudolph et al., 1988; Reiter et al., 1990; Wilson et al., 1990). Luben has demonstrated a slight inhibition of ß-adrenergic agonist binding by PEMF in osteoblasts, and ß-adrenergic agonist induced desensitization has been described in melanoma cells (Ryaby et al.,1990). Electromagnetic fields (EMF) are capable of altering serotonin metabolism and melatonin secretion (Lerchl et al., 1990), and this effect is thought to be under control of the ß-adrenergic system (Pangerl et al., 1990; Guerrero et al., 1990).

The results in chapter 1 demonstrated an association between the ability of isoproterenol to stimulate adenylate cyclase and the response to pulsing electromagnetic fields. In this chapter, we employ both cell lines to delineate the role of adrenergic sensitivity in the PEMF response. The first, the Cloudman melanoma system, is slightly sensitive to ß-adrenergic stimulation. The characteristics of this line have been described in previous chapters. The second system is the MC3T3 osteoblast cell line, which possesses high sensitivity to ß-adrenergic stimuli, and

provide a direct comparison with the melanoma cells due to the presence of parathyroid hormone stimulated adenylate cyclase activity. The important properties of this osteoblastic cell line include: the capacity to differentiate in culture (Kodama et al., 1981); form matrix and calcify in vitro (Sudo et al., 1983); respond to osteotropic hormones and growth factors (Kumegawa et al., 1987; Noda et al., 1986); and synthesize cytokines in response to bone active agents (Horowitz et al., 1989).

In this study, evidence is presented that adrenergic receptors (both αAR and ßAR) may regulate sensitivity to PEMF; and this may involve both homologous and heterologous desensitization mechanisms.

MATERIALS AND METHODS

ELECTROMAGNETIC EXPOSURE APPARATUS

The pulsing electromagnetic field (PEMF) waveforms used in these studies were generated with an exposure system consisting of two parallel circular Helmholtz-aiding coils (18 cm or 10 cm diameter) 8 cm apart or 4.5 cm apart, respectively (Figure 1-2). The waveform was monitored with a coil probe amplified in a Tektronix Model 5441 Storage Oscilloscope, as described previously (McLeod et al.,1985). The probe was designed to provide for a 10 fold amplification of the induced voltage when placed parallel to the plane of the coils. The EMF employed was a 5 msec burst consisting of 21 pulses having 200 μsec main and 20 μsec opposite polarity repeating at 15 hz. dB/dt in the main polarity was 0.1 G/μsec corresponding to an induced electric field of 1mV/cm. This corresponded to an average induced current in the culture dish of approximately 5μA/cm^2 (figure 1-1) (McLeod et al., 1984).. All experiments reported herein were performed with the tissue culture dishes or plates oriented parallel to the plane of the coils.

TYROSINASE ASSAY

Cloudman S91 cells (American Type Culture Collection CCL 53.1 clone M-3) were grown to confluency in Ham's F10 medium (Gibco, or Mediatech) supplemented with 15% horse serum / 2.5% fetal calf serum (Hyclone USA) and

glutamine (2mM [final]) (56). No antibiotics were used during either maintenance or experimental culture. Cells were subcultured by incubating briefly with 1mM EDTA in calcium and magnesium-free Hanks solution and diluted 1:5 into 75 or 150 cm flasks according to need. For tyrosinase experiments, cells were seeded at a density of 75,000 (±1%) per 35 mm culture dish (Nunc) in 2 ml of culture medium. Cells were counted in a Coulter ZM cell counter calibrated with 14.8 μm latex particles using a 100 μm aperture and a threshold setting eliminating cells smaller than 8.0μm. Tyrosinase experiments were conducted in glass culture jars (resin reaction vessels) whose ground glass stoppers were sealed with vacuum grease (Figure 1-). An atmosphere of 20% 0_2, 75% N, and 5% $C0_2$ at saturated humidity in a warm room at 37°C or a 10 cubic foot incubator under the same conditions were used for the experiments. Control cultures grown under these conditions showed growth and tyrosinase response to MSH and insulin identical to that reported in the literature (38,56). Tyrosinase relative activity (monophenol monooxygenase; monophenol, dihydroxy-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) (cpm released per 24 hours from L-[3,5-^{3}H] tyrosine, Amersham) was measured by the method of Pomerantz (57). Cells were grown under control conditions for 24 hours, then incubated with serum free media for 24 hours with or without isoproterenol (ISO, Sigma). HF10 growth media was then added (+/- ISO, as above) containing 0.3μCi/ml [^{3}H] Tyrosine. At the end of the 24 hour interval, the media was removed and free $^3H_20$ is separated from ^{3}H-Tyrosine by activated charcoal/Dowex 50W chromatography and counted by LSC. Data was presented as cpm $^3H_20$ per culture.

ADENYLATE CYCLASE ASSAY

Adenylate cyclase activity was performed according to the method of Salomon (1979). Confluent CS91 melanoma cultures (see above) were subcultured into 35 mm petri dishes($9.6cm^2$), or 4 and 24 well plates (16 mm diameter,$2cm^2$) at a density of 20,000 cells/cm^2 (determined by Coulter Counter as above) and grown under control conditions for a minimum of 24 hours before the start of an experiment. For comparison of serum-free and normal growth conditions, cells were incubated for an additional 24 hours in fresh medium respectively. Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, α-melanocyte stimulating hormone , forskolin, isoproterenol , or under control conditions for the times indicated. The PEMF and MSH costimulated group was achieved by placing cultures first in the PEMF apparatus then immediately adding the MSH stock. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography. For analysis of the ^{3}H-cAMP formation, cultures were thawed at room temperature and transferred to 12 X 75mm glass tubes containing 100 μl of nucleotide carrier solution (5mM [final] of Adenine, Adenosine, AMP, ADP, ATP, and cAMP (Sigma) ; to prevent non-specific binding of ^{3}H-cAMP or ^{14}C-cAMP), 50 μl of ^{14}C-cAMP (Amersham) standard (3000 cpm/tube), and then 50 μl of 4N KOH was added and the mixture

is vortexed. Samples were applied to the column by inversion and 4 ml volumes were collected from the alumina columns in scintillation vials. Fourteen ml of Hydrofluor was added and counted in a LKB-Wallac scintillation counter with internal standard. Data was collected directly onto disk with backup hardcopy printout. The data was edited with Epsilon text editor, and determination of ^{3}H-cAMP performed with PC-Matlab (The Mathworks). Data was expressed as cpm/10^5 cells or cpm/culture.

cAMP DEPENDENT PROTEIN KINASE ASSAY

Confluent CS91 melanoma cultures (see above) were subcultured into 35 mm petri dishes(9.6cm^2), at a density of 20,000 cells/cm^2 (determined by Coulter Counter as above) and grown under control conditions for a minimum of 24 hours before the start of an experiment. For comparison of serum-free and normal growth conditions cells were incubated for an additional 24 hours in fresh medium, respectively. Cultures were then exposed to either PEMF, MSH or other agonists as described. At the end of the timed incubations, cultures are washed 1X in ice cold PBS, then frozen rapidly by immersion into liquid N_2. Cultures were then prepared for analysis by sonication in an ice bath for two 5 second intervals at a power setting of 3 using a microtip (Heat Systems-Ultrasonics Model W385, Farmingdale, NY). cAMP-dependent protein kinase (PKA) was assayed by a combination of the methods of Livesey and Martin (1988) and Levin et al (1988). Kemptide (Bachem) was used as the phosphoacceptor substrate and gamma ^{32}P-ATP (Amersham) as the phosphate

donor. The assay buffer contained 150 μm kemptide (Bachem), 1mM EDTA, 10mM MgAcetate, 15 μM BSA (fraction V), 125 μM ATP, and 0.25 - 1.0 μCi gamma ^{32}P-ATP. The incubations were performed in 40μl total volume in assay plates (Falcon, polystyrene), containing either plus or minus cAMP (6.25 μm) for 15 minutes at 30°C. The assay plates were floated in the water bath with a styrofoam frame. The reaction was stopped by the addition of 10 μl of a 1mM EDTA solution (Mg2+ is a required cofactor for kinase activity). 10 μl samples were then spotted onto Whatman P81 phosphocellulose paper, air dried, then washed with 75mM phosphoric acid 4X for 5 minutes, followed by one 5 minute wash with 95% EtOH, and dried with a infrared lamp. Samples were subsequently counted in 15 ml of H_2O in a LKB Wallac scintillation counter . Soluble protein was measured by the Coomassie blue method of Bradford (1979).

STATISTICAL ANALYSIS

In the experiments reported here a minimum of three experiments were performed with a minimum of four culture wells/point/experiment (5 culture wells/point/experiment when using 35 mm dishes). The mean and standard deviation were evaluated for each sample group. Paired two-tailed Student's t test was employed to test the percentage differences for statistical significance. Significance was accepted at $P \leq 0.05$.

RESULTS

Isoproterenol pretreatment of melanoma cells demonstrated a decrease in PEMF stimulation of adenylate cyclase compared to control. Figure 4-1 shows PEMF increased adenylate cyclase activity after 5 minutes exposure from 243 cpm/10^5 cells in the control group to 401 cpm/10^5 cells, an increase of 65 %. This level of stimulation is comparable to that reported in chapter 1. ISO pretreatment for 3 hours prior to PEMF exposure demonstrated no response compared to control. However, after 6 hours of ISO pretreatment, the PEMF stimulated adenylate cyclase increase was reduced to 19% compared to control. This led us to question whether PEMF stimulation required either ß or α adrenergic receptor (ßAR, αAR) to see a stimulatory effect of PEMF.

The data in figure 4-2 illustrates the ability of the stimulation regimen to regulate its own responsiveness. In this series of experiments, short term pretreatment of cells was performed for 2 hours with either MSH, PEMF, or ISO. Cells were then assayed for their response to a subsequent 5 minute stimulation. In the control group (no preexposure), MSH stimulated adenylate cyclase over 6 fold compared to control (3145 vs. 500 cpm). PEMF and ISO both stimulate adenylate cyclase by the same amount, approximately 70%. Pretreatment of cultures for 2 hours with MSH decreased MSH stimulation by 51% (from 6.1 to 3 fold), whereas no effect was observed on either PEMF or ISO stimulation. PEMF pretreatment decreased the basal cyclase by 20%, but had no effect on MSH stimulation. In fact,

FIGURE 4-1

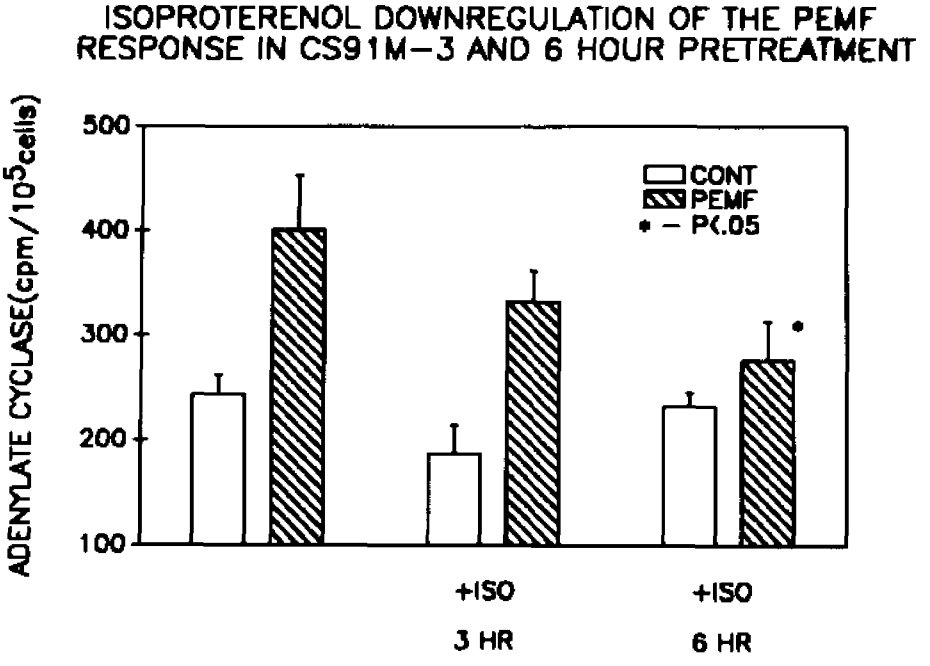

Figure 4-1. Isoproterenol (ISO) pretreatment effect on adenylate cyclase activity in response to PEMF in serum free media. Melanoma cells were exposed to PEMF for 5 minutes post ISO pretreatment. CONT, Control; PEMF, Pulsing electromagnetic field (as described in methods); ISO, isoproterenol, 1 μM. Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

FIGURE 4-2

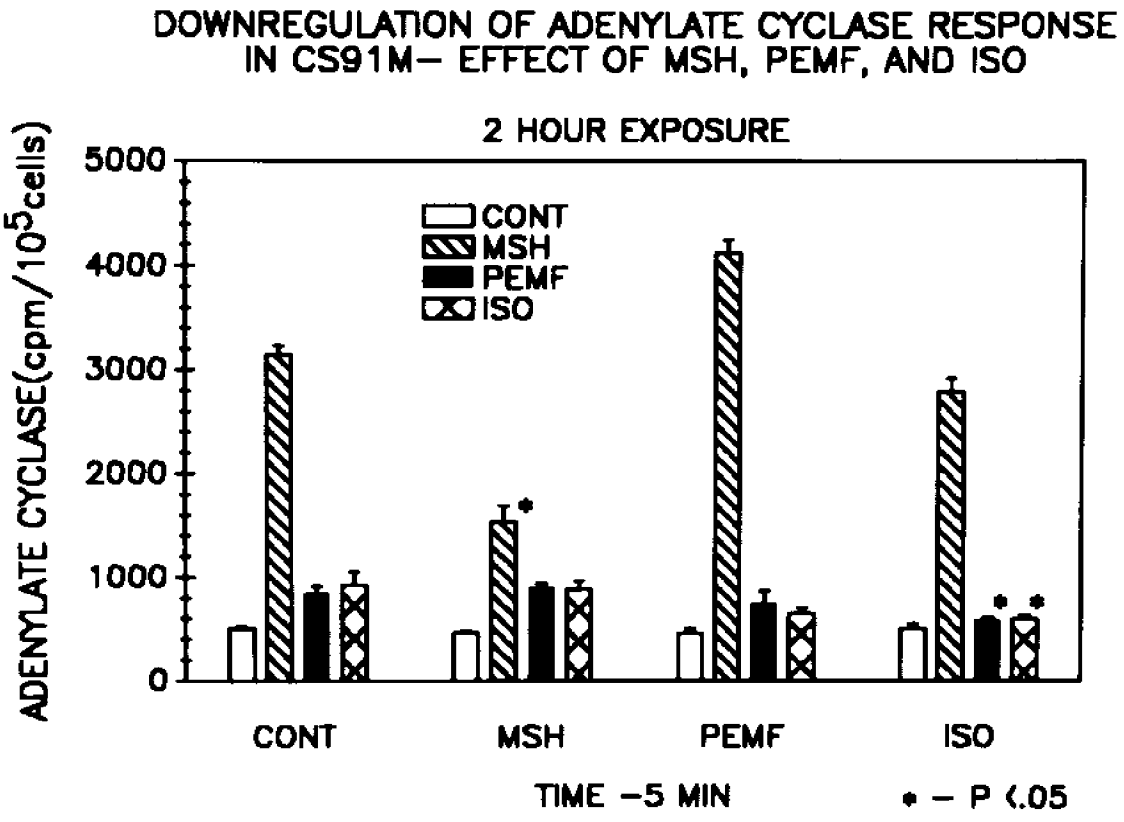

Figure 4-2. Homologous/Heterologous desensitization experiment. Effect of 2 hour pretreatment with α-melanocyte stimulating hormone (MSH), pulsing electromagnetic field (PEMF), or isoproterenol (ISO) on adenylate cyclase activity in response to MSH, PEMF, and ISO in serum free media. Melanoma cells were exposed to MSH, PEMF, and ISO for 5 minutes post 2 hour stimuli pretreatment. CONT, Control; MSH, 10 nM; PEMF, as above; ISO, 1 μM. Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, MSH, ISO or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

MSH stimulation increased 30% in the PEMF pretreatment compared to MSH alone. The response to PEMF was not affected, whereas the ISO response was decreased to a 43% stimulus above control. The most significant results were obtained in the ISO pretreatment group, in which the PEMF and ISO stimulation of cyclase decreased to 30% compared to control, which effectively reduced the stimulation by 60% in comparison to the control group (no pretreatment). No effect of ISO pretreatment was observed on MSH activation of cyclase. These same results, without the MSH group, are presented in higher resolution in figure 4-3.

The time required to observe submaximal desensitization (above) led us to question whether epinephrine (EPI) would exhibit similar results. Figure 4-4 demonstrated the effect of a 24 hour EPI and ISO pretreatment on PEMF and MSH stimulated adenylate cyclase. Following 5 minutes of exposure in the control group, both MSH and PEMF demonstrated strong stimulation of adenylate cyclase, with values of 2.9 and 2.4 fold, respectively. Note that both EPI and ISO pretreatment decreased the response to subsequent stimulation by a 5 minute PEMF exposure. The decrease was 53% of maximal stimulation for the EPI group, and 73% of maximal for the ISO group. The MSH response was not affected by either EPI or ISO pretreatment.

A series of experiments were run at 72 hours to provide for maximal heterologous desensitization. The results from 72 hours of ISO pretreatment are shown own in figure 4-5. Once again,both PEMF and MSH stimulated adenylate cyclase by 2.2 and 2.7 fold, respectively. ISO pretreatment totally inhibited the PEMF

FIGURE 4-3

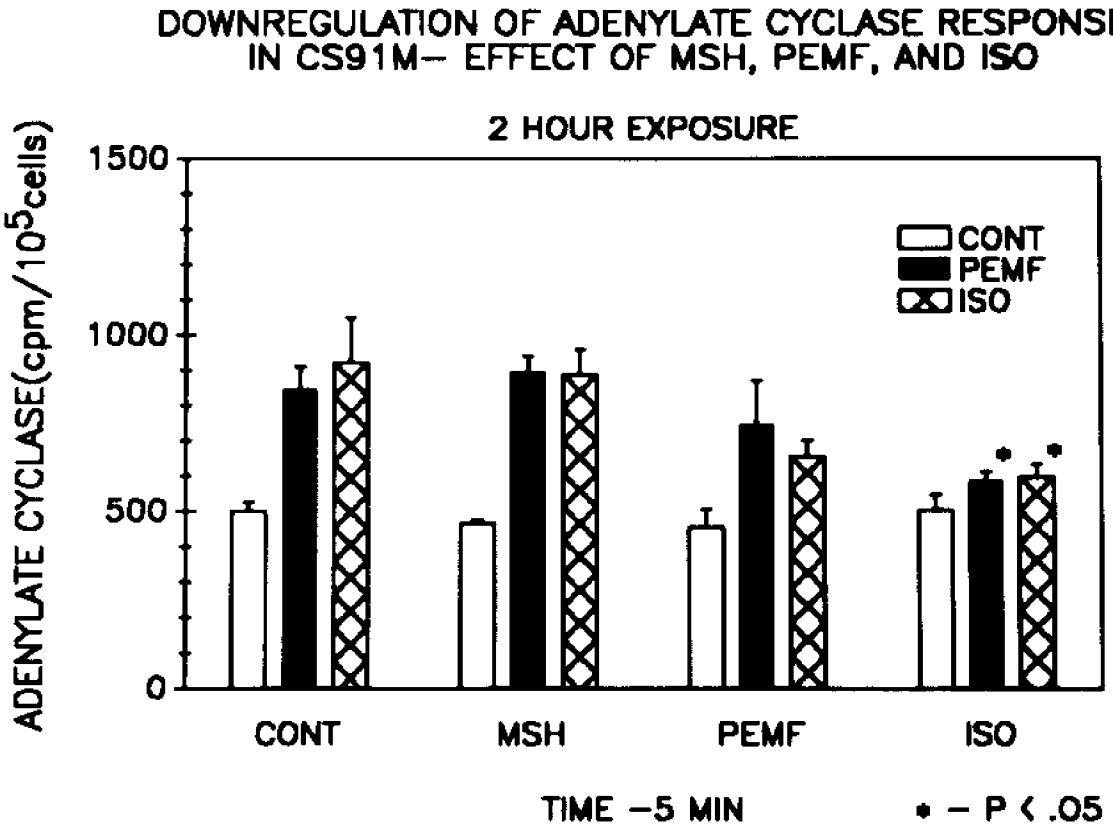

Figure 4-3. Homologous/Heterologous desensitization experiment. Stimuli pretreatment effect on adenylate cyclase activity in response to MSH, PEMF, and ISO in partially synchronized cultures. Melanoma cells were exposed to MSH, PEMF, and ISO for 5 minutes post 2 hour stimuli pretreatment. CONT, Control; MSH, 10 nM; PEMF, as above; ISO, 1 μM. Same data from figure 4-2 presented in expanded scale.

FIGURE 4-4

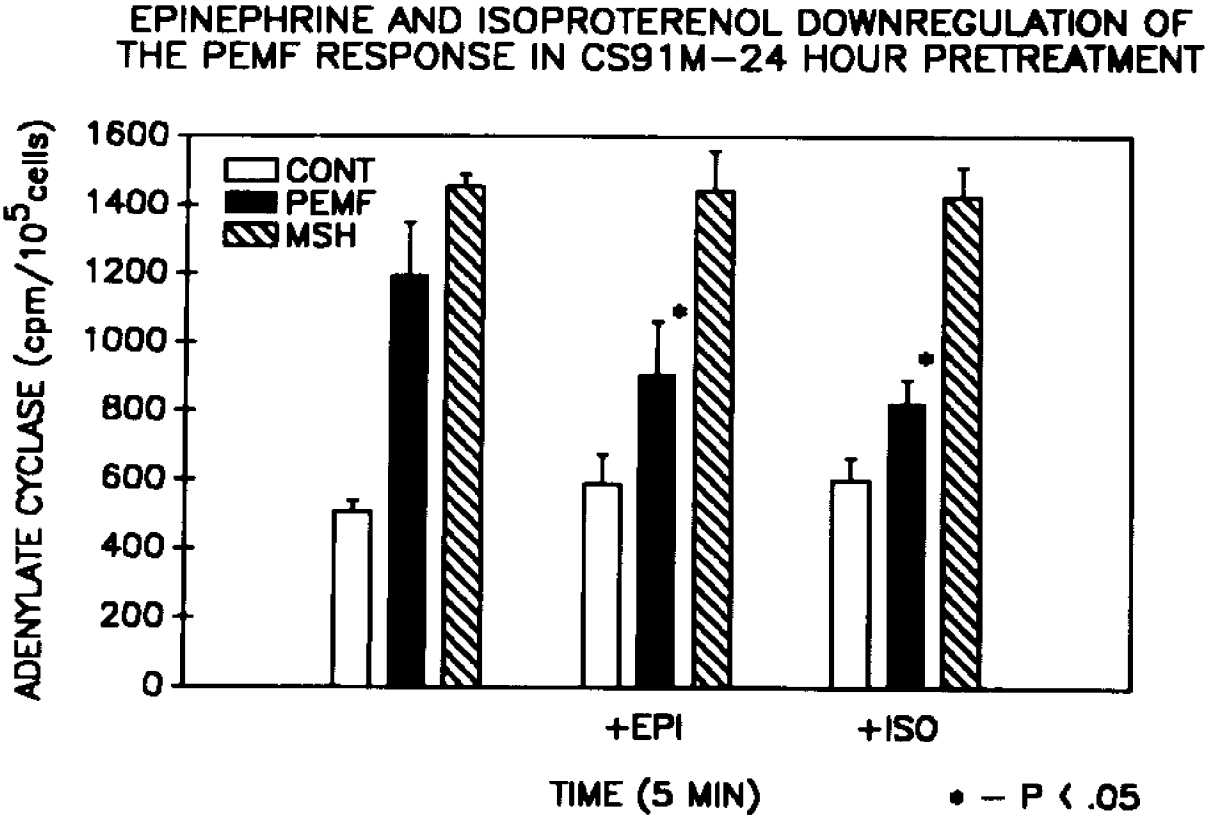

Figure 4-4. 24 hour isoproterenol (ISO) and epinephrine (EPI) pretreatment effect on adenylate cyclase activity in response to MSH and PEMF in partially synchronized cultures. Melanoma cells were exposed to PEMF and MSH for 5 minutes post 24 hour ISO and EPI pretreatment. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, pulsing electromagnetic field (as above). Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, MSH or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

FIGURE 4-5

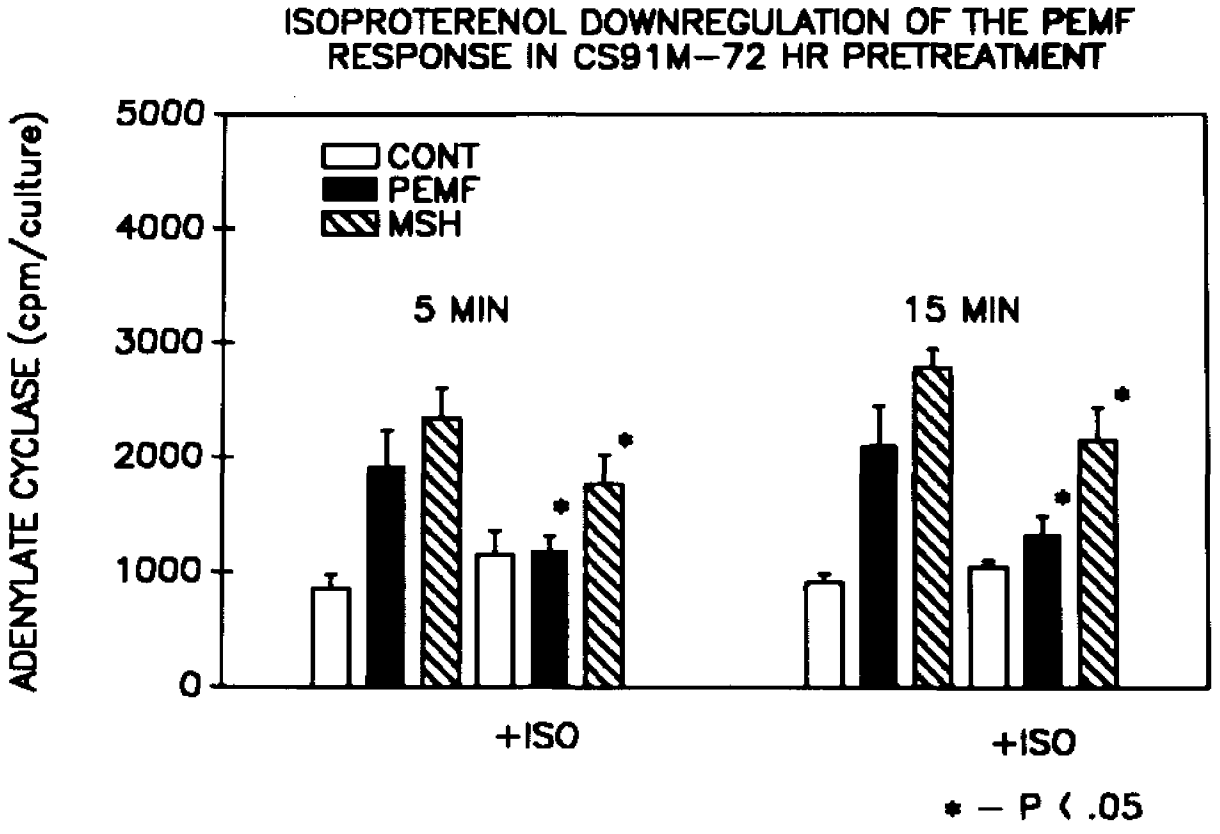

Figure 4-5. 72 hour isoproterenol (ISO) pretreatment effect on adenylate cyclase activity in response to MSH and PEMF in partially synchronized cultures. Melanoma cells were exposed to MSH and PEMF for 5 and 15 minutes post ISO pretreatment. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, pulsing electromagnetic field (as above). Cells were incubated in serum free HF10 containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, MSH or under control conditions for the times indicated. The incubation is terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

stimulation at five minutes (1177 compared to 1148 cpm) and decreased it by 77% at 15 minutes. Of great interest, ISO pretreatment also inhibited the MSH stimulation by 70% at 5 minutes and 50% at 15 minutes. These results indicated that ISO is capable of heterologous desensitization of the MSH response.

Experiments were also performed to address regulation at the level of tyrosinase activity. ISO pretreatment was performed for 48 hours and The results of ISO pretreatment are shown in figure 4-6. Both the MSH and PEMF stimulation of tyrosinase was decreased after ISO pretreatment. The MSH response demonstrated a 2.5 fold stimulation of tyrosinase and ISO pretreatment decreased this to 2 fold, or 34%. PEMF stimulation was diminished even further, from 68% to 24% above control (a 65% decline). The response to FOR, a positive control, was not affected by ISO pretreatment. The final experiments were performed to test the universality of this ISO mediated PEMF response in another cellular model system. The response of MC3T3 osteoblasts to ISO pretreatment was compared to the parathyroid hormone (PTH) stimulation of adenylate cyclase. In Figure 4-7, a decrease in the ISO and PTH response is seen with ISO pretreatment. The ISO desensitization was large, with ISO in the control group (figure 4-8; no pretreatment) demonstrating a 43 fold stimulation at 3 minutes, 56 fold at 5 minutes, and 61 fold at 15 minutes. Pretreatment with ISO (homologous desensitization) for 72 hours decreased this stimulation to 1.9, 1.9, and 1.5 fold respectively. ISO pretreatment decreased PTH response by an mean of 40% compared to PTH alone. PEMF does not stimulate cyclase in this cell line, therefore no effect of ISO pretreatment is observed.

FIGURE 4-6

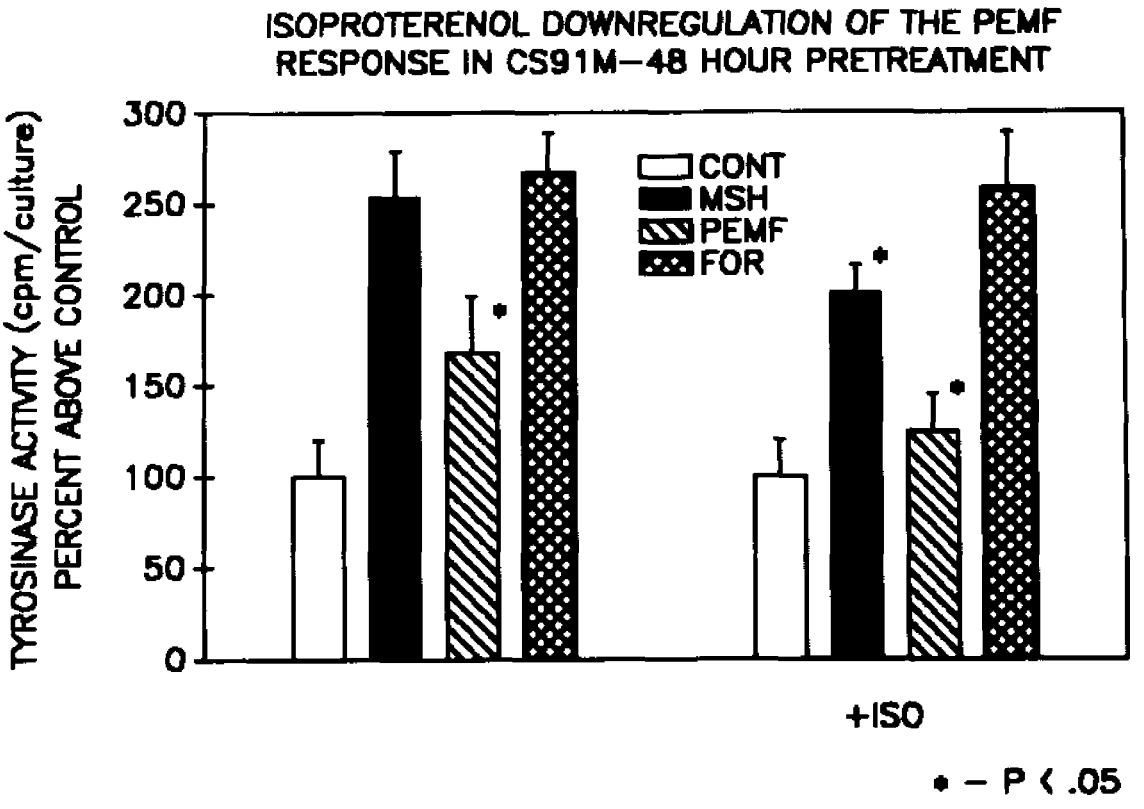

Figure 4-6. 48 hour isoproterenol (ISO) pretreatment effect on tyrosinase activity in response to MSH, PEMF, and FOR. Melanoma cells were exposed to stimuli for 24 hours coincident with ISO treatment. CONT, Control; MSH, α-melanocyte stimulating hormone, 10 nM; PEMF, pulsing electromagnetic field (as above), FOR, forskolin, 1 μM; ISO, isoproterenol, 1 μM. Cultures were incubated with HF10 growth media containing 0.3μCi/ml [^{3}H] Tyrosine; and media was collected after each 24 hour interval. Free ^3H_2O in the culture media was separated from ^{3}H-Tyrosine by activated charcoal/Dowex 50W chromatography and quantified by LSC (Pomerantz,1969).

FIGURE 4-7

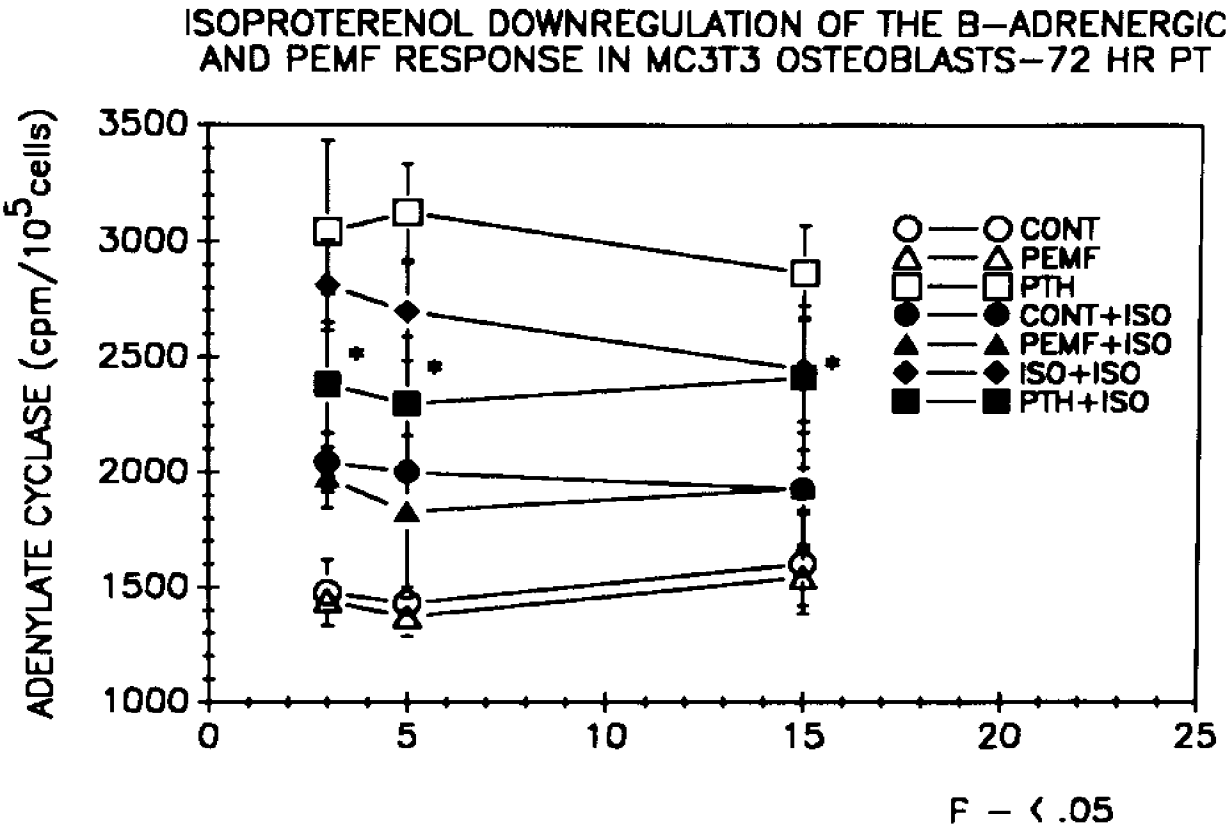

Figure 4-7. 72 hour Isoproterenol (ISO) pretreatment effect on adenylate cyclase activity in response to PTH, PEMF, and ISO in partially synchronized cultures. MC3T3 osteoblast cells were exposed to PTH, PEMF, and ISO for 3, 5 and 15 minutes post ISO pretreatment. CONT, Control; PTH, parathyroid hormone, 10 nM; PEMF, pulsing electromagnetic field (as above); ISO, isoproterenol, 1μM. Cells were incubated in serum free αMEM containing 1 μCi/ml 8-3H-Adenine (Amersham) for 2 hours, then the media was replaced with media containing 200 μM isobutylmethylxanthine (IBMX) for 20 minutes. Cells were then exposed to either PEMF, PTH, ISO or under control conditions for the times indicated. The incubation was terminated by the addition of 0.5 ml of 20% TCA and the plates were then frozen for subsequent Dowex/Alumina chromatography as described in Methods (Salomon, 1979).

FIGURE 4-8

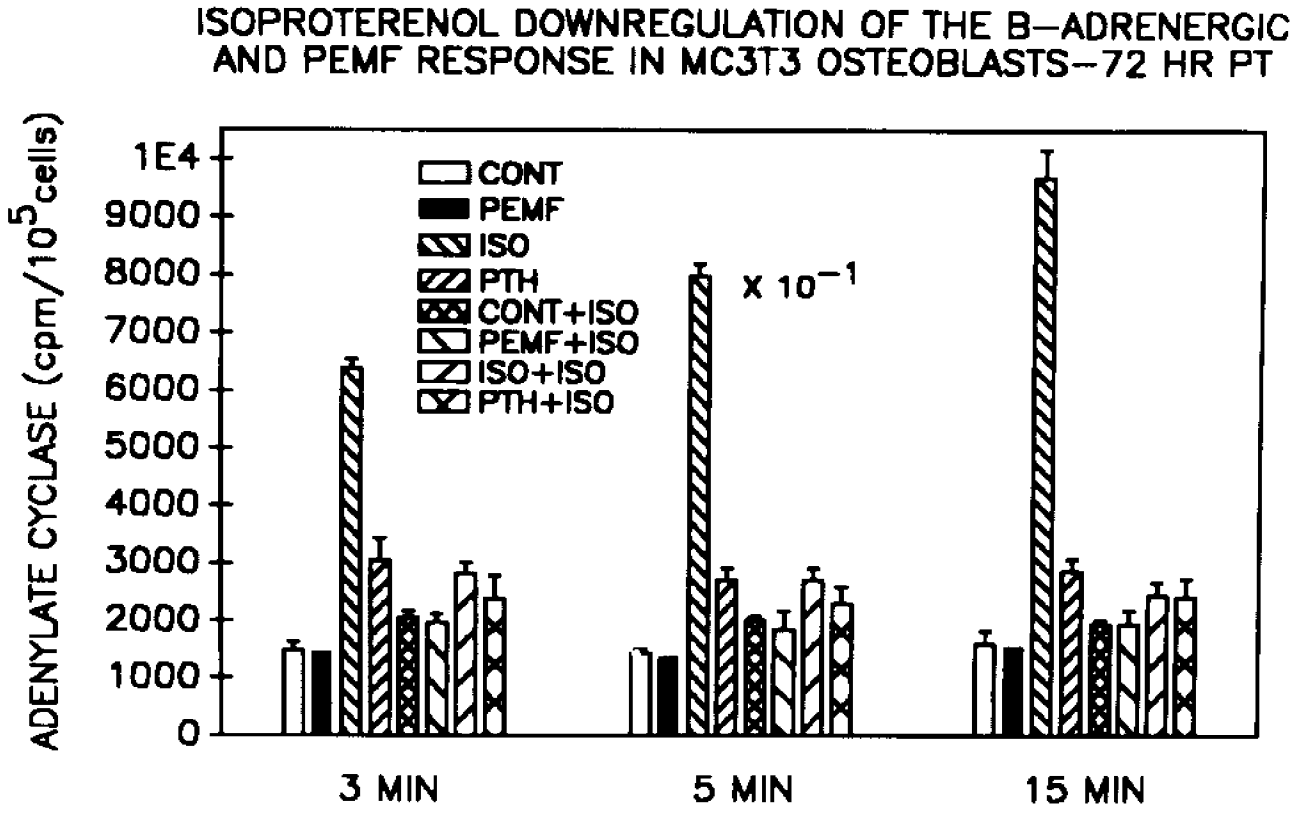

Figure 4-8. Isoproterenol (ISO) pretreatment (72 hour) effect on adenylate cyclase activity in response to PTH, PEMF, and ISO in serum free media. MC3T3 osteoblast cells were exposed to PTH, PEMF, and ISO for 3, 5 and 15 minutes post ISO pretreatment. CONT, Control; PTH, 10 nM; PEMF, as above; ISO, 1 μM. Same data from figure 4-7 presented in expanded scale to illustrate ISO results.

DISCUSSION AND CONCLUSION

The major question addressed by this study is which specific receptor system linked to adenylate cyclase was responsible for the mediation of PEMF sensitivity. The results in chapter 1 demonstrated that the ability of PEMF to directly stimulate adenylate cyclase (i.e., without additional agonist or hormone) correlated with the cells' response to ISO. In this chapter we further characterized this response by employing the homologous and heterologous desensitization mechanisms as tools to probe the possible receptor specificity of PEMF responsiveness.

The mechanism of homologous desensitization has been extensively studied by Lefkowitz and collaborators (Hausdorf et al., 1990). The initial event in desensitization is a decreased adenylate cyclase response to continuous agonist exposure. Dependent upon the receptor level for agonist, this diminished response can either begin to occur within minutes of exposure to agonist, or take several hours or even days. This long term desensitization can also be elicited by cross reactivity of agonists for binding to specific receptors (e.g., binding of epinephrine to both α and ß-AR, Levitzki, 1988). The other means of eliciting long term desensitization is through the heterologous mechanism, in which the loss of response to one agonist leads to the loss of response to other stimuli, including hormones (Bates et al., 1991; Murphy and Majewski, 1990). In this study, our results suggest an interplay between both homologous and heterologous desensitization mechanisms.

The short term results with ISO pretreatment demonstrated a requirement for active ßAR to obtain a PEMF response. The inability of MSH or even PEMF pretreatment to decrease the subsequent response to PEMF is striking, particularly when viewed (figure 4-2) within the framework of homologous desensitization. MSH pretreatment for 2 hours decreased the subsequent MSH response by more than 50%, as did ISO pretreatment/ISO response, suggestive of a homologous desensitization mechanism. PEMF pretreatment increased MSH response with little effect on ISO or PEMF itself. The only pretreatment regimen which demonstrated this cross- specificity with PEMF was the ISO group. This result, and the required time for heterologous desensitization to occur, leads us to believe that the PEMF interaction in the short term is linked to ßAR activity. However, the magnitude of the decrease does not drive the response down to 0% stimulation. The possibility therefore remains open to involvement of other receptor types or signal transduction pathways.

The results at 24 hours demonstrated an interplay between EPI and ISO, suggesting that αAR may also be involved. However, since EPI does bind to the ßAR (with lower affinity than ISO), the EPI effect may also be to downregulate the ßAR. Response to MSH is not affected by either EPI or ISO, indicating that 24 hours may not be sufficient time to observe heterologous desensitization in this system. In addition, this result implies that a functional response to MSH does not necessarily correlated with a response to PEMF.

Heterologous desensitization was observed in the experiments performed for 72

hours, again demonstrating the loss of PEMF responsiveness following ISO pretreatment. Note that the response to MSH is diminished by an average of 60% following this long term pretreatment. The decrease of PEMF response to almost the same extent as the ISO response was consistent with the continuing role of the ßAR in PEMF responsiveness. The tyrosinase experiment was performed at 48 hours, as this was the time point where the small MSH effect MSH had been observed in chapter 1. Pretreatment with ISO decreases the PEMF and MSH response at the tyrosinase level, corresponding well with the data on adenylate cyclase activity. FOR, an activator of cyclase independent of receptor stimulation , was not affected by ISO pretreatment. This result is to be expected because both homologous and heterologous desensitization operates at the receptor level, not at the level of adenylate cyclase (Levitzki, 1988). Our prediction from these studies would be if a cell system exhibited responsiveness to ßAR stimuli, then the system would respond to PEMF. The results with the MC3T3 osteoblastic cell line indicated this not to be the case. The results with ISO pretreatment in this series demonstrated desensitization of both the ISO and PTH response (homologous and heterologous, respectively). We observed no response at all with the PEMF signal, suggesting that functional ßAR is not the only prerequisite for PEMF sensitivity. A positive response to adrenergic stimuli does not per se, therefore, predict a positive response to an electromagnetic stimulus. Perhaps our results are dependent on the weak sensitivity of melanoma cells to ßAR stimuli, compared to the high degree of sensitivity observed in the osteoblastic cells. Or, there could be a difference in ßAR subtype in

the osteoblastic cells (β_1AR versus β_2AR) compared to the melanoma cells (as reported in other cell types; Caron et al., 1987). Further work is required to determine the feasibility of this hypothesis.

The first report on desensitization in PEMF studies was by Luben et al. (1982) who demonstrated that PEMF exposure could decrease the PTH response in bone cells. Farndale and Murray (1986) were the first to propose the analogy between heterologous desensitization and the observed effects with PEMF. Their studies established a decrease in cAMP levels with long term exposure of 7 days, and also an inhibition of PGE_2 stimulated cAMP elevation. Cain and Luben (1985) proposed a putative uncoupling of receptor to G protein to explain the inhibition of PTH stimulation, based on studies suggesting PEMF exposure did not affect equilibrium binding of PTH to the PTH receptor. However, the authors stated that equilibrium binding would not necessarily reflect dynamic interactions between PEMF and the PTH receptor.

Recent work by Luben (1989, 1990) in conjunction with the work presented in this chapter, provide a general framework for interactions of PEMF on the receptor-adenylate cyclase system. Luben has reported on the ability of PEMF to decrease the binding of ICYP (Iodocyanopindolol), a βAR ligand, by 50% after exposure to PEMF. Furthermore, binding of monoclonal antibodies to the PTH receptor were inhibited by PEMF in the G protein interaction site region. A homology of over 70% exists between the PTH and βAR (with virtual identity in the G protein interaction domain), suggesting that the PEMF response may act on a homologous domain in

both the PTH and ßAR (Luben and Duong, 1990; Benovic et al., 1989). The convergence of these studies is based on the fact that adrenergic and related receptor systems, such as the PTH receptor, are closely associated with PEMF effects. Further, these studies provide the foundation for future work on the molecular mechanism of PEMF mediated regulation and activation of adenylate cyclase.

CHAPTER 5

SUMMARY CHAPTER

The question to be addressed in this thesis is what is the role of the cyclic AMP signal transductive pathway in the biological effects of low energy, low frequency electromagnetic fields at the cellular level?

To this end, the first objective is to characterize the short term response of adenylate cyclase to PEMF, to MSH, and to combinations of the two. The results demonstrate that PEMF can induce adenylate cyclase by up to 80% above control, and this is approximately one fifth the level attained with MSH at saturation levels (10^{-8}M). A comparison of PEMF activation with that expected with physiological levels of MSH (10^{-10} - 10^{-11}) demonstrate essentially the same degree of activation (Pawelek et al., 1975). The significance of these results provide direct evidence for coupling of PEMF to adenylate cyclase activation. Furthermore, the direct stimulatory effect of both PEMF and ISO is observed only after preincubation in serum free media, as opposed to MSH which demonstrates activation under both serum free and serum containing growth conditions. Two explanations can be proposed for these results: 1) both PEMF and ISO act only when the cultures are quiescent and partially synchronized, perhaps due to expression of a common receptor pathway at this Go/G1 juncture; this is not observed with MSH, and 2) growth factors or other compounds in the serum interfere with the ability of these stimuli to elicit their effects. The two above explanations are not mutually exclusive, as they perhaps suggest that PEMF and ISO might function through the same receptor pathway, and also indicate that this receptor pathway is distinctly different

from that of MSH. In reference to 2) above, the rationale underlying the desensitization of the ISO response may be the presence of compounds with ß-agonist properties capable of downregulating the ß-adrenergic receptor (ßAR). Epinephrine is an example, due to low affinity ßAR binding activity. We believe that epinephrine may be the link indicating the AR as a site of interaction of PEMF. Investigations of the crosstalk between αAR and ßAR receptor kinase pathways (Sibley et al., 1987) and coordinate regulation through G proteins (Hausdorf et al., 1989) support this speculation.

These results did not clearly delineate receptor specificities for the PEMF response, and other potential mechanisms remain to be discussed. In particular, MSH receptors are maximally expressed during the G_2 phase of the cell cycle (Abdel Malek et al.,1989). There is no information regarding the percentage of cells which respond positively to the hormone at a given time in the results above with MSH activation, since the assay method only provides the summated response over the entire cellular population. In the case of PEMF, serum free conditions may enhance the percentage of cells capable of responding to this stimuli (providing a better signal to noise ratio) by upregulating receptor classes which are sensitive to PEMF stimuli.

The second objective is to determine whether GTP binding proteins are involved in the PEMF and MSH response. GTP binding proteins function as regulators of the receptor stimulated adenylate cyclase (Simon et al.,1991). When hormone binds to receptor, the receptor associates with a G protein, which then activates the G

protein. This activated G protein binds GTP (releasing GDP), and undergoes a conformational change which allows the G protein to activate adenylate cyclase.

In this project we identified the inhibitory G protein, Gi, as a regulatory component of the PEMF stimulatory pathway. A comparison between PT pretreated and control cultures was performed as previously with MSH or PEMF. Removal of the Gi tonic inhibition with PT results in increased hormone responsiveness in several cell types. Katada et al. (1979) reported on the increase in adenylate cyclase stimulation by glucagon after PT pretreatment. Extension of this work by Katada et al.(1982) illustrated this same phenomenon with isoproterenol, a ßAR agonist, and Wilson et al.(1986) reported on inhibition of αAR induced desensitization with PT pretreatment. Cerione et al.(1985) indicated a role for Gi in a reconstitution assay system, providing evidence that not only does Gi inhibition increase response to ßAR agonists but Gi is also required for maximal stimulation of cyclase (due to suppression of basal activity). Our results demonstrate that interaction of PEMF stimulated adenylate cyclase with PT is increased 2 fold versus PEMF alone, indicating a strong interaction between Gi activity and the ability of PEMF to elicit a response. The same result was seen at the level of cAMP-dependent protein kinase (PKA). The MSH response atter PT pretreatment was also increased versus hormone alone, although only by 30%. This data on MSH may indicate that the more potent the hormonal stimulus the less attenuation exhibited by Gi. The role of toxin catalyzed ADP ribosylation of Gi and Gs in cell lysate preparations demonstrates an inhibition of PT catalyzed phosphorylation of Gi by MSH and PEMF, indicating a

downstream regulatory role for Gi. Cain et al. (1987) asserted no role for G proteins in their parathyroid hormone (PTH) bone cell experiments, based on the finding that the response to forskolin was not affected. Forskolin, at the concentration (1 - 100 μM) used in their studies, activates cyclase directly by promoting interaction with Gs, the stimulatory G protein (Seamon and Daly,1987). Since PEMF did not affect the forskolin response, it can be interpreted that cyclase was not directly affected by PEMF, or assumed that Gs was not involved, yet no provision of data on Gi was presented. We provide in this experiment direct evidence for a role for Gi, however the role of Gs remains to be answered.

The third major question addressed by this study is which specific receptor system linked to adenylate cyclase was responsible for the mediation of PEMF sensitivity. We have demonstrated that the ability of PEMF to directly stimulate adenylate cyclase (i.e., without additional agonist or hormone) correlated with the cells' response to ISO. By employing both homologous and heterologous desensitization mechanisms as tools we probed the possible receptor specificity of PEMF responsiveness.

The mechanism of desensitization has been extensively studied by Lefkowitz and collaborators (Hausdorf et al., 1990). The initial event in desensitization is a decreased adenylate cyclase response to continuous agonist exposure. Dependent upon the receptor level for agonist, this diminished response can either begin to occur within minutes of exposure to agonist (homologous), or take several hours or even days (heterologous). Heterologous desensitization is elicited by cross reactivity

of agonists for binding to specific receptors (e.g., binding of epinephrine to both α and ß-AR, Levitzki, 1988). or by the loss of response to one agonist leading to the loss of response to other stimuli, including hormones (Bates et al., 1991; Murphy and Majewski, 1990). Our results suggest an interplay between both homologous and heterologous desensitization mechanisms. This is based on the results with ISO pretreatment demonstrating a requirement for active ßAR to obtain a PEMF response. The only pretreatment regimen which demonstrated this cross-specificity with PEMF was the ISO group. This result, and the required time for heterologous desensitization to occur, leads us to believe that the PEMF interaction in the short term is linked to ßAR activity. Longer term results demonstrated an interplay between EPI and ISO, suggesting that αAR may also be involved. However, since EPI does bind to the ßAR (with lower affinity than ISO), the EPI effect may be to downregulate the ßAR. In addition, we demonstrated that a functional response to MSH does not necessarily correlated with a response to PEMF. Our prediction from these studies would be if a cell system exhibited responsiveness to ßAR stimuli, then the system would respond to PEMF.

In conclusion, these studies along with recent work by Luben and Duong (1990) provide a general framework for interactions of PEMF on the receptor-adenylate cyclase system. Luben has reported on the ability of PEMF to decrease the binding of ICYP, a ßAR ligand, by 50% after exposure to PEMF. A homology of over 70% exists between the PTH and ßAR (with virtual identity in the G interaction domain), suggesting that the PEMF response may act on a homologous domain in both the

PTH and βAR (Benovic et al., 1989). The convergence of these studies is based on the fact that adrenergic and related receptor systems, such as the MSH and PTH receptor, are closely associated with PEMF effects. Further, these studies provide the foundation for future work on the molecular mechanism and receptor regulation of PEMF mediated activation of adenylate cyclase.

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