Testosterone- and Estradiol-Induced Nongenomic Signaling through Surface Receptors in Macrophages

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CONTENTS

1 INTRODUCTION

1.1 Sex Hormones and Immunity

It is now rather a long time ago since the recognition of a striking gender difference in diseases, especially in autoimmune diseases with females having a greater propensity than males (Tab. 1). Conversely, infectious diseases often reveal a higher propensity in males than females, in general, males appear to be more susceptible to various infectious agents such as viruses, bacteria, fungi and parasites (Tab. 2) (Alexander and Stimson, 1988; Roberts *et al.*, 1996). Besides these epidemiological and clinical data, evidence from different experimental animals has also shown this sexual dimorphism. In BUF rats, for example, the female to male ratio is 3:1 in spontaneous thyroiditis (Rose *et al.*, 1977). In C57BL/10 mice, females are able to self-heal *Plasmodium chabaudi* malaria infections, while these infections are fatal in male mice (Wunderlich *et al.*, 1988; Benten *et al.*, 1991). The gender-dependent preponderance for both autoimmune and infectious diseases reflects a difference between the immune responsiveness of males and females. Several lines of evidence have suggested that these gender-related differences are mainly based on the difference of sex hormones (Grossman, 1984; Grossman, 1989; Schuurs and Verheul, 1990; Da Silva, 1995; Olsen and Kovacs, 1996; Verthelyi, 2001), though hormone-independent factors may also play a role, such as genes of the MHC complex and non-MHC genetic background (Weinstein *et al.*, 1984; Wunderlich *et al.*, 1988; Wunderlich *et al.*, 1991) as well as X chromosome-related genes (Rhodes *et al.*, 1969; Grundbacher, 1972).

Both androgens and estrogens have been reported to have profound influences on the immune system and the outcome of various diseases. For example, (1) treatment of mice with androgen or estrogen resulted in thymus involution, whereas gonadectomy led to thymus enlargement (Olsen *et al.*, 1994). (2) Systemic lupus erythematosus (SLE)-like disease was accelerated after 17β-estradiol (E_2) administration in castrated male MRL-lpr/lpr mice (Carlsten *et al.*, 1990). (3) The self-healing capability of the female mice in *Plasmodium chabaudi* malaria infection was abrogated by testosterone (T) treatment (Benten *et al.*, 1991) (Tab. 3).

Moreover, there is vast literature available that T and E_2 have profound influences on different immune organs including spleen, thymus and immune cells including T cells (Ansar Ahmed *et al.*, 1985; Benten *et al.*, 1991), B cells

(Sthoeger *et al.*, 1988; Kincade *et al.*, 1994a; Kincade *et al.*, 1994b) and macrophages (Deshpande *et al.*, 1997; Wichmann *et al.*, 1997). Indeed, it has been reported that, the ratios of CD4+ and CD8+ T cells have been changed by the hormone treatment (Benten *et al.*, 1991); the production of autoimmune-related antibodies of B cells has been changed after the application of sex hormones (Roubinian *et al.*, 1979).

Table 1. Female preponderance of autoimmune diseases

Autoimmune diseases	Female to male ratio		
	Ansar Ahmed *et al.*, 1985	Beeson, 1994	Jacobson *et al.*, 1997
Hashimoto's thyroiditis	25-50:1	5.6:1	17.5:1
Systemic lupus erythematosus	9:1	9:1	7.5:1
Sjögrens's syndrome	9:1	19:1	14.9:1
Multiple sclerosis	1-5:1	1.5:1	2:1
Scleroderma	3-4:1	4:1	12:1
Rheumatoid arthritis	2-4:1	2:1	3:1
Idiopathic adrenal insufficiency	2-3:1	--	--
Myasthenia gravis	2:1	3:1	3:1

Table 2. Male preponderance of infectious diseases

Infectious agents	Male to female ratio	References
Virus		
Rabies virus	7-8:1	Müller, 1989
Mumps virus	3-4:1	Stehr, 1976
Hepatitis B virus	2:1	Tripatzis *et al.*, 1976
Bacteria		
Actinomyces isralii	4:1	Lerner, 1979
Mycobacterium tuberculosis	3:1	Anonymus, 1977
Yersina pestis	3:1	Burnet, 1971
Listeria monocytogenes	2-3:1	Kampelmacher and van Noorle Jansen, 1980
Fungi		
Paracoccidiodes brasiliensis	15-16:1	Restrepo, 1979
Cryptococcus neoformans	3:1	Müller, 1989
Protozoan		
Entamoeba histolytica	9:1	Biagi and Beltran, 1969
Plasmodium falciparum	3-4:1	Weise, 1979
Plasmodium vivax	〃	〃
Plasmodium ovale	〃	〃
Plasmodium malariae	〃	〃

Table 3. Effects of sex hormones on immunity of some experimental models

Mice	Models	Manipulation of sex hormones and outcomes	References
C57BL/6	Immunity	Castration of male leads to enlargement of thymus; T-treatment results in an involution of thymus	Olsen *et al.*, 1994
C57BL/10	Malaria infection	Female mice can survive the malaria; for male it is lethal; T-treatment converts female mice from resistance to susceptibility	Wunderlich *et al.*, 1988; Benten *et al.*, 1991
SJL	EAE	Females have a higher incidence and severity; T-treatment reduced the severity of EAE in females	Dalal *et al.*, 1997
NZBxNZW	SLE	Spontaneous SLE affects females more frequently than males, T-treatment improves survival of females	Ahmed *et al.*, 1987
B/W	SLE	Females have an accelerated SLE-like disease and die earlier than males, estrogen treatment increases the mortality rate, while androgen treatment decrease it	Roubinian *et al.*, 1977
MRL-lpr/lpr	SLE	Estrogen administration accelerates the SLE-like disease in castrated male mice	Carlsten *et al.*, 1990

SLE = systemic lupus erythematosus; EAE = experimental autoimmune encephalomyelitis

In particular, macrophages are versatile cells and play a wide variety of important roles in both innate and acquired immunity. They function not only as simple phagocytes and antigen presenting cells (APCs), but also through secreting numerous bio-active cytokines, such as interleukin-1 (IL-1), IL-6, IL-10, IL-12, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and other active molecules, such as nitric oxide (NO), reactive oxygen intermediates (ROI) and arachidonic acid metabolites including prostaglandin E2 (PGE2), platelet-activating factor (PAF), to modulate both humoral- and cell-mediated immune responses (Hume, 1985; Seljelid and Eskeland, 1993). The versatile macrophages can be influenced by numerous environmental signals including sex hormones (Miller and Hunt, 1996). Both T and E_2 have been reported to be able to exert their immuno-regulatory effects through macrophages. Indeed, it has been reported that E_2 can inhibit LPS-induced expression of some inflammatory cytokines in macrophages, including IL-1β (Deshpande et al., 1997; Ruh et al., 1998), IL-6 (Deshpande et al., 1997), TNF-α (Deshpande et al., 1997; Srivastava et al., 1999). This inhibition is probably through suppressing activation of the transcription factor NFκB (Deshpande et al., 1997). E_2 can also induce NO production in macrophages (Stefano et al., 1999). T suppresses macrophage function after trauma-hemorrhage (Wichmann et al., 1997), which is probably through the inhibition of cytokine secretion of IL-1 and IL-6 in macrophages, and castration of the male experimental animals

could prevent this suppression of macrophage activation (Kahlke *et al.*, 2000; Angele *et al.*, 2000). Also, T has been reported to decrease expression of Fcγ receptor (Gomez *et al.*, 2000) and LPS-induced NO-production (Savita and Rai, 1998; Friedl *et al.*, 2000). Moreover, previous work from our group also showed that T treatment increases the infection levels of the protozoan Leishmania donovani in bone marrow-derived macrophages (BMM) (Zhang *et al.*, 2001) and inhibits apoptosis of BMMs infected with Leishmania donovani (Qiao *et al.*, 1999).

Although a large body of evidence has indicated that sex hormones such as T and E_2 have profound influences on macrophages, the precise mechanism through which sex steroids trigger and/or influence the signaling events in macrophages remains unclear.

1.2 LPS- and FCS-induced Signaling in Macrophages

As a mostly used model, stimulation of macrophages with lipopolysaccharide (LPS), an outer membrane component of the cell wall of Gram-negative bacteria, triggers the activation of macrophages (Sweet and Hume, 1996). Although the precise mechanism of LPS signaling remains to be fully elucidated, it has been reported that LPS, by exerting most of its biologic effects through the lipid A moiety, activates numerous signal transduction pathways in macrophages. It is generally accepted that LPS can bind a variety of serum proteins, among them LPS binding protein (LBP) is well characterized. The LPS/LBP complex binds to the specific surface receptor on monocyte/macrophage - CD14, a glycophosphatidylinositol-linked cell surface glycoprotein (Tobias *et al.*, 1994). Subsequently this binding complex interacts with a transmembrane protein named toll-like receptor-4 (TLR4), which has been suggested to be a receptor for LPS (Chow *et al.*, 1999; Beutler, 2000). This interaction leads to the activation of several signaling pathways, including protein tyrosine kinases (PTKs)-ras-raf-mitogen-activated protein kinase (MAPK) cascade, activation of protein kinase C (PKC), ceramide, and heterotrimeric G-proteins (Stefanova *et al.*, 1993; Sweet and Hume, 1996). It is already known that LPS stimulation can result in the rapid induction of Ets-2, Elk-1, PU.1, and AP-1 transcription factors including c-Fos (Collart *et al.*, 1987; Xie *et al.*, 1993), c-Jun, Jun-B (Fujihara *et al.*, 1993), Egr-1 (Coleman *et al.*, 1992) in macrophages. LPS stimulates activation of the transcription factor NF-κB, which is thought to mediate the production of most pro-inflammatory

cytokines, such as IL-1, IL-6, TNF-α (Baeuerle, 1991; Grilli *et al.*, 1993). Normally NFκB is tightly associated with its inhibitor IκBα. After stimulation of macrophages with LPS, IκBα is phosphorylated and degraded allowing the translocation of NFκB into nuclei. Subsequently free NFκB binds to the corresponding NFκB response elements on the regulatory regions of the target genes (Henkel *et al.*, 1993; Donald *et al.*, 1995). LPS stimulation could also lead to an increased iNOS expression resulting in a high level of NO production, which is believed to function as a cytotoxic effector to kill the intracellular pathogens (Hibbs, Jr. *et al.*, 1988; MacMicking *et al.*, 1997).

Among these LPS-activated signaling events, the activation of *c-fos*, a widely studied immediate early gene (IEG), has been thought to be one of the earliest genotropic alterations following LPS stimulation (Introna *et al.*, 1986; Introna *et al.*, 1987a; Introna *et al.*, 1987b). Activation of *c-fos* is important for the modulation of functional activities in macrophages (Collart *et al.*, 1987). Since the *c-fos* gene product, c-Fos protein, can serve as a transcription factor of AP-1 family (Karin *et al.*, 1997), it modulates the secondary gene expression and may have profound impact on macrophage functions.

LPS has been reported to induce activation of all the three MAPK families, ERK1/2, SAPK/JNK and p38 MAPK (Weinstein *et al.*, 1992; Feng *et al.*, 1999). All of these three MAPKs are activated through dual phosphorylation of threonyl and tyrosyl residues separated by a single amino acid. The members of these MAPK families are involved in many important cellular processes (Robinson and Cobb, 1997). Among them, ERK1/2 (p44/p42 MAPK) function in protein kinase cascades which play a critical role in cell growth and differentiation. It can be activated by different growth factors, cytokines, hormones etc. (Hill and Treisman, 1995a; Lewis *et al.*, 1998; Cobb, 1999). p38 MAPK participates in kinase cascades controling cellular responses to cytokines and stresses (Raingeaud *et al.*, 1995). It can be activated by a variety of cellular stressful stimuli including osmotic shock, inflammatory cytokines including IL-1 and TNF-α, as well as LPS (Brewster *et al.*, 1993; Han *et al.*, 1994; Nick *et al.*, 1996; Ridley *et al.*, 1997; Read *et al.*, 1997). SAPK/JNK function in a protein kinase cascade transducing cellular stress signals, and can be activated by UV light, inflammatory cytokines, LPS and other stresses (Sluss *et al.*, 1994; Galcheva-Gargova *et al.*, 1994; Derijard *et al.*, 1994; Coso *et al.*, 1995; Gupta *et al.*, 1996; Minden and Karin, 1997).

NO production is another important consequence of LPS stimulation and has been studied intensively as an index for macrophage activation following pathogen invasion. NO is

known to mediate many nonspecific cytotoxic and inflammatory responses of macrophages following infection with pathogens. It is generated by a family of NO synthase (NOS) isozymes that convert L-arginine to L-citruline and NO (Torreilles, 2001). Many experiments have shown that LPS triggers the expression of an inducible NO synthase (iNOS) resulting in a high output of NO production from macrophages. It was reported that in mouse J774 macrophages, LPS-induced iNOS expression and NO production is mediated through the activation of p38 MAPK (Chen *et al.*, 1999a).

Serum is required for LPS stimulation because it provides LBP for the interaction of LPS with CD14 (Tobias *et al.*, 1994). Besides this, serum itself is also an important stimulus for almost all kinds of cells including macrophages. Although serum has been widely applied in most of tissue cultures and used as an important stimulus in the study of cell growth and proliferation for many years, the exact mechanism of serum signaling is still not completely elucidated. Since serum is a complex mixture of numerous peptide factors including various growth factors, it is generally accepted that the signals derived from serum are mainly transmitted through the growth factor-induced signaling pathway, which is mainly mediated through the receptors belonging to the class of receptor tyrosine kinase (RTK) (Porter and Vaillancourt, 1998). These single transmembrane-spanning receptors, dimerize and autophosphorylate on tyrosine residue upon ligand binding. The RTK receptors, through a set of adaptors such as Shc, Grb2 and Crk, can activate a guanine nucleotide exchange factor such as Sos and C3G. Subsequently the activation signal is transduced to small GTP binding proteins such as Ras (Margolis and Skolnik, 1994), which in turn activate the cascade of a Raf-MEK-ERK1/2 (Marshall, 1994; Stokoe *et al.*, 1994). The activated ERK1/2 can regulate the cytosolic targets directly and also translocate into the nucleus where, through phosphorylation of a variety of transcription factors, e.g. Elk-1, it regulates the expression of some genes which maybe closely related to the cell growth and proliferation. For instance, the IEG *c-fos* is known to contain a serum response element (SRE) in its promoter region, when the cells were stimulated with serum, ERK1/2 is activated through RTK pathway as described above, then one of the ternary complex factor (TCF) e.g. Elk-1 is phosphorylated (Treisman, 1994). Through interaction with serum response factor (SRF), phosphorylated Elk-1 binds to SRE, this binding induces the activation of *c-fos* promoter and transcription of *c-fos* gene (Hill *et al.*, 1994). These IEG products, in turn, act as transcription factors to modulate the expression of the secondary ("late") genes, which are responsible for the phenotypic alterations of the cells such as growth, proliferation and differentiation.

1.3 Mechanisms of Sex Hormones Action

Sex hormones are synthesized mainly in the sexual glands and adrenal cortex, then they circulate in the blood and enter their target cells by simple diffusion. According to the traditional model, sex hormones like other steroids function exclusively through the intracellular receptors belonging to the steroid/thyroid/retinoid/orphan receptor superfamily (Beato and Klug, 2000). These nuclear receptors act as intracellular transcription factors, which are structurally organized in different domains for nuclear localization, transactivation, dimerization, ligand-binding, etc. (Beato, 1989; Kumar and Tindall, 1998; Kumar and Thompson, 1999). Upon binding of the sex steroids to their cognate receptors, the steroid hormone-receptor complex translocate from the cytoplasm into the nuclei and binds as homo- or heterodimers to palindromic DNA sequences termed hormone response element (HRE) of the target genes. Through the regulation of HRE steroid modulates the transcription of the corresponding gene positively or negatively (Evans, 1988; Beato, 1989; Fuller, 1991; Beato and Klug, 2000).

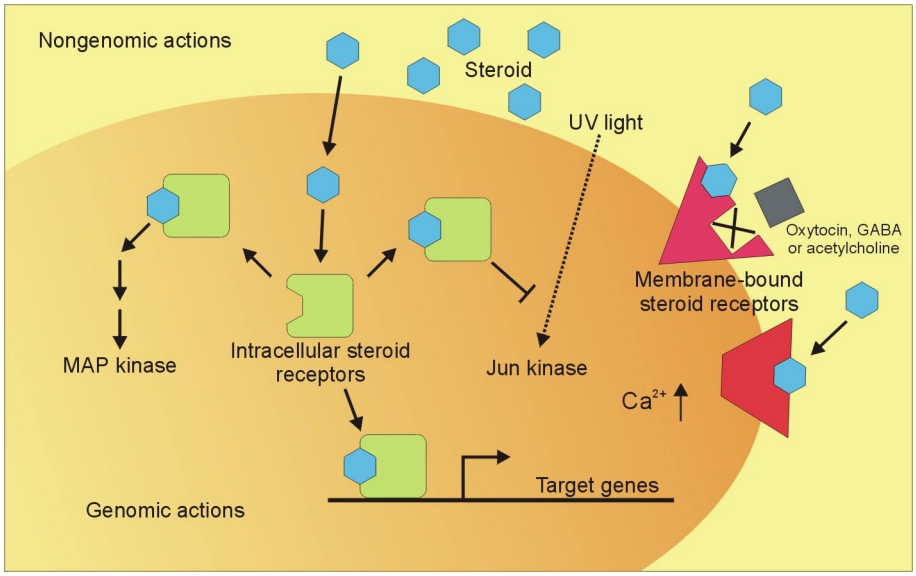


Figure 1. Emerging diversities of the mechanisms of steroids. Steroids can exert their effects through both genomic and nongenomic pathways. Genomic actions are mediated through the classical nuclear steroid receptors acting as transcription factors, while nongenomic actions are reported to be mediated through both intracellular and membrane steroid receptors. (modified from *Picard, 1998*, ***Nature***, 392, 437-438).

In addition to the well-characterized genomic effect, increasing evidence has been accumulated that sex steroids can also induce rapid "nongenomic" effects. These can be transmitted by the same nuclear receptors used in the genomic pathway through direct protein-protein interactions (Improta-Brears *et al.*, 1999; Peterziel *et al.*, 1999). By contrast, there is also information available that nongenomic effects can be mediated through specific membrane receptors for steroids (Brann *et al.*, 1995; Wehling, 1997; Picard, 1998; Watson and Gametchu, 1999; Falkenstein *et al.*, 2000). Usually these nongenomic effects of steroid hormones manifest themselves as rapid responses such as increase of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and/or activation of MAPK (Wehling, 1997) (Fig. 1).

1.3.1 Actions of Testosterone

T, first isolated in 1935 by David and his colleagues, was initially considered as a critical ingredient in organotherapeutic treatments (Shahidi, 2001). Now it has been recognized that T is not only required for the differentiation and development of male sex organs, the maintenance of male phenotype, but also exerts a wide array of effects on non-reproductive tissues including the immune system as described above (1.1).

As a member of the steroid hormones, the male sex hormone T has been considered traditionally to exert its function through the classical intracellular androgen receptor (AR), a member of the nuclear receptor superfamily. The AR is a 110 kDa protein, structurally organized in different domains: a variable N-terminal region, a central, highly conserved, cysteine-rich DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD). These domains are responsible for androgen binding, DNA-binding, nuclear localization, dimerization, and transactivation, respectively (Zhou *et al.*, 1994). T enters the cells by simple diffusion and bind to the ARs, which are mostly located in the cytoplasm at equilibrium. After binding of T with AR at the LBD, the T-AR complexes translocate from the cytoplasm into the nuclei and bind specifically to the androgen response elements (ARE) in the regulatory region of the target genes. Previously reported ARE shares the same response element with glucocorticoid response element (GRE), mineralocorticoid response element (MRE), progesterone response element (PRE) and is characterized as 5'-GGTACAnnnTGTTCT-3' in mouse mammary tumor virus (MMTV) DNA (Beato, 1989). Recently, another two specific recognition sites of AR have been identified. They are 5'-

GGTTCTTGGAGTACT-3' in probasin gene promoter (Claessens *et al.*, 1996) and 5'-GGAACGGAACA-3' identified from a random sequence selection assay (Zhou *et al.*, 1997). Through the binding of T-AR complex to ARE, T can regulate the transcription of the target genes positively or negatively. In addition, AR has also been reported to be able to interact with other transcription factors on the target gene promoters without direct binding to DNA. For example, AR can inhibit activator protein-1 (AP-1) activity by competing the co-activator CRE binding protein (CREBP) (Fronsdal *et al.*, 1998). On the other hand, AR can be activated by a ligand-independent manner, for instance, through a protein kinase A pathway in the absence of androgen (Nazareth and Weigel, 1996).

T has also been described, like other steroids, to exert nongenomic actions through intracellular AR (iAR). For instance, T can stimulate the activation of MAPK and p21-activated kinases through iAR in different cell types such as LNCaP cells, osteoblasts, osteocytes, embryonic fibroblasts and HeLa cells (Cato and Peterziel, 1998; Kousteni *et al.*, 2001; Yang *et al.*, 2001). On the other hand, nongenomic actions of T can also be mediated through membrane androgen receptors (mAR) on cell surfaces, which has been described as increases in $[Ca^{2+}]_i$. This intracellular Ca^{2+} signaling often varies with cell-type, for example, in murine splenic T-lymphocytes, mAR mediated T-induced Ca^{2+} influx through non-voltage gated, Ni^{2+}-blockable Ca^{2+}-channels (Benten *et al.*, 1997; Benten *et al.*, 1999a). In rat osteoblasts, T induces both influx of extracellular Ca^{2+} via voltage-gated Ca^{2+} channels and Ca^{2+} release from intracellular stores through G-protein coupled receptors (GPCR) activating phospholipase C (PLC) via a pertussis toxin (PTX)-sensitive G-protein (Lieberherr and Grosse, 1994).

1.3.2 Actions of Estradiol

Estrogens with its most active form E_2 are required not only for development of the female phenotype, but also exert a series of both beneficial and adverse effects on human health. For instance, estrogens have been described to be protective in diverse neurodegenerative diseases (Bruce-Keller *et al.*, 2000; Wise *et al.*, 2001) including Alzheimer's disease (Henderson, 1997), in central nerve system trauma and stroke (Roof and Hall, 2000), in osteoporosis (Termine and Wong, 1998), in heart disease (Grodstein and Stampfer, 1995) and in arteriosclerosis (Guzzo, 2000). Adverse effects are ascribed to the cell proliferative

activity of E_2 promoting diverse cancer forms in breast (Colditz, 1998) and endometrium (Cohen and Rahaman, 1995). Moreover, E_2 is known to increase risk for autoimmune disorders such as SLE and multiple sclerosis as described above (1.1) (Cutolo *et al.*, 1995).

E_2 effects are considered traditionally to be mediated at the cellular level through nuclear estrogen receptors (ER) (Couse and Korach, 1999). There are two types of ER, i.e. ERα and a recently identified ERβ. The ERα is composed of 599 amino acids with an approximately molecular mass of 66 kDa. The human ERα is located in chromosome 6, and the mouse ERα in chromosome 10. The ERβ is composed of 527-530 amino acid with a molecular mass of approximately 60 kDa. A number of variants have been described both for ERα and ERβ, for instance, a conserved insertion of 18 amino acids in the c-terminal region of the ERβ in rat (Chu and Fuller, 1997), human and mouse (Lu *et al.*, 1998). As other members of the nuclear receptor superfamily, both ERα and ERβ are composed of several different functional domains for estrogen binding, nuclear localization, estrogen response element (ERE) binding, and transactivation of the target genes. It has been recognized that most ERs are already localized in nuclei regardless of whether it is complexed with the ligand. The inactive ER exists in a complex consisting of heat-shock proteins. This complex is tightly associated with the specific consensus sequence termed ERE consisting of 15-bp inverted palindromes (5'-AGGTCAnnnTGACCT-3') in the regulatory regions of the target genes. After entering the cell by simple diffusion, E_2 binds to this complex, the heat-shock proteins dissociate from ER, then E_2-ER complex interacts with basal transcription factors, co-regulators and other transcription factors to regulate transcription of the target gene positively or negatively (Beato, 1989).

In recent years, however, the action spectrum of nuclear ER is turning out to be much more complex than originally anticipated. Indeed, evidence is accumulating that ER does mediate not only genotropic, but also a series of nongenomic actions of E_2 (Beato and Klug, 2000; Falkenstein *et al.*, 2000; Coleman and Smith, 2001). For instance, the E_2-ER complex has been described to activate directly MAPK (Migliaccio *et al.*, 1996; Improta-Brears *et al.*, 1999; Razandi *et al.*, 2000; de Jager *et al.*, 2001; Duan *et al.*, 2001) and endothelial NO synthase (eNOS) (Goetz *et al.*, 1999; Chen *et al.*, 1999b), to increase cAMP (Farhat *et al.*, 1996), inositol (1,4,5)-trisphosphate (IP_3) (Favit *et al.*, 1991; Razandi *et al.*, 1999), and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Improta-Brears *et al.*, 1999). However, there is also information available that nongenomic E_2 actions can be independent of the classical nuclear ER. In particular, the E_2-induced rise in $[Ca^{2+}]_i$ has been reported to be mediated

through membrane ER (mER) on the cell surface (Lieberherr *et al.*, 1993; Audy *et al.*, 1996; Benten *et al.*, 1998; Benten *et al.*, 2001). The molecular nature of mER is currently under controversial debate. Some authors suggest the mER to be largely identical with the classical ER (Razandi *et al.*, 1999; Watson and Gametchu, 1999), or that the mER represents larger complexes containing GPCR linked somehow to nuclear ER (Mendelsohn, 2000), whereas other authors emphasize the views that the mER is different to iER and belongs to the class of G-protein coupled receptors (GPCR) (Lieberherr *et al.*, 1993; Filardo *et al.*, 2000; Benten *et al.*, 2001).

1.4 Ca^{2+} Signaling and Gene Expression

The nongenomic actions of T and E_2 through membrane receptors, as described above, often become evident as rapid rises in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). As we know, Ca^{2+} is a most common second messenger in signal transduction of cells. Normally the $[Ca^{2+}]_i$ of cells is by about 100 nM, which is 20,000-fold lower than the extracellular concentration of 2 mM (Clapham, 1995). The increase of cytosolic Ca^{2+} has been observed in numerous important cellular processes, such as muscle contraction and enzyme activation. Usually, the generation of the increase in $[Ca^{2+}]_i$ of cells can be due to the influx of extracellular Ca^{2+} across the plasma membrane and/or release of Ca^{2+} through ion channels from intracellular stores, such as endoplasmic reticulum (Tsien and Tsien, 1990; Pozzan *et al.*, 1994).

Macrophages belong to the non-excitable cells, in which the increase of $[Ca^{2+}]_i$ is believed to be predominately through the relatively slower pathway mediated by IP_3. Two classes of receptors, i.e. GPCR and RTK can trigger the release of IP_3. Among them, GPCRs stimulate PLCβ through activation of Gα and/or Gβγ subunits, while RTKs activate PLCγ, both PLCβ and PLCγ catalyze the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG) (Berridge and Irvine, 1989; Clapham, 1995). Subsequently IP_3 binds to the specialized tetrameric IP_3 receptor spanning the endoplasmic reticular membrane and elicits release of Ca^{2+} from the endoplasmic reticulum (Berridge, 1993). Another mechanism for the increase of $[Ca^{2+}]_i$ in non-excitable cells is the influx of extracellular Ca^{2+} through selective non-voltage-gated Ca^{2+} channels (Clapham, 1995). In excitable cells, besides the mechanisms described above, the voltage-gated Ca^{2+} channels are

used for rapid and dramatic increase of $[Ca^{2+}]_i$. (Clapham, 1995). It is noteworthy that there are more than 30 GPCRs has been reported to initiate Ca^{2+} release through the activation of PLCβ (Sternweis and Smrcka, 1992). Some types of G-proteins are sensitive to pertusiss toxin (PTX), others are sensitive to cholera toxin (CT), and this sensitivity may depend on the different subtypes of Gα (Wess, 1998).

It is no wonder that the increase of $[Ca^{2+}]_i$ is important for various cell functions including adhesion, motility, proliferation, cell cycle, cell death and activation of gene expression (Berridge, 1993; Ghosh and Greenberg, 1995). For instance, PKC is one of the most important kinases and involved in various cellular processes (Valledor *et al.*, 2000). It is known that some types of PKCs, for example, the class of conventional PKCs including α, β, βI, βII, and γ subtypes, need the increase of $[Ca^{2+}]_i$ for activation. Ca^{2+} stimulates CaM kinase-dependent phosphorylation of the cAMP response element-binding protein (CREB) (Sheng *et al.*, 1991). In single T-lymphocytes the anti-CD3 antibody can elicit lacZ reporter gene expression controlled by NF-AT elements of IL-2 gene and this process was dependent on the increase of $[Ca^{2+}]_i$ (Negulescu *et al.*, 1994). In B-lymphocytes, the amplitude and duration of calcium signals has been correlated with differential activation of the pro-inflammatory transcription factors. NFκB and JNK are selectively activated by a large transient $[Ca^{2+}]_i$ rise, whereas NFAT is activated by a low, sustained Ca^{2+} plateau (Dolmetsch *et al.*, 1997; Crabtree, 1999). In particular, the immediate early gene *c-fos* contains different Ca^{2+}-sensitive response elements including CRE in its promoter and related-regulatory regions. It is therefore very sensitive to intracellular Ca^{2+} and has been classified as a strongly Ca^{2+} inducible gene (Karin, 1995; Hill and Treisman, 1995b; Johnson *et al.*, 1997; Van Haasteren *et al.*, 1999).

1.5 Aim of the Work

Previous work from our laboratory and others have already shown that T and E_2 exert rapid nongenomic effects through unconventional surface receptors on different cell types i.e. osteoblast cells and immune cells like splenic T-lymphocytes. It is therefore of interest whether other immune cells like macrophages also have steroid surface receptors. Moreover, as mentioned before, the surface receptor-mediated nongenomic actions of T and E_2 often become evident as rapid rises in $[Ca^{2+}]_i$. These Ca^{2+} signals, though steroid-specific, are in

general low, so that their consequences for cell functioning are not immediately obvious, and these signals are therefore often regarded as meaningless. However, it is possible that even steroid-induced low rises in $[Ca^{2+}]_i$ are able to activate at least Ca^{2+} sensitive genes such as immediate early genes (Roche and Prentki, 1994; Van Haasteren *et al.*, 1999), which in turn may have consequences on secondary gene expression and, thus, cell functioning. The present work is, therefore, addressed to the following questions:

1. Are there surface receptors for T and E_2 in macrophages?
2. Does T and E_2 induce rapid nongenomic responses in macrophages?
3. Have these T- and E_2-induced rapid responses some effects on genotropic signaling in macrophages?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

Mouse anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) E10 Monoclonal Antibody, rabbit anti-p44/42 MAP Kinase antibody, rabbit anti-phospho-p38 MAP kinase (Thr180/182), rabbit anti-p38 MAP kinase, rabbit anti-phospho-SAPK/JNK (Thr183-/Tyr185), rabbit anti-SAPK/JNK, Cell Signaling Technology, New England Biolabs, Beverly, MA, USA. The antibodies IκBα (C-21), NFκB p65 (C-20)-G, JNK1 (F-3), ATF-2 (C-19), Actin (I-19), c-Jun (H-79), c-Fos (K-25)-G, AR (N-20), AR (C-19), ERα (MC-20), ERα (H-18), ERβ (Y-19), CREB-1 (C-21) X Transcruz, anti-phospho-c-Jun, HRP-conjugated goat anti-rabbit IgG, anti-clathrin antibody heavy chain (N-19), anti-caveolin antibody caveolin-1 (N-20), Santa Cruz Biotechnology, Heidelberg, Germany. anti-NFATc, Alexis; anti-phospho-CREB (rabbit polyclonal IgG), anti-phosphotyrosine, clone 4G10, Upstate Biotechnology through Biomol, Hamburg, Germany. Rat anti-mouse F4/80 antibody, a gift from Dr. H. Mossmann, Max-Planck Institute for Immunobiology, Freiburg, Germany. HRP-conjugated rabbit anti-mouse IgG, Biotin-SP-conjugated AffiniPure mouse anti-rat IgG, TRITC-conjugated AffiniPure goat anti-rabbit IgG, Jackson Immuno-Research Laboratories, West Grove, PA, USA. Biotinylated-mouse anti-rat antibody, streptavidin-fluorescein, Amersham Life Science, Braunschweig, Germany. LysoTracker™ Red DND-99, Molecular Probes, Göttingen, Germany; donkey anti-goat-Cy3 amtibody, a gift grom P. Traub, Max-Planck Institute for Cell Biology, Ladenburg, Germany.

2.1.2 Cell Culture

RPMI 1640, Iscove's modified dulbecco's medium (IMDM), GibcoBRL, Karlsruhe, Germany; fetal calf serum (FCS, virus screened, mycoplasma screened, endotoxin tested, sterile-filtered), PAA, Linz, Austria; Sterile filter, Nunc, Wiesbaden, Germany.

2.1.3 Steroids and Derivatives

Testosterone, 17α-estradiol, 5α-dihydrotestosterone-propionate, 5β-dihydrotestosterone-propionate, 1-dehydrotestosterone, Steraloids through Paesel+Lorei GmbH & Co., Hanau, Germany; 17β-estradiol, Serva, Heidelberg, Germany; testosterone 3-(O-

carboxymethyl)oxime:BSA; testosterone 3-(O-carboxymethyl)oxime:BSA-fluorescein isothiocyanate conjugate; β-estradiol 6-(O-carboxymethyl)oxime:BSA-fluorescein isothiocyanate conjugate, 17α-estradiol, dehydroisoandrosterone, Sigma, Deisenhofen, Germany.

2.1.4 Chemical Reagents and Others

1,4-diazabicyclo-[2.2.2]octane (DABCO; Triethylenediamine), BSA, digitonin, Fura-2/AM, lipopolysaccharide from *salmonella typhosa*, N-(1-Naphthyl)ethylenediamine, poly-L-lysine, sodium fluorid, sodium orthovanadate, sulfanilamide (p-Aminobenesulfonamide), pertussis toxin (islet activating protein), pre-stained SDS Molecular Weight Markers, Sigma, Deisenhofen, Germany.

AffinityPak™ Detoxi-Gel™ endotoxin removing gel, Micro BCA™ protein assay reagent Kit, NE-PER™ Nuclear and cytoplasmic extraction reagents, Pierce, St. Augustin, Germany.

Agar, yeast-extract, tryptone peptone, Difco Laboratories, Detroit, USA.

Agarose, SequaGel XR ready buffer, SequaGel XR complete buffer reagent, National diagnostics through Biozym Diagnostik GmbH Hess, Oldendorf, Germany.

Anisomycin; G418 sulfate (Geneticin); PD098059; Calbiochem-Novabiochem, La Jolla, CA, USA.

Antidigoxinin-AP, Fab fragments, Cell proliferation kit II (XTT), CDP-Star™, Expand™ High Fidelity PCR System, Fugene™ 6 Transfection Reagent; Protease inhibitor cocktail tablets (complete, mini), Roche Molecular Biochemicals, Mannheim, Germany.

BAPTA/AM cell permeant, Molecular Probes, Göttingen, Germany.

Concanavalin A (Con A)-rhodamine, Vectashield® mounting medium for fluorescence H-1000, Vector Laboratories Inc. Burlingame, CA, USA.

DNA EcoR I/Hind III Marker, PNK A, PUC Mix Marker 8, MBI Fermentas, St. Leon Rot, Germany.

ECL and ECL-plus Western blotting detection reagents, Hyperfilm™ high performance autoradiography film, Megaprime™ DNA labelling system, Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP, Amersham, Braunschweig, Germany.

Kodak film for ^{32}P exposure, Biomax MS™, Rochester, New York, NY, USA.

M-MLV Reverse Transcriptase, Taq DNA polymerase in Storage Buffer A, Promega, Heidelberg, Germany.

NucleoSpin® plasmid purification kit, Macherey & Nagel GmbH (MN), Düren, Germany.

Paraformaldehyde, extra pure, Merck, Darmstadt, Germany.

Phospha-Light™ Chemiluminescent Reporter Gene Assay System, Tropix, Bedford, Massachusetts, USA.

Protan nitrocellulose transfer membrane, BA 85, 0.45 µm pore size, Schleicher und Schuell GmbH, Dassel, Germany.

Qiagen-Spin pre-Mini, Midi-Plasmid isolation kits, QIAquick™ Spin Nucleotide Removal Kit, PCR purification kit, Qiagen GmbH, Hilden, Germany

Radioactive chemicals, nonidet p-40, ICN Biomedicals Inc., Meckenheim, Germany

Roti®-load (4×concentrated), Roti®-Block (10×concentrated), Carl Roth, Karlsruhe, Germany.

Subcloning Efficiency DH5α™ Competent Cells, TRIzol™ Total RNA Isolation Reagent, Gibco BRL, Karlsruhe, Germany.

U-73122 and U-73343, Biomol Research Laboratory, Plymouth, MA, USA.

2.1.5 Vectors

pGEM-T Easy vector system, Promega, Heidelberg, Germany.

pSEAP vectors, pEGFP-N3 vector, Clontech, Palo Alto, USA.

TOPO TA cloning^{TM} kit, Invitrogen, Leek, NL.

2.1.6 Solutions and Buffers

AA/BA 30% (1:29)	acrylamide/bisacrylamide (1:29), 87.6 g acrylamide, 2.4 g bisacrylamide, add H_2O up to 300 ml, double filtered, kept at 4°C
APS (10%)	10% ammonium peroxodisulfate in H_2O, aliquoted, stored at -20°C
Arginine medium	21 g PPLO-Broth dissolved in 730 ml dH_2O, autoclaved, 100 ml of horse serum (mycoplasma screened), 100 ml of 10% yeast extract, 10 ml of 0.2% phenol red, 50 ml of 20% arginine, pH was adjusted to 6.5-6.6 with 1 M HCl sterilized through a 0.2 µm filter
Binding buffer (5×)	100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% (v/v) Tween-20, 150 mM KCl
BSA buffer (1.5×10^{-3} M)	335 mg BSA dissolved in 5ml sterile PBS^+
Ca^{2+} buffer (200 mM)	294 mg $CaCl_2\times2\ H_2O$ dissolved in 10 ml HEPES buffer
Cell stock medium	20% FCS, 10% DMSO in IMDM
Digitonin buffer	15.2 mg digitonin, dissolved in 250 µl DMSO, added 4.75 ml dH_2O, kept at 4°C, prepared just before use
DTT (1M)	3.09 g DTT dissolved in 20 ml of 0.1M sodium acetate (pH 5.2), sterilized by filtration, dispensed to 1-ml aliquots and stored at -20°C.
E_2 stock solution	10^{-3} M, 0.2724 mg/ml E_2 in ethanol, freshly prepared
E_2-BSA-FITC solution	1.5×10^{-3} M, 5 mg E2-BSA-FITC dissolved in 1.39 ml sterile PBS^+
EGTA buffer (400 mM)	EGTA 3.8 g, dissolved in 3 ml 10 N NaOH, added 15 ml dH_2O, adjust pH to 7.2, then add H_2O up to 25 ml, kept at 4°C

EMSA loading buffer	60% (v/v) 0.5×TBE, 40% (v/v) glycerol, for the loading buffer with bromphenol blue, add 0.2% bromphenol blue
EMSA-PAGE (4%)	4 ml 30% AA/BA, 3 ml 5×TBE, 23 ml H_2O, 45 µl TEMED, 225 µl 10% APS, total gel volume 30 ml
Fura-2/AM stock	1 mg/ml in DMSO, aliquoted and stored at -20°C
Gelatin buffer	1% gelatin in H_2O, sterilized, stored at 4°C
Glucose medium	21 g of PPLO-Broth dissolved in 730 ml dH_2O, autoclaved, 100 ml of mycoplasma-screened horse serum, 100 ml of 10% yeast extract, 10 ml of 0.2% phenol red, 50 ml of 20% glucose, pH was adjusted to 7.0 with 1 M NaOH sterilized through a 0.2 µm filter.
Griess reagent	0.2% N-(1-Naphthyl)ethylenediamine in H_2O and 2% sulfanilamide in 5% phosphoric acid mixed with a ratio of 1:1 before use.
HEPES buffer	20 mM HEPES, 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM Na_2HPO_4, 1 mg/ml glucose, pH 7.2
IPTG	2 g IPTG in 10 ml of H_2O, filtered through a 0.22 µm filter, aliquoted and stored at -20°C
LB-agar	LB-medium with 1.5% agar
LB-medium	10 g tryptone, 5 g yeast extract, 5 g NaCl, add H_2O to 1 L, adjust pH to 7.5
Na^+-HBS	20 mM HEPES, 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM glucose, adjust pH to 7.3 with 1 M Tris base
PBS^-	8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4×2 H_2O, 0.15 g NaH_2PO_4×H_2O, 0.2 g KH_2PO_4, add H_2O up to 1 L, pH 7.2
PBS^+	8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4×2 H_2O, 0.2 g KH_2PO_4, 0.1 g $MgCl_2$×6 H_2O, 0.1g $CaCl_2$×2 H_2O, add H_2O up to 1 L, pH 7.2
PFA (5%)	Paraformaldehyde in PBS^+, adjust to pH 7.2
Poly-L-lysine buffer	0.1 mg/ml poly-L-lysine in sterilized H_2O, aliquoted and stored at -20°C
Ponceau S red buffer	0.2% ponceau S red, 1% (v/v) acetic acid

Resolving gel buffer (4×)	1.5 M Tris-HCl, pH 8.8, 0.4% SDS
RIPA buffer	1% (v/v) NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 50 mM Tris pH 7.4 and 1 tablet/10 ml protease inhibitor cocktail
R_{max} (Calcium imaging)	150 mM NaCl, 5.4 mM KCl, 8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM glucose, 20 mM Hepes, adjust pH to 7.3 with Tris base
R_{min} (Calcium imaging)	130 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 20 mM glucose, 20 mM Hepes, adjust pH to 7.3 with Tris base
Roti-blue staining buffer	20 ml Roti-blue 5×concentrated; 20 ml methanol; 60 ml H_2O
SDS-PAGE loading (4×)	250 mM Tris-HCl, pH 6.8, 8% SDS, 20% mercaptal ethanol, 40% glycerol, bromphenol blue 0.004%, add H_2O up to 50 ml
SDS running buffer (10×)	75.7 g Tris, 360.3 g glycin, 250 ml 10% SDS, add H_2O up to 2.5 L
Sequence gel	32 ml SequaGel XR ready buffer, 8 ml SequaGel XR complete buffer, 300 µl APS (10%)
SOC-medium	2% trypton, 0.5% yeastextract, 10 mM $MgSO_4$×7 H_2O, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$×6 H_2O, 20 mM glucose in H_2O
SSC (20×)	175.4 g NaCl, 88.2 g sodium citrate, adjust pH to 7.2, add H_2O up to 1 L, treated with DEPC, sterilized by autoclave
Stacking gel buffer (4×)	0.5 M Tris-HCl, pH 6.8, 0.4% SDS
Stripping buffer	100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, adjust pH to 6.8
TBE (5×)	54 g Tris-base, 70 g boracic acid, 3.4 g EDTA, add H_2O up to 1 L
TE buffer	10 mM Tris-HCl, pH 7.4, 1 mM EDTA
T stock solution	10^{-3} M, 0.2884 mg/ml T in ethanol, freshly prepared
T-BSA-FITC solution	$1.5×10^{-3}$ M, 5 mg dissolved in 1.39 ml sterile PBS^+
TEN buffer	10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 8.0
Transfer buffer	200 ml methanol, 14.4 g glycin, 3 g Tris base, add H_2O up to 1 L, adjust pH to 8.3 with concentrated HCl
Tris-base (2M)	4.846 g Tris base dissolved in 20 ml H_2O, kept at 4°C
Trypan blue buffer	0.25% in dH_2O

TST (10×)	87 g NaCl, 60 g Tris, 5 ml Tween-20, add H_2O up to 1 L, adjusted to pH 7.5 with concentrated HCl
Washing buffer	0.5% BSA, 0.05% Tween-20 in PBS^+, pH 7.2
X-gal stock solution	X-gal 50 mg, add 1 ml N, N-dimethylformamid

2.2 Methods

2.2.1 Cell Culture

The murine macrophage cell lines RAW 264.7 (ATCC, TIB-71) and IC-21 (ATCC, TIB-186) were maintained in phenol red-free mycoplasma-tested low-endotoxin IMDM containing 5% heat-inactivated mycoplasma-free low-endotoxin FCS in 37°C, 5% CO_2 and humidified atmosphere. The cells were passaged twice per week with a split ratio of 1:5-6 for RAW264.7 cells or 1:3 for IC-21 cells. The cell numbers were determined using a Neubauer chamber with the cell viability of above 95% as assessed by the trypan blue exclusion method. Two weeks before the experiments, FCS was replaced with charcoal stripped FCS. For the experiments in which the cells would be serum-starved, the media were removed first, then the cells were washed twice with 37°C pre-warmed PBS^- and received appropriate volumes of the pre-warmed IMDM without serum.

The stable transfected RAW 264.7 cell lines were maintained in IMDM containing 250 µg/ml G418. For the steroid stimulation experiments cells used were passaged not more than 8 times. For calcium imaging, the cells were inoculated onto the gelatin-coated glass coverslips the day of the measurement.

2.2.2 Mycoplasma Test

In order to monitor a potential contamination of the cell culture with mycoplasma, 1 ml of the cell suspension (~10^6 cells) from the cell culture was inoculated separately into two 10 ml sterile tubes (Greiner, Frickenhausen, Germany) containing 5 ml of arginine medium or glucose medium respectively. The mixtures were incubated at 37°C in a bacteria incubator for 1 week, and the color of the medium was checked daily. The change of the color from yellow-orange to red in arginine medium or from red to yellow in glucose medium indicates the contamination of either of the two types of mycoplasma in the cell culture. All the cells used in the experiments were mycoplasma negative.

2.2.3 Preparation of Charcoal Stripped FCS

In order to eliminate the influence of endogenous steroids in serum, charcoal stripped FCS was prepared. 500 ml FCS was shaken with 1 g active charcoal and 0.1 g dextran for 30 min at 56°C, then centrifuged at 4,000×g for 1 h at room temperature. The supernatants were collected, shaken once again with the same amount of active charcoal and dextran for 30 min at 37°C. After centrifugation at 4,000×g for 1 h, the resulting supernatants were sterilized using a 0.22 μm pore sized filter, then aliquoted and stored at -20°C before use.

2.2.4 Stimulation of Cells with Steroids, FCS and LPS

For the experiments with steroid stimulation alone or those with steroids and charcoal-stripped FCS together, the cells were washed two times with pre-warmed PBS^-, then the cells received pre-warmed IMDM without serum. After 18 h of serum-starvation at 37°C 5% CO_2, the cells were treated with 10% charcoal-stripped FCS (v/v) for the indicated time periods as described in results. For the experiments with LPS stimulation, or steroids treatment combined with LPS, after the PBS^- washing, the cells were incubated in IMDM with 5% charcoal-stripped FCS for 18 h at 37°C, 5% CO_2. Then they were stimulated with 1 μg/ml LPS or LPS together with steroids for the indicated time periods as detailed in results.

2.2.5 Extraction of Total Proteins from Cells

A modified RIPA buffer (see 2.1.6) was used for the extraction of the total cellular proteins from the cells. It should be pointed out that the sodium orthovanadate (Na_3VO_4) used here has been activated for maximum inhibition of protein phosphotyrosyl-phosphatases following a special protocol. Briefly, 10 mM Na_3VO_4 solution was prepared, pH was adjusted to 10 using 1 N NaOH. The yellow solution was boiled until it turns clear, cooled to room temperature and readjusted to pH 10. The procedure was repeated until the solution remains clear and the pH stabilizes at 10, then the solution was aliquoted and stored at -20°C. The cell stimulation was stopped on ice, the media were aspirated from the culture. The cells were washed once with ice-cold PBS^- and lysed by adding ice-cold RIPA buffer. Then the cells were scraped off the plates with a cell scraper and the cell lysates were collected into the pre-chilled microcentrifuge tubes. After incubation on ice for 30 min, the cell lysates were then centrifuged at 14,000×g at 4°C for 15 min. The supernatants were collected into fresh microcentrifuge tubes. An aliquot of the cell lysates was removed for the

protein concentration determinations as described in chapter 2.2.6. The rest of the cell lysates were then boiled for 5 min in a Roti-load sample buffer, cooled down on ice immediately and centrifuged at 14,000×g for 2 min to remove cell debris. The samples will be applied to the sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) directly or stored at -80°C for a later application.

2.2.6 Determination of Protein Concentrations

A detergent-compatible Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL) was used for the determination of the protein concentrations, in which the bicinchoninic acid (BCA) formulation was used for colorimetric detection and quantification of total protein in dilute aqueous solutions. Briefly, the working reagent was prepared by mixing buffer MA, MB and MC with a ratio of 25:24:1, then the samples were mixed with equal volumes of the working reagent. After an incubation at 60°C for 60 min, the mixtures were cooled down to room temperature. The OD_{562} values were measured with a spectrophotometer (Beckman, DU®-640, Müchen, Germany) and the protein concentrations were determined according to the standard curve derived from BSA.

2.2.7 SDS-PAGE of Proteins

The Hoefer® Might small SE 245 dual gel caster (Amersham Pharmacia Biotech, Freiburg, Germany) was used to prepare the mini-SDS-polyacrolamide gels (8×10 cm). The resolving gel and the stacking gel were prepared as shown in Tab. 4. After the gel was completely polymerized (~30 min), it was mounted in a self-made vertical electrophoresis apparatus which matches Hoefer® Might small SE250 gels and the SDS-PAGE running buffer was added in the electrophoresis tank. The Teflon comb was removed carefully, the sample wells were washed with running buffer to remove any unpolymerized acrylamide. After the protein samples were applied to the wells, (usually 20 µl/well), the gel was run at 20 mA/gel, 16°C for 1.5 h with a PowerPac 300 power supply (Bio-Rad, Müchen, Germany). The prestained molecular weight markers (Sigma, St. Louis, MO, USA) were run parallel to monitor the migration of proteins and later to show the molecular weight of the interested protein bands.

Table 4. Solutions for Preparing Resolving and Stacking Gels of SDS-PAGE

Components	Resolving Gel				Stacking Gel
	15%	12.5%	10%	7.5%	5%
30% AA/BA	5.0 ml	4.2 ml	3.3 ml	2.5 ml	750.0 µl
4×Gel buffer*	2.5 ml	2.5 ml	2.5 ml	2.5 ml	1.25 ml
H_2O	2.5 ml	3.3 ml	4.2 ml	5.0 ml	3.0 ml
TEMED	10.0 µl	10.0 µl	10.0 µl	10.0 µl	10.0 µl
10% APS	100.0 µl	100.0 µl	100.0 µl	100.0 µl	20.0 µl

*Resolving or stacking gel buffer respectively

The table shows the solutions required for 2×mini-gels (8×10 cm).

2.2.8 Western blotting

The proteins were blotted with an electrical Fast Blot apparatus (Biometra, Goettingen, Germany) according to a semi-dry blot method (Sambrook *et al.*, 1989). Briefly 3 sheets of gel-sized Whatman 3MM paper (Whatman Ltd., Mainstone, UK) were socked in Transfer buffer (see 2.1.6), then placed on the anode plate. Wetted with deionized water, a piece of nitrocellulose membrane was placed on the stack of Whatman 3MM paper. When the electrophoresis of the SDS-PAGE (see 2.2.7) was completed, the glass plate was carefully removed. The stacking gel was cut away, the resolving gel was rinsed briefly with distilled water, then transferred onto the top of the nitrocellulose membrane. Another 3 layers of wet Whatman 3MM paper were placed on the gel, a glass pippet was rolled over the Whatman 3MM papers to remove air bubbles. The cathode plate was placed on top of the stack. A current of 60 mA/gel for 90 min was applied with the LKB ECPS power supply 3000/150 (Pharmacia Biotech, Freiburg, Germany). When the blotting was over, the membrane was stained with Ponceau S to check the protein transfer efficiency.

For immune detection, the membranes were rinsed briefly with distilled water. The non-specific binding sites were blocked by immersing the membrane in 1×Roti-block Reagent for 1 h at room temperature on an orbital shaker. After blocking, the membranes were incubated with primary antibody dissolved in TST (see 2.1.6) for 2 h at room temperature. Then the membranes were briefly rinsed with TST once and washed once for 15 min, twice for 5 min with fresh changes of TST buffer at room temperature. After 3 times washing, the membranes were incubated with the secondary antibody for 1 h and then washed 3 times with TST as described above. Sufficient detection buffer (0.125 ml/cm^2 membrane) was

prepared by mixing equal volumes of ECL detection solution 1 with solution 2. After the washing of the blots, the excess washing buffer from the membranes was drained and the membrane was placed on a piece of transparence wrap with the protein side up. The ECL detection buffer was added to the protein side of the membrane, so that the reagents were held by surface tension on the membrane. The membrane was incubated with the detection buffer for precisely 1 min at room temperature without agitation. Excess reagent was drained off and the membrane was wrapped between 2 sheets of transparence papers. Air pockets were gently smoothed out and the blots were placed in a Biomax MS^{TM} film cassette (Kodak, Rochester, NY, USA) with the protein side up. Then a sheet of Hyperfilm (Amersham, Braunschweig, Germany) was placed on top of the membrane and exposed for certain periods of time to get the desired signals.

After the first round of the immune detection, the blots could be stripped and reprobed again with other antibodies. The membranes to be stripped were kept wet wrapped in a 4°C refrigerator after each immunodetection. Membranes were submerged in the Stripping buffer (see 2.1.6) and incubated at 50°C for 30 min with occasional agitation, then washed with 50 ml TST twice, each 10 min and blocked with Roti-block for 1 h at room temperature. The subsequent immunodetection protocol was performed as described above.

After stripping and reprobing, the software Quantiscan (Biosoft, Cambridge, UK) was used to determine the quantification of the certain bands, and the activation of the certain kinase was calculated by the ratio of phosphorylated kinase protein/total kinase protein.

2.2.9 Transformation of *E. coli*

Subcloning Efficiency DH5α^{TM} Competent *E. coli* Cells (Gibco BRL, Karlsruhe, Germany) were used for the transformation. Usually they were aliquoted and stored at -80°C, before use, 20 µl of the competent cells were thawed on ice. Normally 5-15 ng ligated plasmid DNA (1/5-1/10 of the ligation mixture) were added into the cells, they were mixed by tapping the tube briefly. The cells were incubated on ice for 30 min, followed by a heat shock for 40 s at 42°C and incubation on ice for 2 min. After the addition of 80 µl SOC-medium, the cells were shaked for 1 h at 37°C, 280 rpm in a bacteria shaker. By using a sterile bent glass rod, the transformed cells were gently spread over the surface of the LB-agar plates which containing 50 µg/ml Ampicillin or other antibiotics with appropriate concentrations. The plate was left at room temperature until the liquid has been absorbed, then it was inverted and incubated at 37°C for 12-16 h.

2.2.10 Preparation of Plasmid DNA

The plasmid DNA was isolated from *E. coli* with the mini- and midi-plasmid preparation kits according to the manufacturer's instructions. Normally after transformation with a certain plasmid, the bacteria were shaked for 1 h at 37°C, then plated onto LB-Ampicillin plates. After an overnight-culture at 37°C, the single colonies were picked out from the plates, and inoculated into LB-medium with 50 µg/ml Ampicillin or other selective antibiotics. Then the bacteria cultures were shaked overnight at 280 rpm, 37°C in a bacteria culture shaker. Usually after the mini-preparations, the plasmids were analyzed with appropriate endonuclease restriction enzymes, then the plasmid samples containing both the predicted size and orientation of the fragments were sequenced as described in chapter 2.2.14.

2.2.11 Elimination of Endotoxin from Plasmid DNA

To eliminate the influence of endotoxin on the transfection experiments with macrophages, an AffinityPak™ Detoxi-Gel™ Endotoxin removing gel was used to remove endotoxin from plasmid DNA according to the manufacturer's instructions. First, the columns were regenerated before use by washing the gel with 5 column volumes (5 ml) of 1% sterile sodium deoxycholate, followed by 3 column volumes (3 ml) of pyrogen-free water to remove the detergent. 5 ml of DNA samples were applied to the gel, and collected with gravity flow, then the columns were regenerated again and the gels were equilibrated with 3 ml pyrogen-free water, sealed and stored at 4°C for a later use. The 5 ml of the flow-through were transferred into a fresh tube, 5 ml of isopropyl alcohol and 500 µl of 3 M sodium acetate were added to precipitate DNA. After incubated at -20°C overnight, the DNA was pelletted by centrifugation at 10,000 rpm (Beckman JS-13.1 Rotor, Müchen, Germany) for 30 min at 4°C. The supernatants were removed. The DNA pellet was washed once with 5 ml of 75% ethanol, vacuum dried, dissolved in endotoxin-free water and stored at -20°C. The concentrations were determined as described in chapter 2.2.12.

2.2.12 Determination of the Amount of Nucleic Acid

Quantification of the DNA and RNA preparations was performed with an ultraviolet spectrophotometer (Beckman DU® 640, Müchen, Germany). The readings were taken at the wavelengths of 260 nm and 280 nm. OD_{260} allows calculation of the concentration of nucleic acid in the samples. The ratio (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic

acid (Sambrook *et al.*, 1989). The concentration was calculated according to the following formulae:

DNA concentration (μg/ml) = OD_{260}×50×dilution factor

RNA concentration (μg/ml) = OD_{260}×40×dilution factor

All the samples used for experiments had OD_{260}/OD_{280} ratios between 1.8 to 2.0.

2.2.13 Agarose Gel Electrophoresis of DNA

The DNA fragments were separated in agarose gels using a standard method (Sambrook *et al.*, 1989). The concentrations of agarose (Biozym, Hess, Oldendorf) used were 0.8-2% depending on the size of the DNA fragments to be separated. The gels were prepared using 1×TBE and the same buffer was also used as the electrophoresis running buffer. The samples were mixed with 6×agarose gel loading buffer (Fermentas, St. Leon Rot, Germany), then applied into the agarose gels containing 0.5 μg/ml ethidium bromide. As the molecular stantards, λ-DNA digested with EcoRI/HindIII and pUC-mix 8 markers were run in parallel to show the sizes of the interested DNA fragments.

2.2.14 DNA Sequencing

Ligation of the inserts into the vectors and the restriction analysis of the plasmids were performed with the standard methods (Sambrook *et al.*, 1989) or according to the manufacturer's instructions. For DNA sequencing, all the PCR products were cloned into TOPO TA Cloning® vector, transformed into One Shot® chemical transformation competent cells (Invitrogen, Leek, NL), and sequenced with a LI-COR sequencer (MWG-Biotech, Ebersberg, Germany). Clones were sequenced with Thermo Sequenase fluorescent-labelled sequencing kit (Amersham) and analyzed with the LICOR sequencer. Briefly, a master mix was prepared: 15 μl of the plasmid DNA from mini preparation (MN), 5 μl H_2O, 4 μl of reverse and forward fluorescent-labeled primers (0.3 pmol/μl, synthesized by MWG-Biotech, Ebersberg, Germany), 2 μl of A, C, G and T were added to the 0.2 ml sequence tubes respectively, and mixed well. Then 5.6 μl of the master mix were added to the sequence tubes, centrifuged for 1 min, then 10 μl of mineral oil were added onto the reactions. The 2-step sequence reaction were performed with a PCR program: (1) 94°C, 1 min; (2) 50°C, 90 s; (3) 94°C, 30 s; (4) go to step (2) for more than 34 times; (5) 20°C, 1 h. After the reaction at 20°C, the sequence tubes were put on ice, then 4 μl of stop buffer were added into each of the reactions. After the centrifugation for 1 min at 5,000 rpm in a

Beckman GPKR centrifuge, the samples were stored at -20°C. 1.5 µl of the reaction were loaded onto the sequence gel and run in the 1×TBE. After electrophoresis, the nuclear sequences were analyzed with a BaseImage IR V 4.0 software (LI-COR).

2.2.15 Preparation of Nuclear Extract

The nuclear fraction of the cells was isolated with NE-PER™ Nuclear and Cytoplasm Extraction kit, which is a modification of Dignam's method (Dignam *et al.*, 1983). The treatments of the cells were stopped on ice, the mediums were removed and ice-cold PBS^- was added onto the cell monolayers. The cells were scraped off the dishes with a cell scraper, collected into ice-cold blue cap 50-ml Falcon tubes, then centrifuged for 5 min at 1,200 rpm and 4°C in a GPKR Beckman centrifuge. The cell pellets were resuspended in 1 ml ice-cold PBS^-, transferred into fresh eppendorf tubes, centrifuged in a microcentrifuge for 2 min at 4,000 rpm and 4°C, to collect the cells. The volumes of the cell pellets were estimated, and 500 µl ice-cold CER I were added to every 50 µl cell pellet. The cell suspensions were vigorously vortexed on the highest setting for 15 s, followed by a 10 min incubation on ice. Thereafter, 28 µl of ice-cold CER II were added into every 500 µl cell suspension with CER I, vortexed for 5 s on the highest setting, incubated on ice for 1 min, vortexed once again for 5 s, and centrifuged for 5 min at 14,000 rpm and 4°C in a microcentrifuge. The supernatants were collected as cytoplasmic fractions. The pellets were resuspended in 250 µl NER, then the mixtures were vortexed for 15 s on the highest setting and incubated for 10 min on ice. The procedure was repeated every 10 min, totally 40 min. After centrifugation for 15 min at 14,000 rpm and 4°C, the supernatants regarded as the nuclear fractions were transferred into the clean pre-chilled 1.5 ml microcentrifuge tubes, aliquoted into 200 µl PCR tubes and stored at -80°C before use.

2.2.16 Construction of *c-fos*-promoter-SEAP Plasmid

The *c-fos* promoter region from -520 to +109 was amplified from the mouse genomic DNA derived form the female C57BL/10 mice with an Expand™ High Fidelity PCR System (Roche). The primers used were *c-fos*-prom (+): 5'-TGA ATT CCA CTG GTG GGA GCT GCA GAG CAG AGC TGG G-3' and *c-fos*-prom (-): 5'-CGA ATT CTC CAG ATT GCT GGA CAA TGA CCC GGG TCT C-3'. The reaction contained 10µl of each primer, 2 µl of 10 mM dNTPs, 200 ng of genomic DNA, 10 µl of 10×Expand™ buffer, 0.75 µl Expand™ enzyme. The PCR program was: first 2 min at 94°C, then 5 cycles of 30 s at 94°C, 30 s at

55°C, 2 min at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 65°C, 2 min at 72°C, and a final extension step at 72°C for 10 min. The PCR products with EcoRI linker, were cloned into pSEAP-basic2 vector (Clontech, Heidelberg, Germany), and the construct was confirmed by sequencing. The plasmids for transfections were purified with a midi-plasmid DNA preparation kit, and the endotoxin contamination was removed with Detoxi-Gel columns (Pierce, St. Augustin, Germany) as described in chapter 2.2.11.

2.2.17 Establishment of Stable Transfected Cell Lines

The stable transfected cell lines were selected with the Neomycin analogue G418. Before the selection, 1×10^6 of the cells were inoculated into 100/20 mm tissue culture dishes (Greiner, Frickenhausen, Germany), cultured overnight at 37°C and 5% CO_2. The transfection was performed with a FuGENE^{TM}6 transfection reagent (Roche) according to the manufacturer's instructions. Briefly, the cells were transfected with 9 µg of *c-fos*-promoter-SEAP plasmid DNA together with 1 µg of GFP-N3 plasmid DNA which contains a neomycin resistant gene. 2 days later 250 µg/ml G418 were added to the culture dishes. The cells were cultured further for 2-3 weeks until the visible colonies appeared. The colonies were picked out with sterile pippets, inoculated into 48 well culture plates and later amplified in 100/20 mm dishes. The stable transfected cell clone stocks were prepared and stored in liquid nitrogen. The different *c-fos* promoter-SEAP stable transfected RAW 264.7 cell clones were screened for an optimal response to charcoal-stripped FCS. The clone 13 which has a maximum response to charcoal-stripped FCS was used for the experiments. The cells for the steroid stimulation experiments were passaged not more than 8 times.

2.2.18 *c-fos*-promoter-SEAP Assay

The *c-fos*-promoter-SEAP stable transfected cells were inoculated in a 24 well plate (No. 662160, Greiner, Frickenhausen, Germany), 5×10^5 cells/well and cultured overnight to allow the cells to adhere to the plates. On day 2, the media were removed and the cells were washed 2 times with 1 ml pre-warmed PBS^-. Then the cells received 1 ml pre-warmed IMDM with or without charcoal-stripped FCS and were cultured overnight at 37°C and 5% CO_2. On day 3, just before the stimulation, 100 µl of the supernatants from all the tested wells were collected as the samples for the relative light unit (RLU) at 0 h point (RLU_{0h}) and stored at -20°C. After 3 h stimulation, another 100 µl of the supernatants from all the test wells were harvested again as the samples for RLU at 3 h point (RLU_{3h}) and stored at -20°C.

SEAP activity was determined using a Phospha-Light kit (Tropix, Weiterstadt, Germany) as described previously (Krücken *et al.*, 1999). Briefly, 100 µl of the supernatants were diluted with 300 µl of the 1×Dilution buffer, then heated at 65°C for 30 min to inactivate the endogenous SEAP and cooled down on ice. The mixtures were centrifuged at 14,000×g for 15 min to remove the cell debris and 100 µl of the resulting supernatants were added to the luminometer tubes with 100 µl/tube. Subsequently 100 µl of Assay buffer were added to each tube and the mixtures were incubated for 5 min at room temperature. 100 µl of Reaction buffer were added to each tube, mixed well and incubated for 20 min. The samples were measured with a Lumat LB 9507 luminometer (EG&G Berthold, Germany). The RLU were calculated by the subtraction of $0.9 \times RLU_{0h}$ from RLU_{3h}, the values, after normalization with the control, represented the relative activity of *c-fos* promoter.

2.2.19 Preparation of Total RNA

TRIzol Reagent was applied to isolate total RNA from culture cells and tissues. Cell monolayers grown in 10 cm diameter dishes were washed once with ice-cold PBS^- and then lysed directly by adding 4 ml of TRIzol Reagent. The cells were homogenized by passing several times through a pipette and tissues (200 mg) were homogenized with an ultra-turrax. The lysates were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.8 ml of chloroform was added to the lysates and mixed by shaking vigorously for 15 s. Then the mixtures were incubated at room temperature for 2 to 3 min and centrifuged at 12,000×g for 15 min at 4°C. The colorless upper aqueous phases containing RNA were transferred to fresh tubes. 2 ml isopropyl alcohol were added to precipitate RNA. After incubated at room temperature for 10 min, RNA was pelletted by centrifugation at 12,000×g for 10 min at 4°C. The supernatants were removed. The RNA pellet was washed once with 4 ml of 75% ethanol, vacuum dried and dissolved in RNase-free water and stored at -80°C.

2.2.20 Reverse Transcription (RT) of RNA

Complementary DNA (cDNA) was synthesized using a reverse transcription reaction, which was setup in a sterile RNase-free 200 µl microcentrifuge tube. The system with a total volume of 20 µl contains 4 µl 5×RT buffer, 11 µl DEPC-H_2O, 1 µl RNase inhibitor (20 U/µl), 1 µl dNTPs (dATP, dGTP, dCTP, dTTP, 10 mM each, Roche Molecular Biochemicals), 1 µl random hexamers (50 µM), 2 µg of total RNA and 1 µl M-MLV reverse

transcriptase (200 U/µl). The reactions were mixed by tapping the tubes briefly and the cDNA were synthesized in a miniCycler™ programmable thermal controller (MJ Research Inc., Biozym Diagnostik GmbH, Hess, Oldendorf, Germany) with the following program: 22°C 10 min for equilibration, 42°C 60 min for reverse transcription, 95°C 5 min for denaturation, 4°C 30 min. The products were kept at -80°C before use.

2.2.21 Amplification of cDNA by PCR

The PCR reaction was used for the cDNA amplification. The following reagents were added into a sterile 0.5 ml microcentrifuge tube: 10 µl of 10×PCR buffer, 2 µl of dNTPs (mixture of dATP, dGTP, dCTP and dTTP, 10 mM each, Roche Molecular Biochemicals, Mannheim, Germany), 10 µl of each primer (1 µM), 0.5 µl of Taq DNA polymerase in storage buffer A (5 U/µl, Promega, Heidelberg, Germany), 5 µl of template cDNA and 56.5 µl of H_2O. The reactions were performed in a PTC-100™ programmable thermal controller (MJ Research Inc., Biozym Diagnostik GmbH, Hess, Oldendorf, Germany) using the following program: 1 min for denaturation at 95°C, 1 min for annealing at 56°C, and 1 min for extension at 72°C for 35 cycles, followed by a further extension at 72°C for 15 min. After amplification, 10 µl of PCR products were mixed with 2 µl 6×DNA gel loading buffer, then loaded on 2% agarose gel containing 0.5 µg/µl ethidium bromide and separated by electrophoresis in 0.5×TBE buffer at 80 mA for 1 h and visualized with an UV transilluminator.

After the separation in 2% agarose gels, the PCR fragments were purified using a PCR product purification Kit (Qiagen, Hilden, Germany) and cloned into TOPO TA cloning vector according to the manufacturer's instructions. Plasmid DNA was prepared using a MN mini plasmid purification kit (MN), then the restriction analysis was performed with EcoRI, and the clones with the correct size of the fragments were sequenced with LI-COR sequencer as described in chapter 2.2.14.

2.2.22 Online Sequence Comparison

After sequencing, the sequences of the PCR products were compared with the original sequences from GenBank database using Blast 2 comparison program (Tatusova and Madden, 1999) from national center for biotechnology information (NCBI), which is available online at http://www.ncbi.nlm.nih.gov/. The sequences of *Mus musculus* AR (GI 7304900), ERα (GI 193179) and ERβ (GI 1912467) were used for the alignment respectively.

Table 5. Primers used in RT-PCR for AR, ERα and ERβ

Receptor	Domain	Name	Sequence 5'-3'
AR	ARS1	P8-2062	GAA TGT CAG CCT ATC TTT CTT AAC G
		M1-2405	TGC CTC ATC CTC ACA CAC TGG C
	ARS2	P6-2084	ACG TCC TGG AAG CCA TTG AGC C
		M1-2622	GAA CCA CTC GAC CAT CTT CGC G
	ARS3	P3-2146	TCC TTT GCT GCC TTG TTA TCT AGC
		M1-2405	TGC CTC ATC CTC ACA CAC TGG C
	ARD1	P5-1543	GAC CTT GGA TGG AGA ACT ACT CCG
		M9-2031	GGT TGG TTG TTG TCA TGT CCG GC
ERα	ERαS1	P2-1434	ACA GGA ATC AAG GTA AAT GTG TGG
		M1-1807	CTC CAG GAG CAG GTC ATA GAG G
	ERαS2	P9-1350	GGC TGG AGA TTC TGA TGA TTG G
		M5-1935	GGG TAT GTA GTA GGT TTG TAA GG
	ERαD1	P16-589	CTA CTA CCT GGA GAA CGA GCC
		M21-1029	GAA GCA CCC ATT TCA TTT CGG C
ERβ	ERβS1	P7-710	CAA TGT GCT AGT GAG CCG TCC
		M4-1209	CTG CTG CTG GGA AGA GAT TCC
	ERβS2	P3-855	CAA GTC CGC CTC TTG GAA AGC
		M1-1160	CAT CTG TCA CTG CGT TCA ATA GG
	ERβD1	P5-224	CTT GCC TGT AAA CAG AGA GAC C
		M4-709	GAC GGC TCA CTA GCA CAT TGG

For each of the steroid hormone receptor, S1, S2, S3 and D1 represented steroid-binding domain 1, steroid-binding domain 2, steroid-binding domain 3 and DNA-binding domain 1, respectively.

2.2.23 Electrophoretic Mobility Shift Assay (EMSA)

The specific oligonucleotides used for the analysis of the major response elements on mouse *c-fos* promoter were synthesized by MWG Biotechnology GmbH, Ebersberg, Germany. They were purified with HPSF method and dissolved in sterile dH_2O forming a stock solution with a concentration of 100 pmol/µl. The sequences used in the experiments have been listed in Tab. 6.

2.2.23.1 Annealing of the Oligonucleotides

Before labeling of oligonucleotides, they were dissolved in sterile dH_2O with a concentration of 100 pmol/µl, and annealed. 5 µl of both sense and antisense single-stranded oligonucleotides were added to 90 µl of TEN buffer and mixed well. The annealing reactions were performed with the following PCR program: 95°C 5 min, 65°C 15 min, 50°C 15 min, 37°C 15 min, 20°C 15 min, 4°C 30 min. The resulting double-stranded oligonucleotides were stored at -20°C before use.

Table 6. Oligonucleotides for c-fos Promoter Analysis

Response Element	Type	Sequence 5'-3'
SIE	wild	GGC GAG CTG TTC CCG TCA ATC CCT CCC
	mutant	GGC GAG CTG **CCA** CCG TCA ATC CCT CCC
	consensus	GTG CAT TTC CCG TAA ATC TTG TCT ACA
SRE	wild	ACA GGA TGT CCA TAT TAG GAC ATC TGC G
	mutant	ACA GGA TGT CCA TAT TA**T T**AC ATC TGC G
	consensus	GGA TGT CCA TAT TAG GAC ATC T
CRE	wild	TTC CGC CCA GTG ACG TAG GAA GTC CAT
	mutant	TTC CGC CCA GTG **TG**G TAG GAA GTC CAT
	consensus	AGA GAT TGC CTG ACG TCA GAG AG
AP-1	consensus	CGC TTG ATG AGT CAG CCG GAA

Oligonucleotides sequences for SIE, SRE, CRE and AP-1 have been listed. For some response elements, 3 types of sequences, i.e. wild, mutant and consesus sequences have been shown. The sequences underlined show the core binding motifs for the certain response elements, the block letters show the mutations of the core binding motifs from the wild type mouse *c-fos* promoter sequences.

2.2.23.2 Labeling of Oligonucleotides

The double-stranded oligonucleotides were labeled using an end labeling kit (Fermentas, St. Leon Rot, Germany) according to the manufacturer's instructions. Briefly, the system had a total volume of 20 µl which contains 4 µl double-stranded oligonucleotides (5 pmol/µl), 2 µl of 10×PNK A buffer, 8 µl H_2O, 5 µl γ-ATP ^{32}P (10 µCi/µl) (activity>4000Ci/mmol, ICN Biochemicals Inc., Meckenheim, Germany) and 1 µl T4 polynucleotide kinase (7.5 U/µl). The reactions were mixed well by gentle pipetting, then incubated at 37°C for 10 min. The unincorporated nucleotides, salts and enzymes were removed with a QIAquick Nucleotide

Removal Kit (Qiagen, Hilden, Germany). Briefly, after reactions, 200 µl buffer PN were added into the 20 µl reaction sample and mixed, then the mixture was applied to the QIAquick column. After 1 min centrifugation at 14,000×g, the column was placed into a clean 2 ml collection tube and the old tube containing the radioactive flow-through was discarded appropriately. The column was washed with 500 µl buffer PE twice, the collection tubes with flow-throughs were discarded. After an additional centrifugation at 14,000×g for 1 min, the column was placed in a clean 1.5-ml microcentrifuge tube. 30 µl H_2O were added to the center of the QIAquick membrane, the column was standed for 1 min, centrifuged for 1 min at 14,000×g to collect the ^{32}P labeled samples. 2 µl of the sample were counted with a Beckman LS Analyzer counter (Beckman, Müchen, Germany) for 1 min. Normally it is about 100,000-600,000 cpm/2 µl ready solution and 80,000 cpm with the volume of 1 µl was used for each gel shift binding reaction.

2.2.23.3 Binding Reaction

Before the binding reaction was setup, the 4-6% native acrylamide gel was prepared using 0.5×TBE. The pre-electrophoresis was carried out for 1 h in pre-cooled 0.5xTBE running buffer. The binding reaction containing a total volume of 20 µl was setup in 1.5 ml microcentrifuge tubes as following: 4 µl of 5×binding buffer (100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% (w/v) Tween-20, 150 mM KCl), 1 µl of poly dI-dC (1 µg/µl), 1 µl of poly-L-lysine (0.1 µg/µl), x µl of distilled H_2O, 5 µg of total nuclear protein and 1 µl γ-ATP ^{32}P-labeled oligonucleotides, mixed carefully by pipetting and incubated for 30 min at room temperature.

For the competitive binding reactions, 10- or 100-fold excess of the unlabeled oligonucleotides were used for the binding reaction. For the experiments with supershift, the nuclear protein was preincubated with the appropriate antibody for 20 min at room temperature and then incubated with ^{32}P labeled oligonucleotides for 30 min as described above.

2.2.23.4 Electrophoresis and Autoradiography

The native PAGE was prepared according to a standard method. The solutions required has been listed in Tab. 7. The binding reaction was stopped on ice, then 5 µl of loading buffer (60% (v/v) 0.5×TBE, 40% (v/v) glycerol) was added to the tube and mixed well with pippeting. Then the samples were applied immediately to the pre-electrophoresed

polyacrolamide gel. Before loading, the sample wells were cleaned from fuzzy pieces of polyacrylamide to insure sample application without diffusion. The gel was run in a pre-cooled 0.5×TBE running buffer at 10V/cm until the bromophenol blue dye reaches 3/4 of the way to the bottom of the plates. The gel was wrapped with fresh plastic membranes, placed into the Kodak film cassettes with a Biomax enhancer screen (Kodak, Rochester, NY, USA), then exposed to a Biomax MS^{TM} film (Kodak) for certain periods of time, it usually takes from 1 h to 1 day depending on the intensity of the signals.

Table 7. Solutions for Native PAGE

Components	Native Polyacrlamide Gel			
	4%	5%	6%	8%
30% AA/BA	4.0 ml	5.0 ml	6.0 ml	8.0 ml
5×TBE buffer	3.0 ml	3.0 ml	3.0 ml	3.0 ml
H_2O	23.0 ml	22.0 ml	21.0 ml	19.0 ml
TEMED	45.0 µl	45.0 µl	45.0 µl	45.0 µl
10% APS	225.0 µl	225.0 µl	225.0 µl	225.0 µl

The table shows the solutions required for 1 gel (18×20 cm), total volume 30 ml

2.2.24 Occurrence of iAR and iER

These experiments were performed with both intact and permeablized macrophages. The latter were fixed with 0.5% PFA in PBS$^+$ at 37°C for 30 min, then diluted to 10^7 cells/ml in PBS$^+$ supplemented with 0.5% BSA and 0.05% Tween-20. Aliquots of 150 µl were centrifuged for 10 min at 12 00 rpm (GPCR, Beckman, München, Germany), and the cell pellets were incubated at room temperature for 1 h with 200 µl of the anti-AR antibody AR (N-20) (1µg/ml; Santa Cruz Biotechnology, Heidelberg, Germany) or the anti-AR antibody AR(C-19) (0.5µg/ml; Santa Cruz Biotechnology), as well as the different ER-antibodies ERα (MC-20), ERα (H-184), and ERβ (Y-19) (all Santa Cruz Biotechnology, Inc.) in concentrations of 2 µg/ml for 1 h at room temperature. Anti rabbit IgG (whole molecule) FITC conjugate (working dilution 1:320; Sigma, Deisenhofen, Germany) and a donkey anti goat-FITC antibody (working dilution 1:100; gift from P. Traub, MPI for Cell Biology, Ladenburg, Germany) were used as secondary antibodies for 45 min. In some experiments, the reactions with the anti-AR and anti-ER antibodies were performed in the presence of 10-

fold blocking peptides AR (N-20)P, AR (C-19) and ER (MC-20)P, repsectively (Santa Cruz Biotechnology, Heidelberg, Germany).

2.2.25 labeling with Hormone-BSA-FITC

Macrophages were washed twice with PBS^+, and incubated at the indicated temperatures for varying periods with $1.5x10^{-5}$ M T-BSA-FITC, E_2-BSA-FITC or with BSA-FITC or BSA alone as controls. For internalization experiments, intact macrophages were incubated at room temperature or at 37°C for 1 min up to 1 h with T-BSA-FITC, E_2-BSA-FITC, BSA-FITC or ConA-rhodamine (1:50) or a rat anti-mouse F4/80 antibody (2 µg/ml, gift from H. Mossmann, MPI for Immunology, Freiburg, Germany) and with Biotin-SP-conjugated AffiniPure mouse antirat IgG (H+L) (1:500 30 min; Jackson ImmunoResearch Laboratories, Inc., WestGrove, PA) as a secondary antibody and streptavidin-fluoresceine (6 µg/10^7 cells 10 min; Amersham Pharmacia Biotech, Braunschweig, Germany). Colocalization was performed in in tact cells using LysoTracker Red DND-99 (10 µM; Molecular Probes, Inc., Göttingen, Germany) or transferrin conjugated with tetramethylrhodamine (20 µg/ml: Molecular Probes). Then, the samples were postfixed with 1% paraformaldehyde (PFA). Cells prefixed with 0.5% PFA were incubated for 1 h with the anticlathrin antibody HC (N-19) (2 µg/ml; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and with a donkey antigoat-Cy3 antibody (1:200; gift from P. Traub, MPI for Cell Biology, Ladenburg, Germany) as secondary antibody, or for 1 h with the anti-coveolin antibody caveolin-1 (N-20) (2 µg/ml; Santa Cruz Biotechnology, Inc.) using a TRITC-conjugated AffiniPure goat antirabbit IgG (H+L) antibody (1:80 for 45 min; ImmunoResearch Laboratories, Inc.). The cells were postfixed with 3% PFA.

2.2.26 Flow Cytometry

Aliquots of 150 µl IC-21, RAW264.7 and RAW-fos13 macrophages (10^7 cells/ml in PBS^+) were centrifuged, and the cell pellets were labeled as described above. Cells were analyzed in a FACScan (Becton Dickinson, Sunnyvale, CA, USA) with a sample size of 10,000 cells gated on the basis of forward and side scatter. The data were stored and processed using the FACScan software as described previously (Benten *et al.*, 1991).

2.2.27 Confocal Laser Scanning Microscopy (CLSM)

Preparation of coverslips: microscopical cover-glasses (Assistant® No.1014, 26×21 mm, 0.17±0.01 mm thick, Germany), were sterilized with brief immerse into 70% ethanol and dried in the sterile air. They were then coated with 500 µl poly-L-lysine buffer for 5 min, then washed 3 times with dH_2O and dried in the air under bacteria-free condition. Afterwards, they were preserved in sterile 35/10 mm culture dishes (Greiner, Frickenhausen, Germany) before use.

IC-21 and RAW 264.7 macrophages (2×10^6 cells/ml) were allowed to adhere onto poly-L-lysine-coated glass coverslips overnight, then labeled as described above, and embedded in a 1:1 (v/v) mixture of glyercol and vectashield (Vector, Burlingame, CA, USA) containing 2% (w/v) 1,4-diazabicyclo-[2.2.2]octane (DABCO) (Merck, Darmstadt, Germany) and sealed with nail polish as described previously (Benten *et al.*, 1999b). The confocal laser scanning microscope Leica TCS NT, version 1.5.451 (Leica Lasertechnik, Heidelberg, Germany) was used for analysis of the specimens with FITC fluorescence excitation at 488 nm or Cy3, rhodamine and TRITC fluorescence at 568-nm, respectively. Z-series optical sections were taken at 0.5 µm intervals and evaluated using Adobe Photoshop 5.0 for Windows (Adobe Systems, Mountain View, CA, USA) and CorelDRAW 8 for windows (Corel, Ottawa, Ontario, Canada).

2.2.28 Cell Proliferation Assay

Quantitative determination of cellular proliferation was performed with a cell proliferation kit II (XTT, Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. Briefly, the cells prepared for the proliferartion were inoculated in 96 well plates at a concentration of 2×10^4 cells/100 µl with 100 µl/well. Thereafter, the cells were incubated at 37°C 5% CO_2 and humidified atmosphere overnight, washed with 100 µl pre-warmed PBS twice, then incubated in IMDM without FCS. After serum starvation for 18 h, the cells were stimulated with T, E_2 and charcoal-stripped FCS, which were dissolved in 50 µl medium, for the appropriate time intervals. XTT labeling reagent and electron-coupling reagent were thawed in 37°C water bath and mixed immediately before use. According to the manufacturer's instructions, 8 ml XTT were mixed with 0.16 ml electron-coupling reagent, the mixture was then applied to the cells in 96-well cell culture plates with 75 µl/well. The spectrophotometrical absorbance of the samples were measured with an Anthos ELISA Reader 2001 (Anthos, Cologene, Germany). The measure-wavelength is 450 nm and

the reference-wavelength is 620 nm, all the experiments were made in triplicate and the results were from 3-4 independent experiments.

2.2.29 Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by using the ratiometric fluorescent indicator dye Fura-2/AM, the membrane-permeant acetoxymethyl ester form of Fura-2. When added to cells, the lipophilic Fura-2 ester diffuses across the plasma membrane and was hydrolyzed to Fura-2 by cytosolic esterases. After hydrolysis, Fura-2 can not cross the plasma membranes and remains in the cytosol. Now the concentration of $[Ca^{2+}]_i$ can be continuously monitored by the fluorescence of Ca^{2+}-bound Fura-2 at 340 nm and Ca^{2+}-free Fura-2 at 380 nm for excitation and 510 nm for emission. The IC-21 and RAW 264.7 macrophages were routinely grown in 100/10 mm culture dishes (Greiner, Frickenhausen, Germany) until 80% confluent, then scraped off the dishes with a cell scraper. They were washed twice with HEPES buffer (see 2.1.6), each time for 10 min at 1,200 rpm and room temperature by using the GPKR centrifuge (Beckman, Müchen, Germany). Then they were loaded with 1 μM Fura-2/AM in the same HEPES buffer at room temperature for 30 min or 1 h respectively. After loading with Fura-2, the cells were kept in HEPES buffer in the dark at room temperature until used for the $[Ca^{2+}]_i$ measurement (within 1 h). Then 2 ml of the cell suspension which containing 5×10^6 cells were placed in the standard 1 cm square measuring quartz-cuvette. The Ca^{2+} measurements were performed in a PE LS 55 Luminescence spectrometer (Perkin Elimer, Weiterstadt, Germany) at room temperature according to the manufacturer's instructions. Usually, measurements start after 5 min of equilibration, when the basal line for $[Ca^{2+}]_i$ becomes stable. The reagents were added directly to the cuvette under continuous stirring. With the FL WinLab software (Perkin Elimer) the $[Ca^{2+}]_i$ was calculated from the ratio of 340:380 nm fluorescence values as described previously (Grynkiewicz *et al.*, 1985) using the following formula:

$$\left[Ca^{2+}\right]_i = K_d \cdot \frac{\left(R_t - R_{\min}\right)}{\left(R_{\max} - R_t\right)} \cdot \frac{S_{f380}}{S_{b380}}$$

The dissociation constant (K_d) for the Fura-2-Ca^{2+} complex was taken as 224 nM. The values for R_{max} and R_{min} were calculated from measurements using 25 μM digitonin and 4 mM EGTA and enough 2 M Tris base to raise the pH to 8.3 or higher as described

previously (Benten *et al.*, 1999b). R_t is the fluorescence intensity ratio for the dye at time *t*. The ratio S_{f380}/S_{b380} is the ratio of 380 nm fluorescent intensities of the free and bound ion probe. It is a factor that is added to the equation to allow it to more readily cancel out any instrument dependent artifacts associated with the equation extremes.

2.2.30 Single Cell Calcium Imaging

2.2.30.1 Preparation of Coverslips

Microscopical cover-glasses (Assistant No. 1001, 42 mm Φ, Germany) were sterilized with briefly immerse into 70% ethanol, dried in the sterile air, then immersed into sterile 1% gelatin, dried in the air under bacteria-free condition, then preserved in 60/15 mm culture dishes (Greiner, Frickenhausen, Germany) before use.

2.2.30.2 Inoculation of the cells

The cells were inoculated onto the coverslips in IMDM medium with a cell concentration of 1×10^6 cells/ml, 1 ml/coverslip. After incubation at 37°C and 5% CO_2 for 2 h, the cells received 1 ml pre-warmed IMDM more and were further incubated at 37°C, 5% CO_2 and humidified atmosphere in a cell culture incubator until use.

2.2.30.3 $[Ca^{2+}]_i$ Imaging

Cells were grown on gelatin-coated glass coverslips until 60% confluence. Then they were washed twice with 2 ml pre-warmed HBS-Na^+ buffer and loaded with 1 μM Fura-2/AM for 1 h at 37°C and 5% CO_2. The coverslips were carefully rinsed with 2 ml of pre-warmed HBS-Na^+ twice, mounted onto the chamber, then received 1.2 ml pre-warmed HBS-Na^+. The cells were observed with an inverse microscope (Axiovert, Zeiss, Oberkochen, Germany) and parallel with a digital Imaging-fluorescence microscope (Photon Technology International Inc., New York, NJ, USA). Single cells were gated for Ca^{2+} imaging and 300 μl of the HBS-Na^+ were removed from the micro-incubator chamber carefully, then 300 μl of the steroids and other reagents dissolved in HBS-Na^+ were added carefully into the chamber. The Image Master 1.333-software for Windows was used for the calcium imaging and analysis. The Fura-2 fluorescence was measured at 340 nm (Calcium bound Fura-2) and 380 nm (free Fura-2) for excitation and 510 nm for emission. The $[Ca^{2+}]_i$ was calculated from the ratio of 340/380 nm ("background corrected Images") fluorescence values as

reported previously (Grynkiewicz *et al.*, 1985). The raw data were then imported into Excel 2000 and the mean and standard derivation (SD) of the fluorescence ratios from different cells were calculated as described recently (Saeger *et al.*, 2001).

2.2.31 Measurement of NO

It has been reported that the primary decomposition product of NO in hemoglobin-free solution is nitrite (Ignarro *et al.*, 1993) and more than 95% of total NO released from activated macrophages will be converted to nitrite in the culture medium (Chang *et al.*, 1998). Therefore nitrite accumulation in the supernatant of cell culture was determined and used as an index of NO production. The Griess reagent was used for the NO measurement as described previously (Krücken *et al.*, 1999). Briefly, the supernatants from the tested cells were centrifuged for 10 min at 14,000×g to remove the cell debris and mixed with an equal volume of Griess reagent. Nitrite concentration was calculated from the A_{540} by reference with a $NaNO_2$ standard curve in culture medium.

2.2.32 Statistical Analysis

Unless otherwise mentioned, each data was normalized to the control value, the mean and standard error of mean (SEM) from at least three experiments were determined. The data were analyzed by using Excel 2000 (Microsoft, US) and SPSS 10.0 for Windows (SPSS Science, Chicago, IL, USA).

3 RESULTS

3.1 Nogenomic T-Signaling in IC-21 Macrophages

3.1.1 Absence of Intracellular AR (iAR)

Initially the macrophage cell line IC-21 was used for the study of T action, because this cell line was derived from C57BL/6 mice, which has a similar genetic background as C57BL/10 mice which we used in the previous studies on T. According to the traditional model, T acts through iAR. In order to detect the iAR in IC-21 cells, both intact as well as permeabilized cells were investigated by flow cytometry using the anti-iAR antibody AR (N-20), which is directed against an epitope corresponding to the amino acids 2-21 mapping at the amino terminus of the iAR. Incubation of intact IC-21 cells with this antibody did not result in any significant labeling of the cells (Fig. 2A). After permeabilization of IC-21 cells, incubation with AR (N-20) resulted in a slight increase in fluorescence intensity. However, this fluorescence was not iAR-specific since it could not be competitively displaced by an AR (N-20)-specific blocking peptide (Fig. 2A). Also, the anti-AR antibody AR (N-20) could not detect any ARs in IC-21 cells using Western blots, though this antibody reacted with the iAR-band at 110 kD in AR-expressing human prostate cancer LNCaP cells (Fig. 2B) (Taplin *et al.*, 1995). Moreover, RT-PCR was used to detect AR mRNA in IC-21 cells and in mouse testes as a control. Using primers spanning the DNA-binding domain and three different regions from the carboxy terminus of the AR, RT-PCR revealed the expected bands in testes, but not in IC-21 macrophages (Fig. 2C). DNA sequencing confirmed that the PCR-fragments derived from testes RNA contained the predicted regions of the AR. Moreover, the RNA isolated from IC-21 cells was intact, since the low abundant mRNA of the single-copy gene *mzfm* (Wrehlke *et al.*, 1999) could be amplified by the same RT-PCR procedure using two different primer pairs (Fig. 2C).

3.1.2 T-Induced Ca^{2+} Mobilization

IC-21 cells were loaded with Fura-2 to determine the effect of T on $[Ca^{2+}]_i$. T at the physiological concentration of 10 nM triggered an immediate spike in $[Ca^{2+}]_i$ which represented a Ca^{2+} increase by about 100 nM (Fig. 3A). Such a spike was also induced when

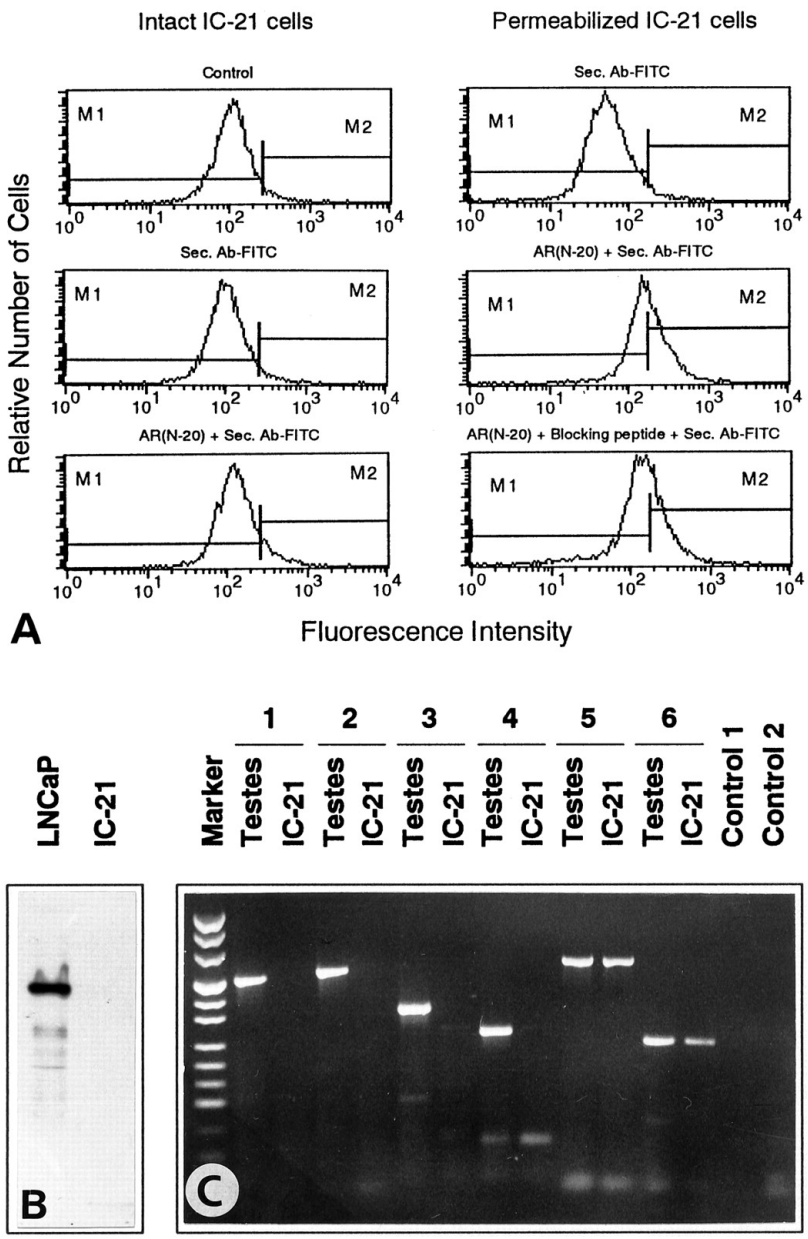


Figure 2. Absence of iAR in IC-21 cells. (**A**) Flow cytometry of intact and permeabilized IC-21 cells incubated for 1 hour with the anti-iAR antibody AR (N-20) and secondary fluorescent antibody (Sec. Ab-FITC). In permeabilized cells, the blocking peptide AR (N-20)P cannot competitively displace the slight increase in fluorescence of AR (N-20). (**B**) IC-21 cells and LNCaP cells as a control were subjected to Western blotting using the anti-iAR antibody AR (N-20) and the ECL detection system. Only LNCaP cells reveal the AR band at 110 kDa. (**C**) RT-PCR with RNA isolated from mouse testes and IC-21 cells. The primer pair ARD1 (1) spanned a 511 bp region of the DNA-binding domain of iAR. The primer pairs ARS2 (2), ARS1 (3), and ARS3 (4) spanned regions of the carboxy terminus of the iAR with 560 bp, 365 bp and 281 bp, respectively. The expected bands were only revealed in testes. The primer pairs mzfm-P1/mzfm-M1 (5) and mzfm-P2/mzfm-M2 (6) yielded bands of 640 bp and 253 bp, respectively, of the low abundant mRNA of the gene *mzfm* in both testes and IC-21 cells. Control 1: RT-PCR without primers; control 2: PCR with the primer pair ARD1 without RNA.

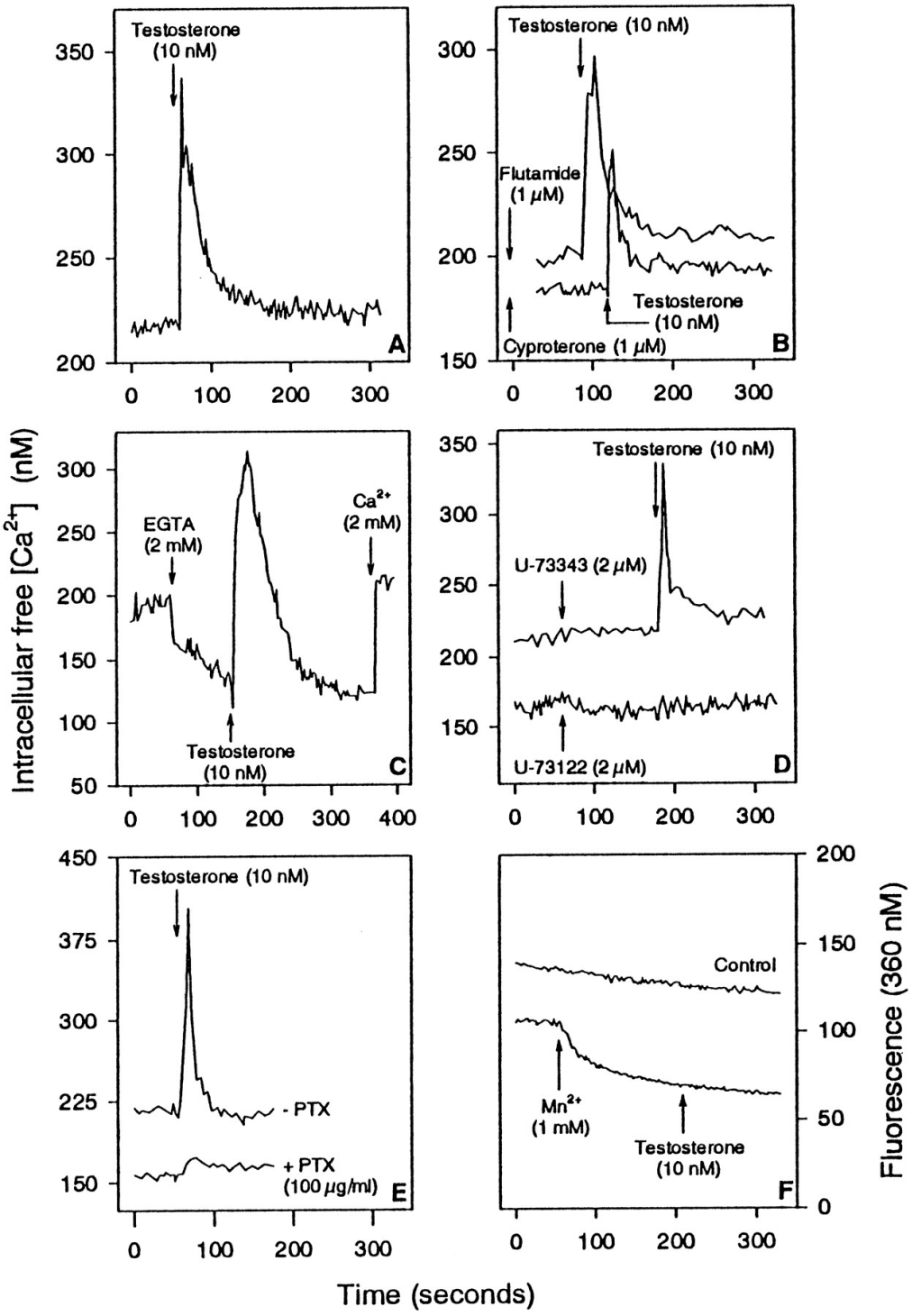


Figure 3. T-induced Ca^{2+} mobilization in IC-21 cells. (**A**) T causes an immediate Ca^{2+} spike. (**B**) Cells were treated with cyproterone for 30 min or flutamide for 60 min, before adding T. (**C**) Cells were incubated with EGTA for 90 s before addition of T. (**D**) Cells were treated with the phospholipase C inhibitor U-73122 or with the inactive control compound U-73343 for 2 min before adding T. (**E**) Cells were pretreated with pertussis toxin for 16 h (+ PTX) before addition of T. (**F**) Cells were incubated with Mn^{2+} for 2 min before adding T. Arrows indicate addition of substances to IC-21 cells.

the cells were preincubated with the iAR-blockers cyproterone and flutamide in excess (Fig. 3B). The Ca^{2+} increase may be due to influx of extracellular Ca^{2+} and/or release of Ca^{2+} from intracellular Ca^{2+} stores. To test this, extracellular Ca^{2+} was removed by EGTA, before T was added. T was still able to induce the Ca^{2+} spike (Fig. 3C). However, the T-induced Ca^{2+} spike was totally abolished by the direct phospholipase C inhibitor U-73122, but not by the inactive control compound U-73343 (Figure 3D). Also, the Ca^{2+} spike could be inhibited by pertussis toxin (PTX), an inhibitor of G-proteins (Figure 2E). Mn^{2+} did not induce any quenching after T treatment (Fig. 3F). These data indicate that a PTX-sensitive G-protein coupled to PLC is involved in the Ca^{2+} response to T.

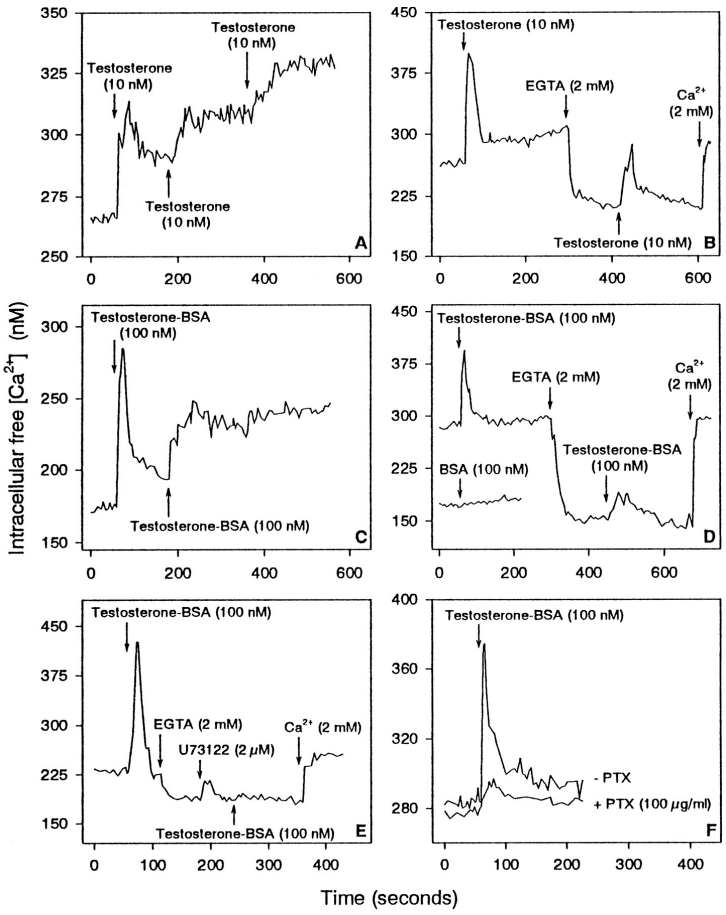


Figure 4. Ca^{2+} responses of IC-21 cells to T and T-BSA. (**A**) A second or third addition of T shortly after the first treatment induces a sustained increase in $[Ca^{2+}]_i$ rather than a Ca^{2+} spike. (**B**) Cells were treated for 4 min with T, then for 2 min with EGTA before the second addition of T that induced only a Ca^{2+} spike. (**C**) The first addition of T-BSA induced a Ca^{2+} spike whereas the second addition induced a sustained Ca^{2+} increase. (**D**) BSA alone had no effect on $[Ca^{2+}]_i$ (lower line). After incubation with T-BSA for 2 min, cells were treated with EGTA before the second addition of T-BSA. (**E**) After incubation of cells with T-BSA, the cells were pretreated with both EGTA and U-73122 before second addition of T-BSA. (**F**) Cells were treated with pertussis toxin for 16 h (+ PTX) before adding T-BSA. Arrows indicate addition of substances to IC-21 cells.

However, when T was added for a second or third time shortly after the first addition, it induced a prolonged elevation of $[Ca^{2+}]_i$ instead of a Ca^{2+} spike (Fig. 4A). This prolonged elevation in $[Ca^{2+}]_i$ was due to both Ca^{2+} release and Ca^{2+} import, since, after removal of extracellular Ca^{2+}, treatment with T resulted only in a Ca^{2+} spike instead of a prolonged elevation (Fig. 4B).

T coupled to bovine serum albumin (BSA), which is not freely permeable through the cell membrane, had the same effects on $[Ca^{2+}]_i$ as free T. It induced first a Ca^{2+} spike, whereas a second addition caused a prolonged elevation of $[Ca^{2+}]_i$ (Fig. 4C). The Ca^{2+} spike was only due to Ca^{2+} release from intracellular Ca^{2+} stores, whereas the prolonged elevation of $[Ca^{2+}]_i$ was due to both Ca^{2+} release and Ca^{2+} import (Fig. 4D and E). Moreover, pertussis toxin blocked the T-BSA-induced mobilization of intracellular Ca^{2+} (Fig. 4F).

The amounts of released Ca^{2+} induced by the first addition of both T and T-BSA increased with increasing concentrations, reaching apparently saturation at about 10 nM T and 100 nM T-BSA, respectively (Fig. 5A). Moreover, cells responded to a second addition of T again with a Ca^{2+} spike, when the period between first and second addition exceeded at least 10 min (Fig. 5B).

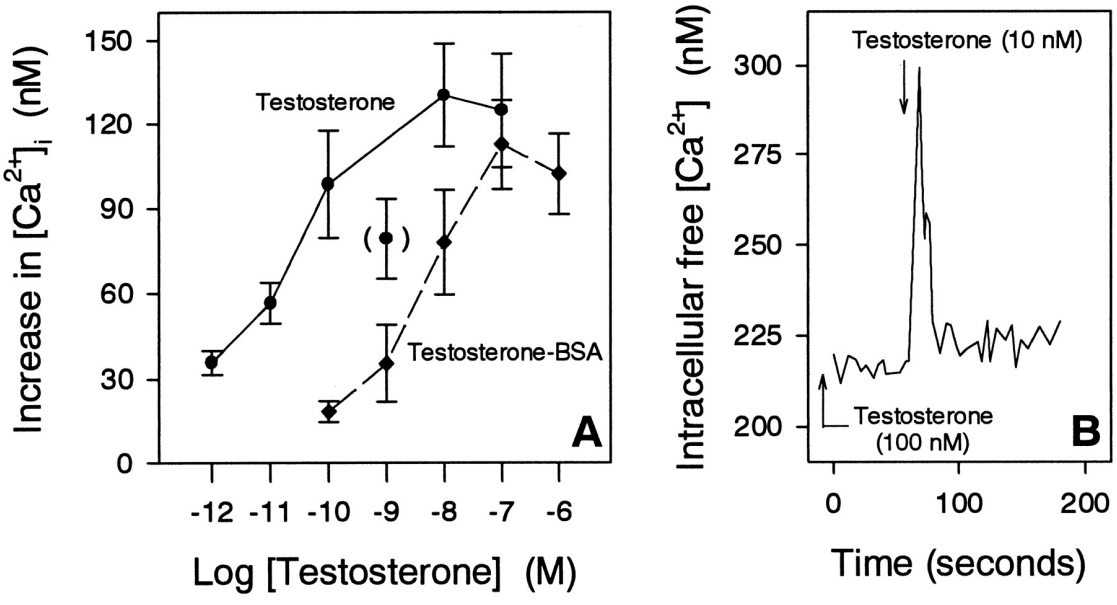


Figure 5. Effects of different concentrations of T and T-BSA and T pretreatment on $[Ca^{2+}]_i$ of IC-21 cells. (**A**) Increase in $[Ca^{2+}]_i$ with increasing concentrations of T and T-BSA, respectively. (**B**) Cells were pretreated with T for 60 min before adding T for a second time.

3.1.3 Surface Binding of T

T binding sites were identified on the surface of intact IC-21 cells. When cells were incubated with the impeded ligand T-BSA coupled to fluoresceine isothiocyanate (FITC) for 5 s, flow cytometry revealed an increase in fluorescence intensity (Fig. 6A). Incubation with BSA-FITC alone or together with free T did not result in any significant labeling in comparison to unlabeled control cells (cf. also Fig. 7A). Confocal laser scanning microscopy (CLSM) detected the fluorescence of the bound T-BSA-FITC exclusively on the surface of IC-21 cells (Fig. 6B). The same labeling pattern on the surface showed the plasma membrane marker ConA-rhodamine (cf. also Fig. 7B). There is some evidence that the sex hormone binding globulin (SHBG) can bind to specific receptors on the plasma membranes, which are able to mediate rapid effects of T and estradiol (Rosner *et al.*, 1998). However, the surface of IC-21 cells had not bound any significant amounts of SHBG as identified by flow cytometry and CLSM using an anti-SHBG antibody (data not shown).

3.1.4 Selective Internalization of Surface membrane T receptors (mARs)

There is evidence that G-protein-coupled surface receptors can be sequestered (Koenig *et al.*, 1997). In order to identify such a possible sequestration of mARs, IC-21 cells were incubated with T-BSA-FITC between 5 s and 1 h and analyzed by flow cytometry and CLSM. Flow cytometry revealed an increased labeling with progressive incubation periods (Fig. 6A). When incubation lasted for 5 s or 1 min, more than 80% of the cells were labeled with T-BSA-FITC. After 5 min, however, the percentage of labeled cells was increased to more than 95%, and the fluorescence intensity of the cells was higher. Thereafter, the number of fluorescent cells remained about the same, whereas the fluorescence intensity of the cells still increased with progressing incubation times, reaching a maximum after about 1 h. Obviously, cells bound increasing amounts of T-BSA-FITC with progressing incubation times. In parallel with the increase in fluorescence intensity, CLSM revealed an increasing punctate fluorescence inside cells (Fig. 6B). Whereas the fluorescence was exclusively localized on the cell surface after 5 s and 1 min, punctate weak fluorescence emerged after 5 min inside cells at their periphery, besides surface fluorescence. After 15 min and 1 h, the punctate fluorescence was increased in intensity and was distributed throughout the whole cytoplasm. The internalization of T binding sites was selective. BSA alone or BSA-FITC did not induce any sequestration (Fig. 7A). Also, when cells were incubated with free T together with BSA-FITC for 15 min, there was no sequestration, though sequestration was observed when cells were incubated in parallel with T-BSA-FITC (Fig. 7A and B). Moreover,

internalization occured neither with surface-bound ConA-rhodamine nor with the macrophage specific surface marker F4/80 identified by a rat monoclonal antibody against F4/80. Even if the surface labeling of IC-21 cells was performed in the presence of T, there was no internalization of ConA-rhodamine and F4/80 (Fig. 7B).

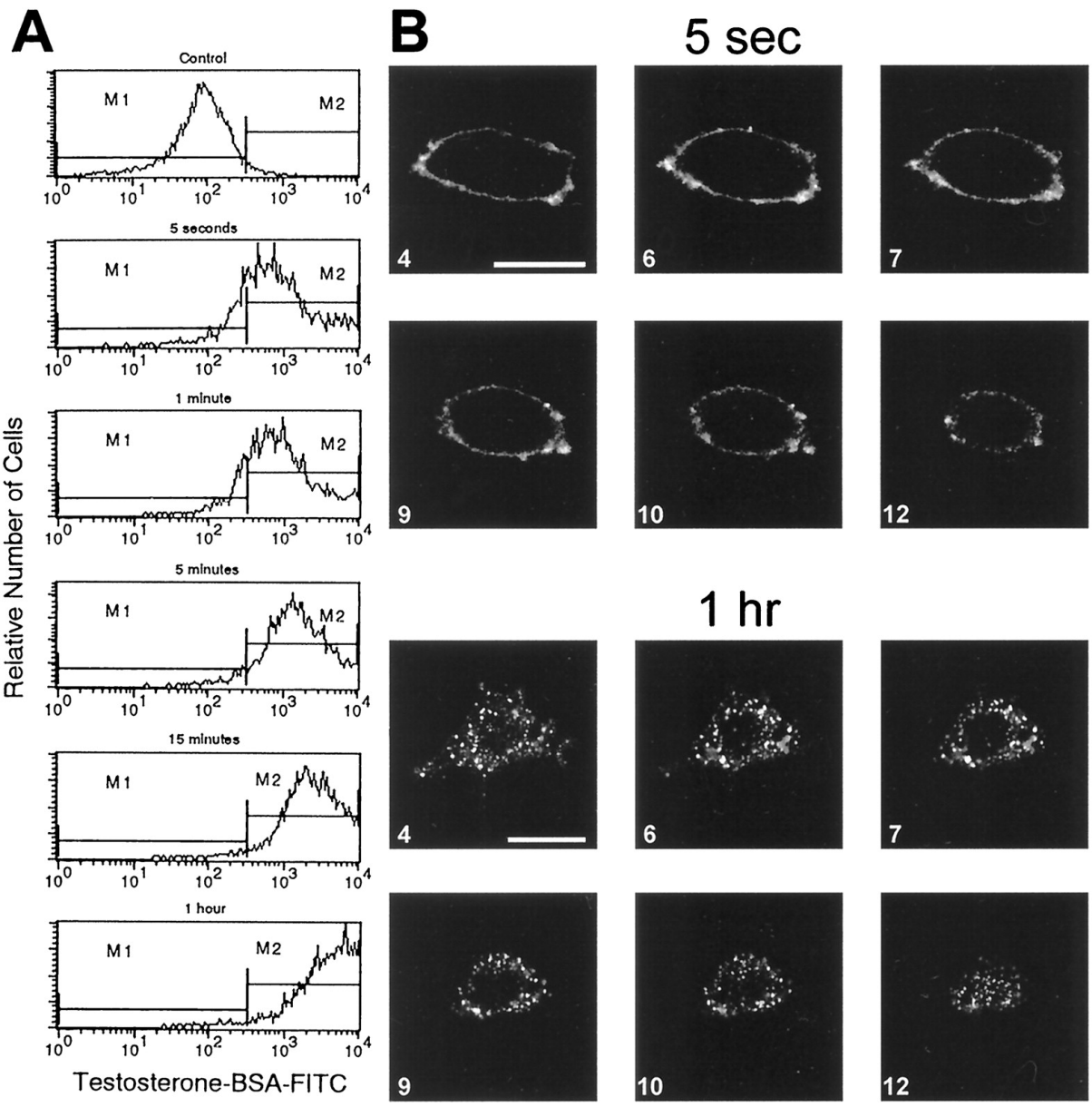


Figure 6. Surface binding sites of T and Their internalization in intact IC-21 cells. (**A**) Cells were incubated with T-BSA-FITC for various periods between 5 seconds and 1 hour and then analyzed by flow cytometry. (**B**) CLSM of cells incubated with T-BSA-FITC for 5 seconds and 1 hour. Bars indicate 10 µm.

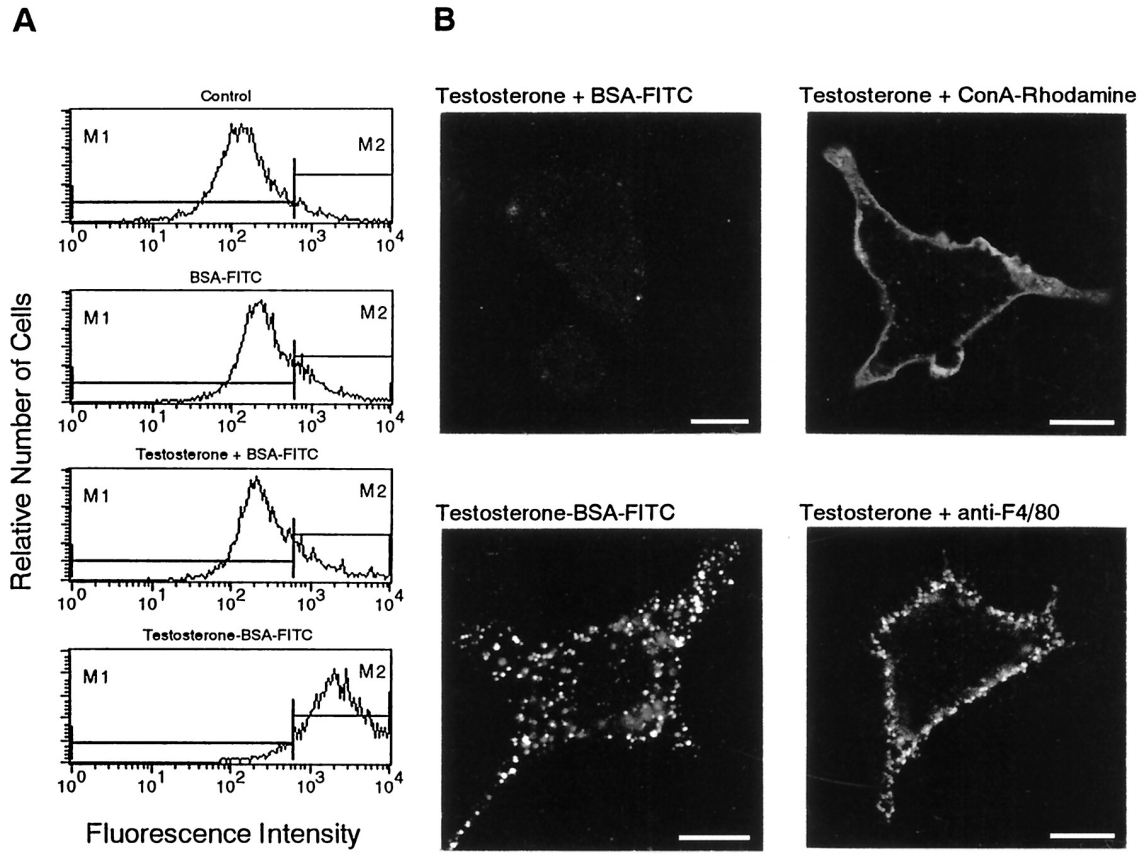


Figure 7. Selective internalization of surface binding sites of T. (**A**) Flow cytometry of IC-21 cells treated with the indicated substances for 15 min. (**B**) CLSM of cells incubated for 15 min with the indicated substances and the corresponding FITC-labeled secondary antibody. Note the difference between the smooth uniform surface labeling with Con A-rhodamine and the granular surface fluorescence of the F4/80 antigens. Bars indicate 10 µm.

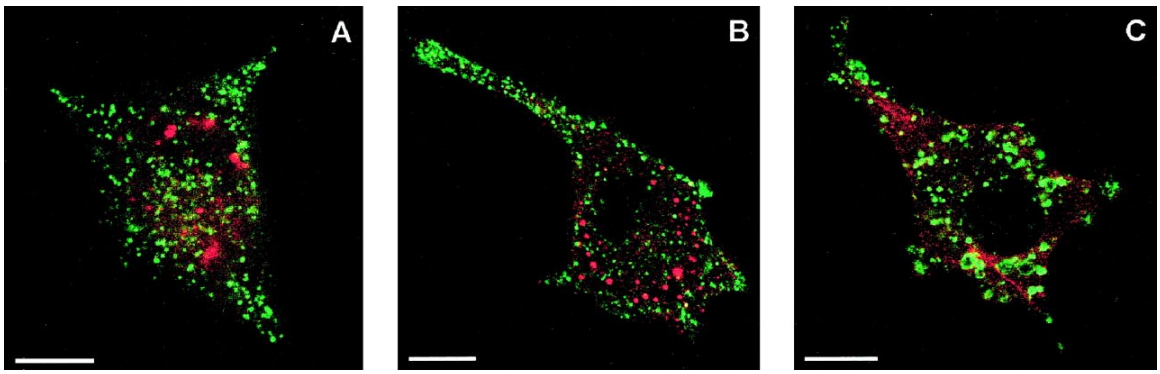


Figure 8. CLSM colocalization of the sequestered surface binding sites of T. (**A**) Cells were incubated in parallel with T-BSA-FITC and LysoTracker™ Red DND-99 at 37°C for 1 h. T-BSA-FITC did not colocalize with acidic vesicles stained with LysoTracker™ Red DND-99. (**B**) T-BSA-FITC was not sequestrated within clathrin-coated vesicles as detected by anti-clathrin antibodies and Cy3-labeled corresponding secondary antibody. (**C**) Internalized punctate fluorescence of T-BSA-FITC was not labeled with an anti-caveolin antibody and its corresponding secondary TRITC-conjugated antibody. Bars indicate 10 µm.

The sequestrated T-BSA-FITC was not contained in acidic vesicles. The latter were identified by CLSM using LysoTracker™ Red DND-99. The vesicles stained with LysoTracker™ Red DND-99 did not colocalize with the green punctate fluorescence of T-BSA-FITC (Fig. 8A). Also, the sequestrated T binding sites did colocalize neither with clathrin as detected by anti-clathrin antibodies (Fig. 8B) nor with caveolin as monitored by anti-caveolin antibodies (Fig. 8C).

In order to determine the effect of various parameters on receptor internalization, intact IC-21 cells were incubated with T-BSA-FITC for 15 min and subsequently fluorescence intensity was analyzed by flow cytometry and fluorescence localization by CLSM. Figure 9A shows that internalization of T-BSA-FITC could be competitively reduced by T, but not by the structurally similar compound 1-dehydrotestosterone. Moreover, internalization of surface-bound T-BSA-FITC was largely inhibited at temperatures below about 16°C, whereas temperature did not affect binding of T-BSA-FITC to the cell surface (Fig. 9B). Depletion of ATP by sodium azide resulted in a decrease of fluorescence intensity by approximately 40% (Tab. 8). This fluorescence was localized almost exclusively on the cell surface. Internalization of surface-bound T-BSA-FITC could be also abolished by preincubation with

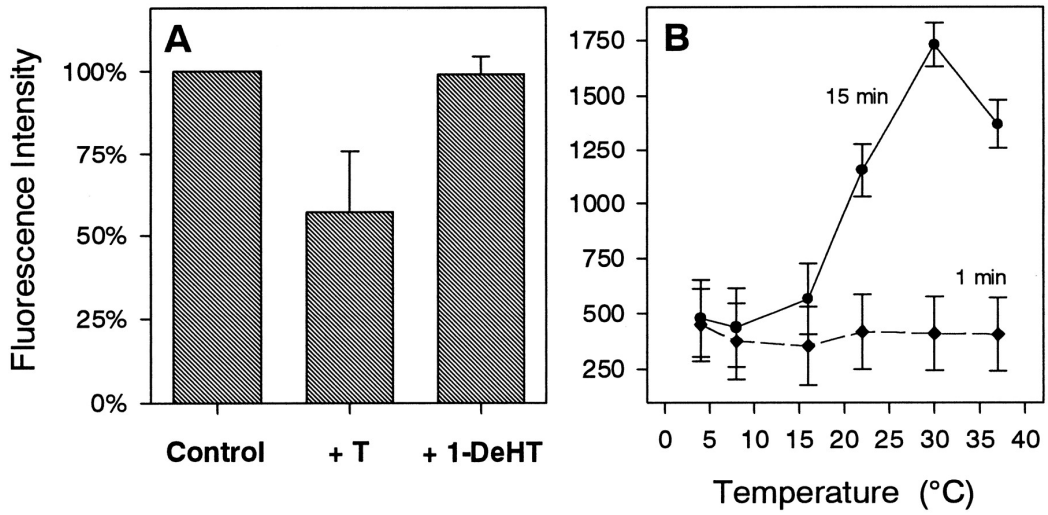


Figure 9. Flow cytometry of the internalization of surface-bound T-BSA-FITC in IC-21 cells. (**A**) Cells were incubated for 15 min with T-BSA-FITC (10^{-6} M) in the absence (Control) or in the presence of a 10-fold excess of unlabeled T (+ T) or 1-dehydrotestosterone (+ DeHT). Values normalized to controls are given as means ± SD from five different experiments. (**B**) Cells were equilibrated for 30 min at the indicated temperatures and then incubated with 1.5 x 10^{-5} M T-BSA-FITC for 1 min or 15 min at the same temperatures. Values represent means ± SD from at least two different experiments.

the G-protein inhibitor pertussis toxin (Tab. 8). The phospholipase C inhibitor U-73122, but not the inactive compound U-73343, also blocked internalization since preincubation with 2 µM U-73122 for 2 min resulted in complete surface localization of T-BSA-FITC, whereas controls have internalized T-BSA-FITC as revealed by the approximately 10% higher fluorescence intensity (Tab. 8). Finally, internalization obviously involved cytoskeletal elements. Both the tubulin-blocker nocodazole and the microfilament-blocker cytochalasin B inhibited internalization, but not surface binding of T-BSA-FITC (Tab. 8). Control cells revealed higher fluorescence intensities by approximabely 25% due to internalization of surface-bound T-BSA-FITC.

Table 8. Effect of various substances on sequestration of surface-bound T-BSA-FITC

Substance	Fluorescence intensity (%)	Surface-bound fluorescence	Internalized fluorescence
Control	100	+	+
NaN_3 (0.04%, 30 min)	62.9 ± 6.5	+	-
Pertussis toxin (500 ng/ml, 16 h)	70.4 ± 0.5	+	-
U-73122 (2 µM, 2 min)	89.2 ± 5.8	+	-
U-73343 (2 µM, 2 min)	101.8 ± 5.4	+	+
Nocodazole (2.5 µg/ml, 15 min)	72.0 ± 5.5	+	-
Cytochalasin B (10 µg/ml, 15 min)	72.2 ± 5.4	+	-

Intact IC-21 cells (1.5 x 10^6) were incubated with the different substances at the indicated concentrations for the indicated periods, before T-BSA-FITC was added for 15 min. Thereafter, cells were fixed and fluorescence intensity was evaluated by flow cytometry and fluorescence localization by CLSM. Values normalized to controls are given as means ± SD from at least two different experiments.

3.1.5 Insensitivity of IC-21 macrophages to different activation stimuli

In order to examine a possible role of T on activation of transcription factors and gene expression, IC-21 cells were stimulated with 10 nM T, then different transcription factors and kinases were tested. There was no response in terms of MAPK activation, e.g. ERK1/2 (Fig. 10A) and transcription factor activation, e.g. NFκB, NFATc (data not shown). Moreover, LPS and PMA, the mostly used activators for macrophages, also failed to induce any detectable responses. To figure out the reason, another macrophage cell line RAW264.7 was treated in parallel. In Fig. 10B, after stimulation of cells with LPS and PMA, no significant activation can be observed in IC-21 cells, whereas dramatic activation can be

observed in RAW264.7 cells by both stimuli. This is in accordance with a previous report showing that different macrophage cell lines have different responsiveness to LPS (Barbour *et al.*, 1998) and suggests that in terms of kinase and transcription factor activation IC-21 cells are less sensitive than RAW264.7 cells to extracellular stimuli, which might also include sex hormones. Therefore, the following experiments were performed only with RAW264.7 cells.

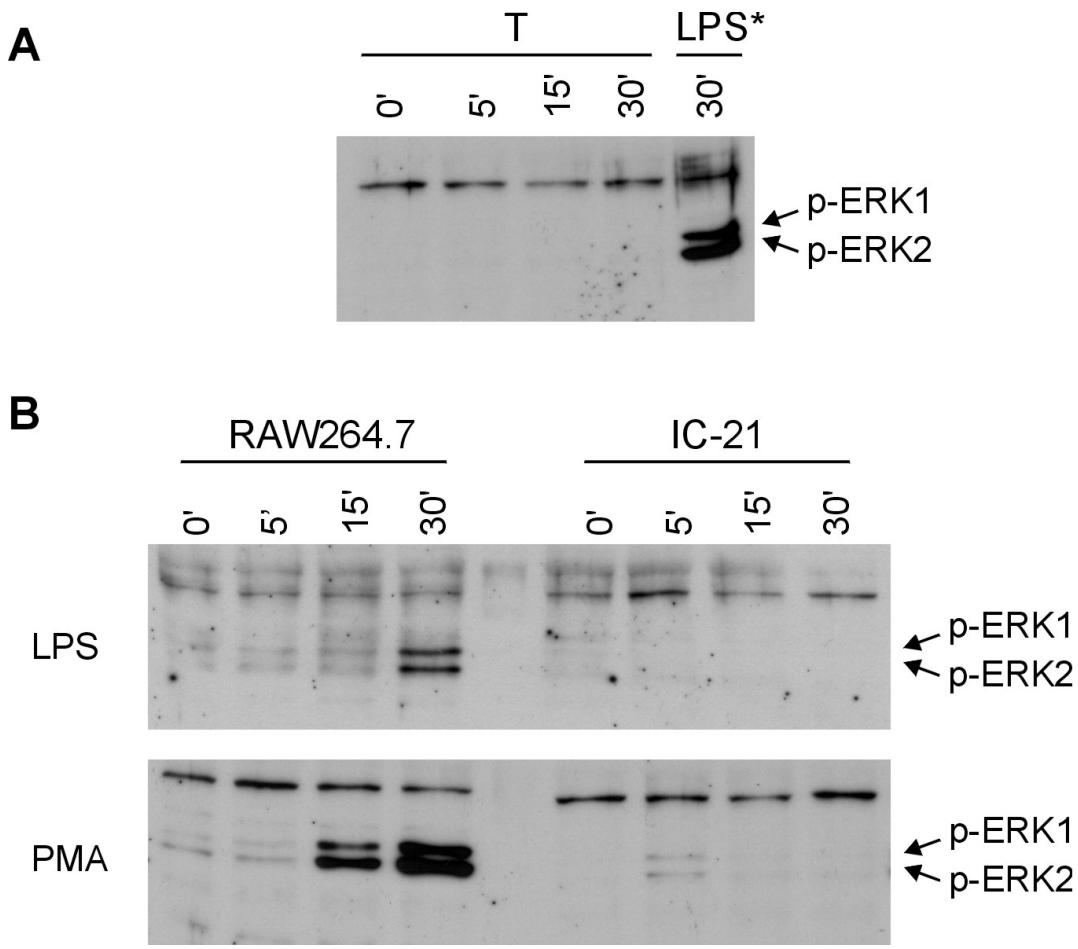


Figure 10. Insensitivity of IC-21 macrophages to different activation stimuli. (**A**) IC-21 cells were treated with 10 nM T for the indicated time periods. No activation of ERK1/2 can be observed. Stimulation of RAW264.7 cells with LPS (LPS*) served as a control for ERK1/2 activation. (**B**) Comparison of the sensitivity of IC-21 and RAW264.7 macrophages to LPS and PMA stimulation. The cells were treated with 1 μg/ml LPS and 10^{-7} M PMA for the indicated intervals, respectively. Protein extracts were subjected to Western-blot with anti-phospho ERK1/2 antibody as described in **Materials and Methods**.

3.2 Construction of a T/E_2-sensitive *c-fos* Promoter Stable Cell Line

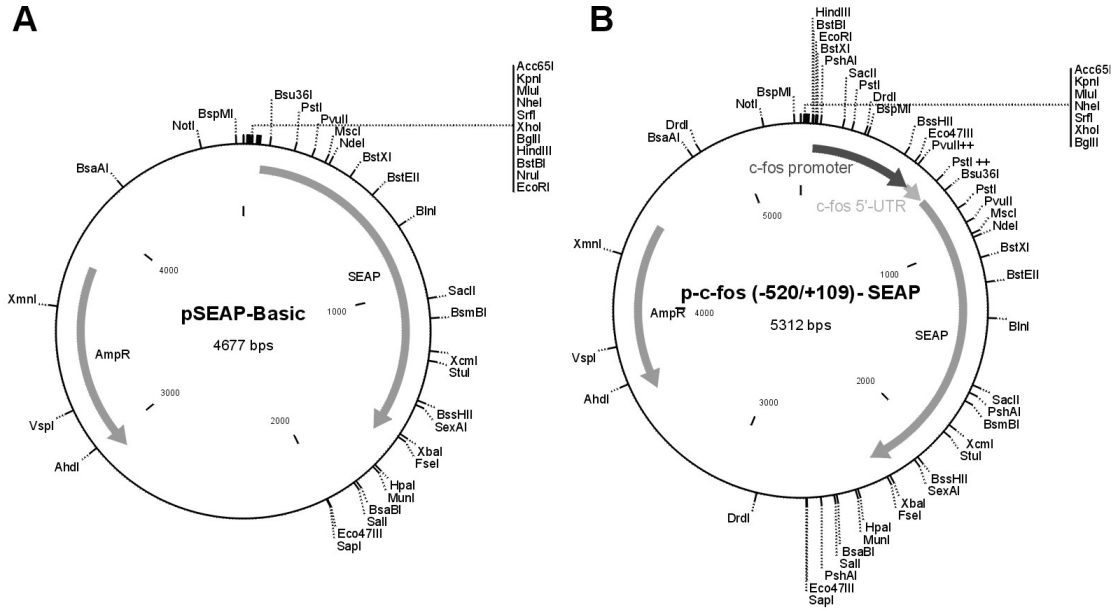
As an immediate early gene, *c-fos* can be regulated at transcriptional level by numerous extracellular stimuli, including steroids, and the activity of *c-fos* promoter has been regarded as an early and sensitive index for the genotropic change of the cells (Hill and Treisman, 1995; Karin, 1995). In order to study the effects of T/E_2 on signal transduction in macrophages, the mouse *c-fos* promoter from –520 to +109 was inserted into the pSEAP-basic2 vector at the EcoRI site (Fig. 11A and B). The construct was verified by sequencing with a 100% identity to the sequence from GenBank (GI: 50399) (Fig. 11C). The murine RAW264.7 macrophages were then transiently or stably transfected with this *c-fos* promoter-SEAP plasmid using FUGENE6 (Material and Methods). Stable transfection was achieved by a co-transfection of the cells with pEGFPN3 (Clontech) followed by the selection of the cells using 250 μg/ml G418. Totally 36 clones were obtained from 2×10^7 cells and screened for an optimal response to 10% charcoal stripped calf serum. 16 clones showed response to CSCS, among them, the clone No.13 (RAW-fos13) that does not express detectable EGFP and has a maximum response to T, thus was used for the experiment.

3.3 Effects of T on RAW-fos13 and RAW 264.7 Macrophages

3.3.1 Absence of iAR and presence of mAR

We have shown the existence of mAR and the absence of iAR in IC-21 macrophages. Therefore we have examined the occurrence of mAR and iAR in macrophage cell lines of RAW 264.7 and RAW-fos13. These cells do not express any significant amounts of iAR as proven by RT-PCR (Fig. 12A). When primer pairs flanking a region of the DNA-binding domain or three different regions of the steroid-binding domain were used, all four predicted PCR products could be amplified using RNA from mouse testes. Expression of iAR could not be detected with RNA from RAW 264.7 or RAW-fos13 cells, although the quality of the RNA and the cDNA from these cell lines was proven by amplification of the intracellular estrogen receptor α (ERα) using two different primer pairs (Fig. 12A).

For detection of mAR, the plasma membrane-impermeable T-BSA-FITC conjugate was used to label the cells for 1 min. This resulted in an increase of fluorescence intensity of cells



C

Mouse c-fos promoter from -520 to +109

```
-520  tccagattgc tggacaatga cccgggtctc atcccttgac cctgggaacc
-470  gggtccacat tgaatcaggt gcgaatgttc gctcgccttc tctgcctttc
-420  ccgcctcccc tcccccggcc gcggccccgg ttcccccccc gcgctgcacc
-370  ctcagagttg gctgcagccg gcgagctgtt cccgtcaatc cctccctcct
-320  ttacacagga tgtccatatt aggacatctg cgtcagcagg tttccacggc
-270  cggtccctgt tgttctgggg gggggaccat ctccgaaatc ctacacgcgg
-220  aaggtctagg agacccccta agatcccaaa tgtgaacact cataggtgaa
-170  agatgtatgc caagacgggg gttgaaagcc tggggcgtag agttgacgac
-120  agagcgcccg cagagggcct tggggcgcgc ttcccccccc ttccagttcc
-70   gcccagtgac gtaggaagtc catccattca cagcgcttct ataaaggcgc
                                +1
-20   cagctgaggc gcctactact ccaaccgcga ctgcagcgag caactgagaa
+30   gactggatag agccggcggt tccgcgaacg agcagtgacc gcgctcccac
+80   ccagctctgc tctgcagctc ccaccagtgg
```

Figure 11. (**A**) The map of plasmid pSEAP-Basic2. (**B**) Insertion of the *c-fos* regulatory region from –520 to +109 into the EcoRI site of the pSEAP-Basic2. (**C**) The nucleotide sequence of mouse *c-fos* promoter from –520 to +109, the arrow with +1 indicates the start of the primary transcript.

as analyzed by flow cytometry (Figure 12B). No binding was detectable with BSA-FITC (Fig. 12B). The fluorescence of the bound T-BSA-FITC was localized exclusively on the cell surface as revealed by CLSM (Fig. 12C). The surface binding of T-BSA-FITC was also corroborated by colocalization with the red fluorescent cell surface marker ConA-rhodamine (Fig. 12C).

3.3.2 T-induced rise in $[Ca^{2+}]_i$

As we have observed in IC-21 cells, T has also shown to induce a rapid rise in $[Ca^{2+}]_i$ of Fura-2-loaded RAW-fos13 cells. With fluorescence spectrometry the increase in $[Ca^{2+}]_i$ amounted to about 40-70 nM at the physiological concentration of 10 nM T (Fig. 13A). Remarkably, 5α-dihydrotestosterone (5α-DHT) could also induce an increase in $[Ca^{2+}]_i$ by about the same amount as T, whereas 5β-dihydrotestosterone (5β-DHT) and 1-dehydrotestosterone (1-DeHT) were ineffective to raise $[Ca^{2+}]_i$ (Fig. 13A, B). Moreover, the T-induced rise in $[Ca^{2+}]_i$ could not be prevented by either the iAR-blocker cyproterone or by raloxifene, tamoxifen and ICI 182,780 - inhibitors of the iER, through which T could have been possibly acting after aromatization to 17β-estradiol (Fig. 13C, D). An effective rise in $[Ca^{2+}]_i$, was also inducible by the plasma membrane-impermeable T-BSA, but not by BSA alone (Fig. 13E).

The T-induced Ca^{2+} increase may be due to influx of extracellular Ca^{2+} and/or release of Ca^{2+} from intracellular Ca^{2+} stores such as endoplasmic reticulum. When extracellular Ca^{2+} was removed by EGTA, T was still able to elevate $[Ca^{2+}]_i$ by about the same extent as without EGTA (Fig. 13F). This indicates that in the RAW-fos13 cells, the T-induced rise in $[Ca^{2+}]_i$ is predominantly due to intracellular Ca^{2+}-mobilization rather than Ca^{2+}-influx. Consistent with this view, the T-induced rise in $[Ca^{2+}]_i$ could be prevented by the PLC inhibitor U-73122, but not by the inactive component U-73343 (Fig. 13G). Also, the T-caused rise in $[Ca^{2+}]_i$ was abolished by preincubation of cells with PTX (Fig. 13H). These data indicate that a PTX-sensitive G-protein coupled to PLC is involved in the Ca^{2+} response to T.

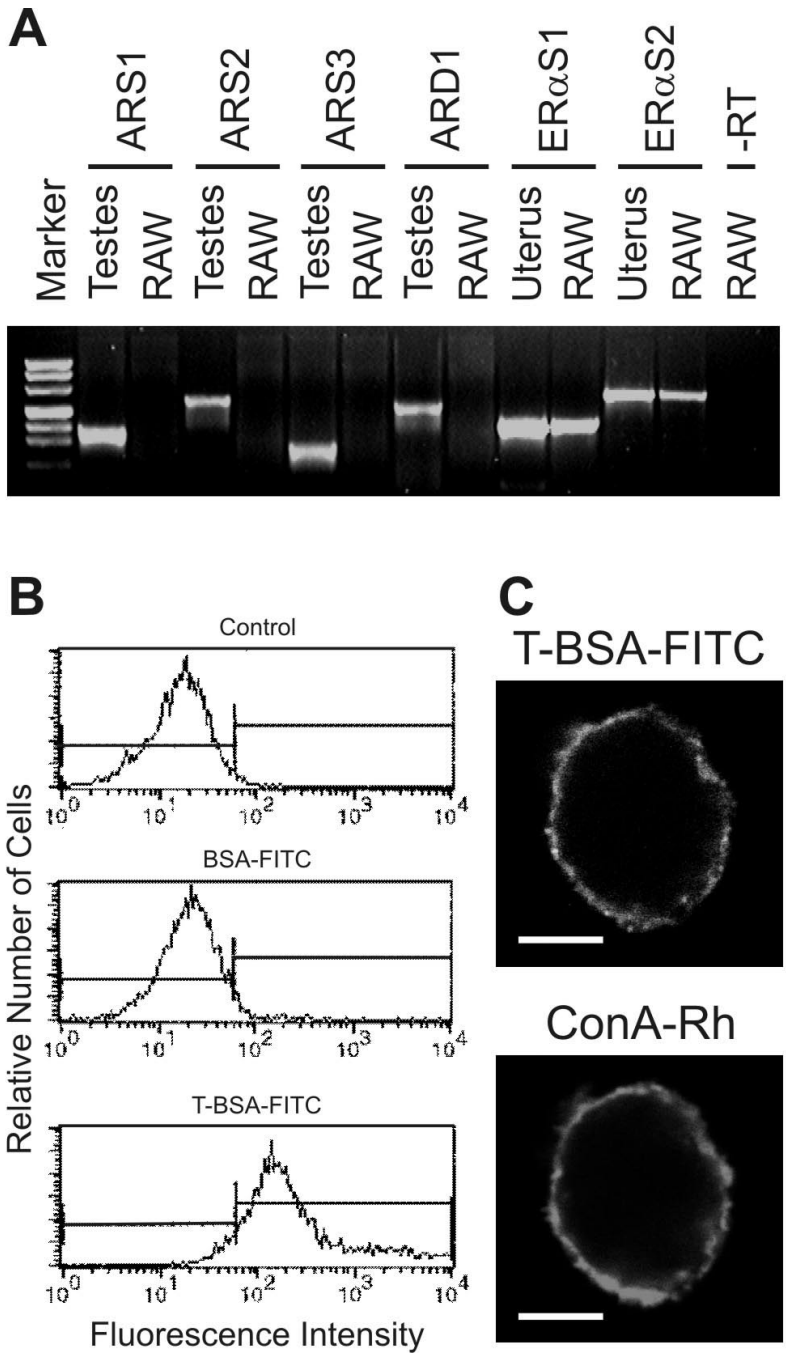


Figure 12. Detection of iAR and mAR in RAW-fos13 cells. (**A**) Absence of iAR in RAW-fos13 cells. RT-PCR was performed with the primer pairs ARS1, ARS2, ARS3 spanning 365, 560, 281 bp of the steroid binding domain of iAR, respectively, and the primer pair ARD1 spanning 511 bp of the DNA binding domain of iAR. The predicted bands in a 2% agarose gel were only revealed in RNA from murine testes. The primer pairs ERαS1 and ERαS2 amplified iERα-fragments of 385 and 608 bp, respectively, with RNA from murine uteri. The negative control -RT was with RNA from RAW-fos13 cells and the primer pair ERαS1 without reverse transcriptase. (**B**) Flow cytometry of intact RAW-fos13 cells labeled with T-BSA-FITC and BSA-FITC. (**C**) CLSM of RAW-fos13 cells labeled with T-BSA-FITC or ConA-rhodamine (ConA-Rh). Bars represent 10 µm.

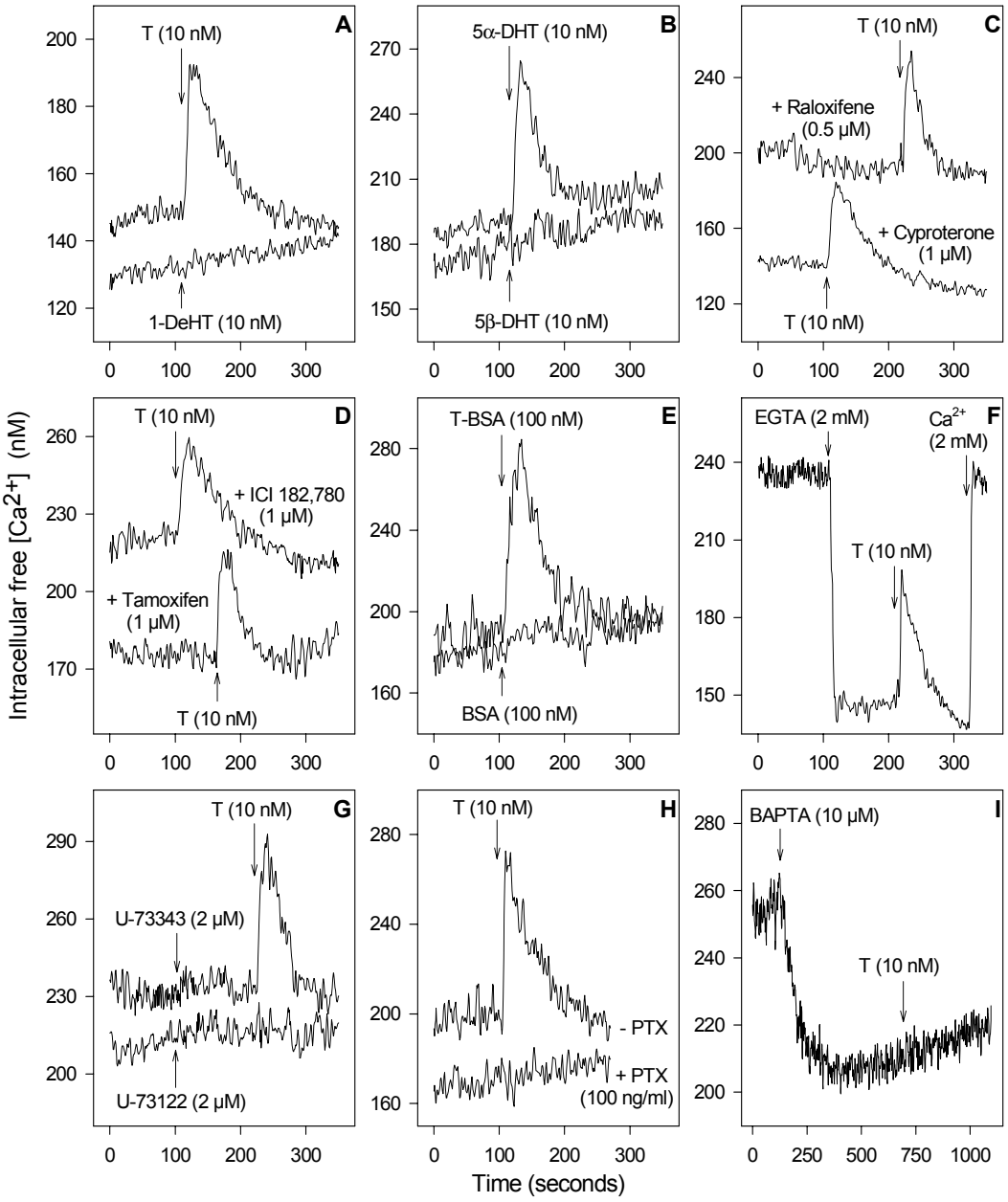


Figure 13. T-induced rise in $[Ca^{2+}]_i$ of RAW-fos13 cells. (**A**) T elicits an immediate Ca^{2+} spike, while 1-DeHT is ineffective. (**B**) Cells stimulated with 5α-DHT or 5β-DHT. (**C**) Cells preincubated with cyproterone or raloxifene for 1 h before T application. (**D**) Cells incubated with ICI 182,780 or tamoxifen for 1 h before addition of T. (**E**) Cells stimulated with T-BSA or BSA. (**F**) Cells incubated with EGTA for 100 s before addition of T. (**G**) Cells pretreated with PLC inhibitor U-73122 or the inactive control compound U-73343 for 2 min before T stimulation. (**H**) Cells preincubated with or without PTX for 16 h before T application. (**I**) Cells pretreated with BAPTA for 10 min before T addition. Arrows indicate addition of reagents to RAW-fos13 cells. Representative experiments are shown and the results were verified in at least four independent experiments.

In addition to fluorescence spectrometry with cell suspensions, single cell calcium imaging was also employed with adherent RAW 264.7 and RAW-fos13 macrophages. Similar results were obtained. Following T treatment, an increase in $[Ca^{2+}]_i$ was observed within seconds and reached its peak between 60 and 100 s. It is noteworthy that although most of the treated cells had the calcium response, the individual cells showed different amplitudes of response, from the basal ratio [340/380] level of about 0.8-0.9 to the maximum of about 0.95-1.0 (Fig. 14), few cells even had no response to T stimulation. The mechanism for this heterogeneity of the responsiveness is not clear, it may reflect the variant expression level of the T membrane receptors among different individual cells.

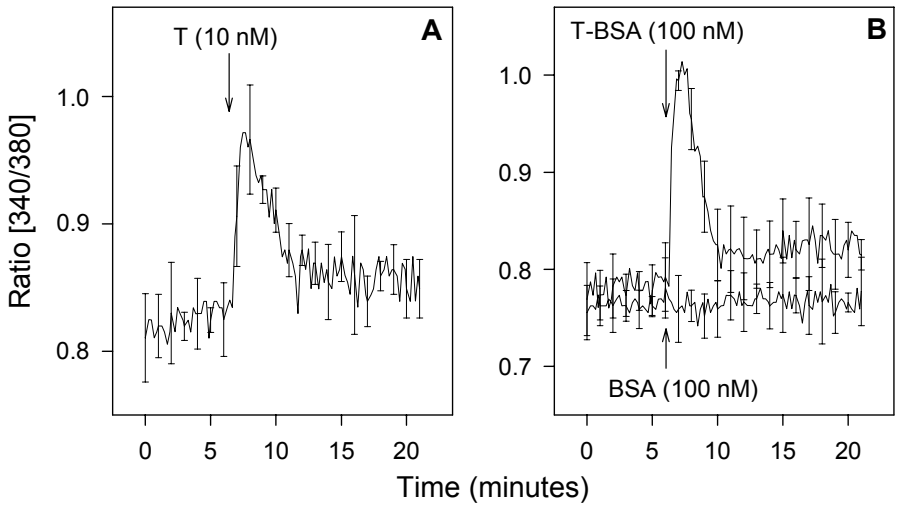


Figure 14. T-induced calcium response measured by single cell calcium imaging. (**A**) T elicits an increase in $[Ca^{2+}]_i$ (n=4, "n" indicates number of cells). (**B**) Cells were stimulated with T-BSA (n=4) and BSA (n=5), respectively. Arrows indicate addition of reagents to RAW-fos13 cells. Representative experiments are shown and the results were verified in at least two independent experiments.

3.3.3 T-unresponsiveness of MAPK and *c-fos*

It has been reported that androgens are able to activate ERK1/2 in different cell types (Pieterziel *et al.*, 1999; Kousteni *et al.*, 2001). Therefore we have tested whether T can activate MAPK. However,T was unable to induce any direct activation of the three MAPK families p38, ERK1/2 and JNK/SAPK (Fig. 15A). The reason for this T-unresponsiveness of the three MAPK families is not an inherent failure of the cells since anisomycin (Kyriakis *et al.*, 1994; Hazzalin *et al.*, 1998) stimulates phosphorylation of all the three kinases (Fig. 15A; cf. also Fig. 16). In order to detect a possible direct effect of the T-induced rise in

$[Ca^{2+}]_i$ on expression of at least Ca^{2+} sensitive genes such as the immediate early gene *c-fos*, we have studied the effect of T on the activation of the *c-fos* promoter, which is used here only as a molecular marker for early genotropic signaling. RAW 264.7 cells were transiently transfected with a *c-fos* promoter linked to a SEAP reporter gene and then stimulated with 10 nM testosterone for 3 h. However, this stimulation is not sufficient to induce any significant response of the *c-fos* promoter in comparison to non-stimulated control cells (Fig. 15B). Also, T is not able to induce any change in *c-fos* promoter activity in the cell clone RAW-fos13 which was derived from RAW 264.7 through a stable transfection with the same reporter construct (see **Materials and Methods**) (Fig. 15C). This unresponsiveness is not due to a possible defect in stimulatibility of the transfected *c-fos* promoter construct, since LPS causes a significant induction in both transiently and stably transfected cells (Fig. 15B, C).

3.3.4 Down-regulation of LPS Signaling by T

3.3.4.1 T attenuates activation of p38 MAPK induced by LPS

LPS-stimulation of macrophages is known to activate numerous signaling events including the three MAPK families p38, ERK1/2, and JNK/SAPK (Hambleton *et al.*, 1996; Sweet and Hume, 1996; Feng *et al.*, 1999; Beutler, 2000; Cheng and Chen, 2001). In accordance, 1 µg/ml LPS activates all these three MAPKs in RAW-fos13 cells after 15 min (Fig. 16). Activation of JNK/SAPK and p38 MAPK declined after 60 min, whereas the decline of the ERK1/2 activation is obvious only after 180 min. Conspicuously, however, co-stimulation with 10 nM T significantly attenuates LPS-induced activation of p38 MAPK. The maximal inhibition is about 50% after 15 min. By contrast, T does not affect the LPS-induced activation of ERK1/2 and JNK/SAPK at all (Fig. 16). The attenuation of LPS-induced activation of p38 MAPK is dependent on the T-induced rise in $[Ca^{2+}]_i$ since it is completely prevented by the intracellular Ca^{2+}-chelator BAPTA (Fig. 17) under conditions blocking the T-induced Ca^{2+} increase (Fig. 13I). BAPTA does not influence the LPS-induced activation of p38 MAPK (Fig. 17).

3.3.4.2 T attenuates LPS-activation of the transiently transfeced c-fos promoter

Besides the MAPK, LPS is also known to activate the immediate early gene *c-fos* in macrophages (Collart *et al.*, 1987). In accordance, we can show that 1 µg/ml LPS induces

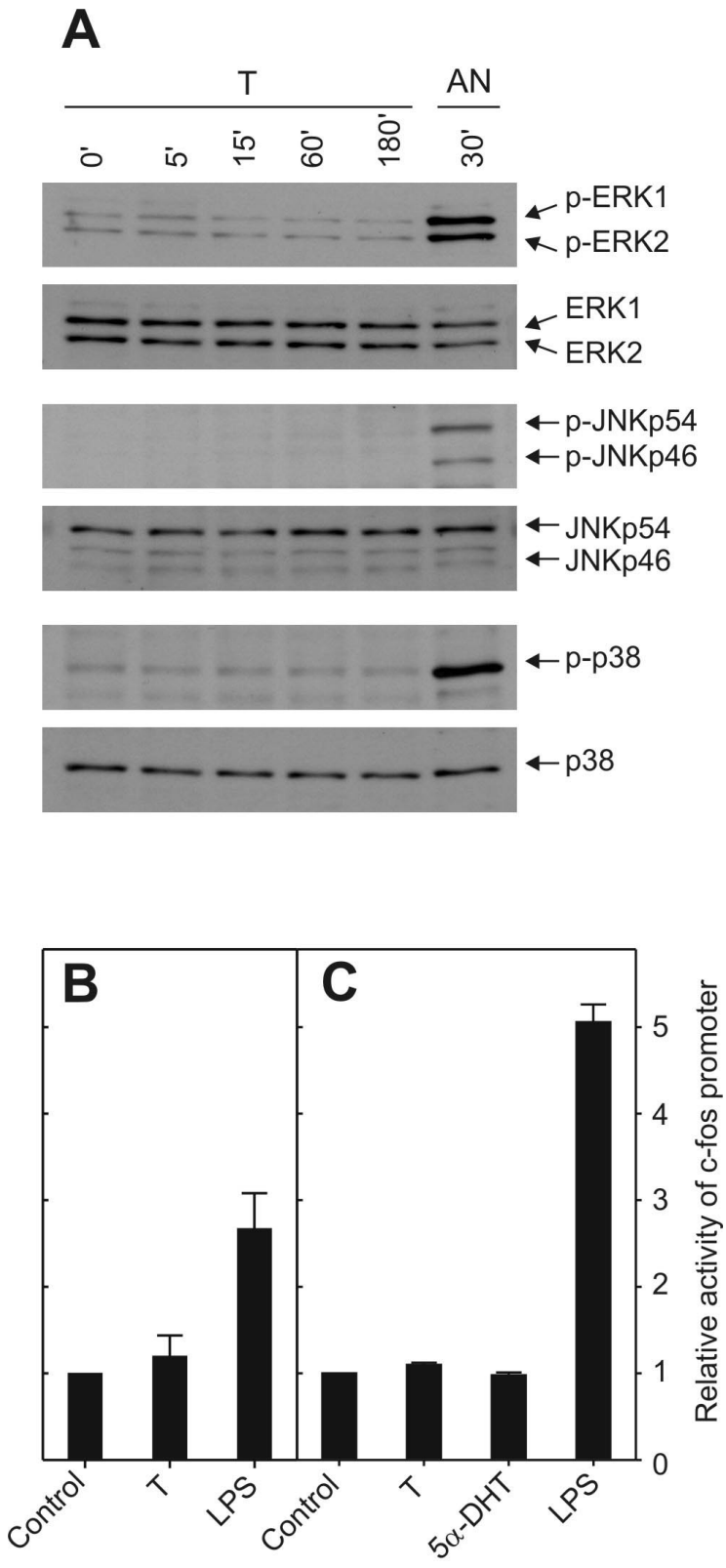


Figure 15. T-unresponsiveness of MAPK and *c-fos* promoter. **(A)** RAW 264.7 cells were treated with 10 nM testosterone (T) for different periods. Protein extracts were subjected to immuno-determination as described in Materials and methods. The upper panels represent the activated forms of p-ERK1/2, p-JNK/SAPK, or p-p38MAPK as detected by anti-phospho-antibodies. The lower panels indicate total ERK1/2, JNK/SAPK, or p38MAPK as detected with anti-total MAPK antibodies. A 30 min stimulation with 10 µg/ml anisomycin (AN) was used as positive control. Representative blots are shown and the results were verified by at least two independent experiments. **(B)** RAW 264.7 macrophages were cultured in IMDM medium containing 5% stripped FCS for 18 h, then transiently transfected with *c-fos* promoter-SEAP and stimulated with 10 nM T or 1 µg/ml LPS for 3 h. The *c-fos* promoter activity of each group was determined relative to the activity of vehicle-treated control. The results shown are means ± SEM of at least three independent experiments performed in triplicate. (**C**) RAW-fos13 cells stably transfected with *c-fos* promoter-SEAP construct were stimulated with 10 nM T, 5α-DHT or 1 µg/ml LPS for 3 h.

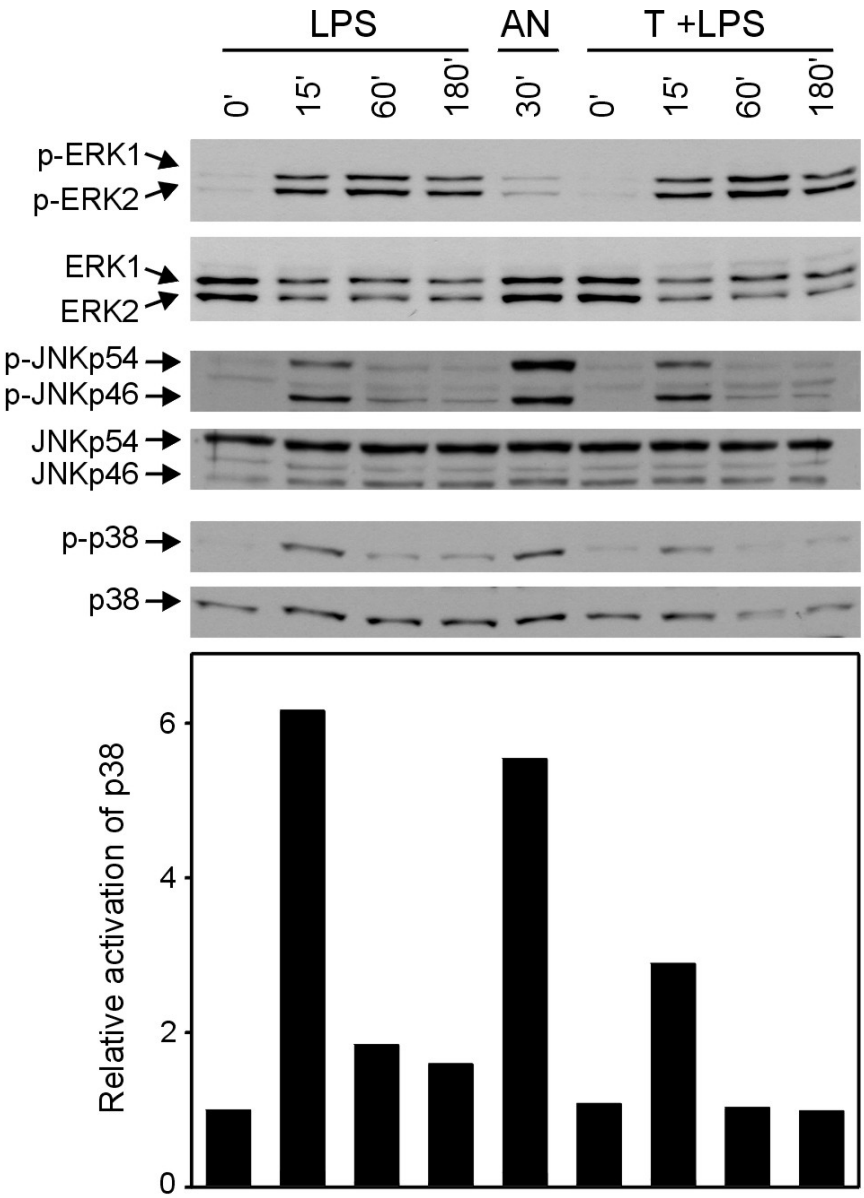


Figure 16. Effects of T on LPS-activation of MAPKs. (**A**) T attenuates activation of p38 MAPK induced by LPS. The RAW-fos13 cells were stimulated with 1 µg/ml LPS and 10 nM T for different periods. Protein extracts were subjected to immuno-determination as described in Materials and methods. The upper panels for p-ERK1/2, p-JNK/SAPK, or p-p38 MAPK represent the phosphorylated forms, the lower panels total ERK1/2, JNK/SAPK, or p38 MAPK, as detected with the corresponding antibodies. Representative blots are shown. Stimulation of cells with 10 µg/ml anisomycin (AN) was used as positive control. Relative activation of p38 was densitometrically evaluated. The results were verified in at least two independent experiments.

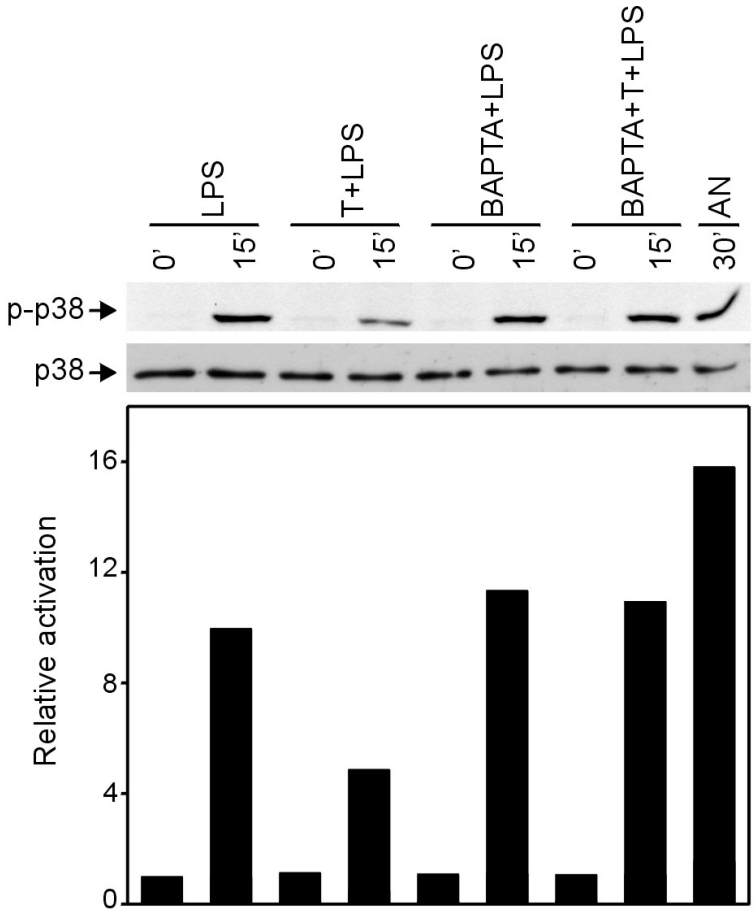


Figure 17. Requirement of intracellular calcium mobilization for Te-induced depression of p38 MAPK activation by LPS. RAW-fos13 cells were preincubated with 10 µM of the intracellular Ca^{2+} chelator BAPTA for 10 min, then stimulated for 15 min with 1 µg/ml LPS combined with 10 nM T. Results are presented as in **Fig. 16.**

activation of the *c-fos* promoter-SEAP construct transiently transfected in RAW 264.7 cells. Similar to the situation with p38 MAPK, 10 nM T attenuates the LPS-induced activation of *c-fos* promoter by approximately 35% (Fig. 18A). This depressive effect of T is specific since, under the same experimental conditions, 17β-estradiol (E_2) causes an increase in the *c-fos* promoter activity. In addition, we can exclude that the opposite effects of T and E_2 are due to their possible effects on translation and/or secretion of SEAP. For, neither T nor E_2 influence the transport of SEAP into medium when SEAP is under control of the SV40 promoter (Fig. 18B).

3.3.4.3 T attenuates LPS-activation of the stably transfeced c-fos promoter

The T-attenuated LPS-response of *c-fos* promoter is also observed in stably transfected RAW-fos13 cells, which are still more sensitive to LPS- and T-stimulation. Incubation of RAW-fos13 cells with 1 µg/ml LPS for 3 h resulted in a 5-fold induction of *c-fos* promoter activity (Fig. 18C). This induction, however, is significantly reduced by about 60% upon co-incubation with 10 nM testosterone (Fig. 18C). About the same reduction can be induced with 10 nM 5α-DHT, whereas 10 nM 5β-DHT and 10 nM 1-DeHT were ineffective to influence the LPS-induced increase in *c-fos* promoter activity.

3.3.4.4 T-attenuated LPS-activation of the c-fos promoter through p38 MAPK

To delineate the possible signaling pathway through which T exerts its attenuative effect on LPS-activation of *c-fos* promoter, we have used different inhibitors. The iAR-blocker cyproterone and the iER-blocker ICI 182,780 cannot prevent the depressive testosterone-effect on LPS-stimulated *c-fos* promoter activation (Fig. 18C). By contrast, when RAW 264.7 macrophages transiently transfected with the *c-fos* promoter were pre-incubated with the intracellular Ca^{2+}-chelator BAPTA, T lost its ability to exert a depressive effect on *c-fos* promoter (Fig. 18D). Also, the PLC inhibitor U-73122 and the G-protein inhibitor PTX can block the T-effect (data not shown). This indicates that the depressive T-effect requires a rise in $[Ca^{2+}]_i$, just as the attenuating effect of T on LPS-induced p38 MAPK phosphorylation. To find out whether the Ca^{2+}-dependent downregulation by T of LPS-induced *c-fos* promoter activation involves the p38 MAPK pathway, we have used SB 203580, a specific inhibitor of p38 MAPK. At 50 µM SB 203580, LPS-stimulation of p38 is reduced by about 50%, and the remaining promoter activity is no longer regulated by T (Fig. 18D). These results indicate that p38 is involved in the LPS-induced activation of *c-fos* promoter and that the T-attenuation of the LPS-stimulated *c-fos* promoter activity is achieved predominately - if not exclusively - by interference with p38 MAPK phosphorylation. In addition, GF109203X, an inhibitor of Ca^{2+}-sensitive PKC can also inhibit the depressive T-effect suggesting the possible involvement of PKC (data not shown).

3.3.5 Downregulation by T of LPS-stimulated NO-production through p38 MAPK

Activation of p38 MAPK is known to be important for induction of genes associated with macrophage activation manifesting itself as, for example, increased production of the key

immune effector molecule NO (Chen *et al.*, 1999a). We therefore suspected that the T-induced rise in $[Ca^{2+}]_i$ could also influence NO-production.

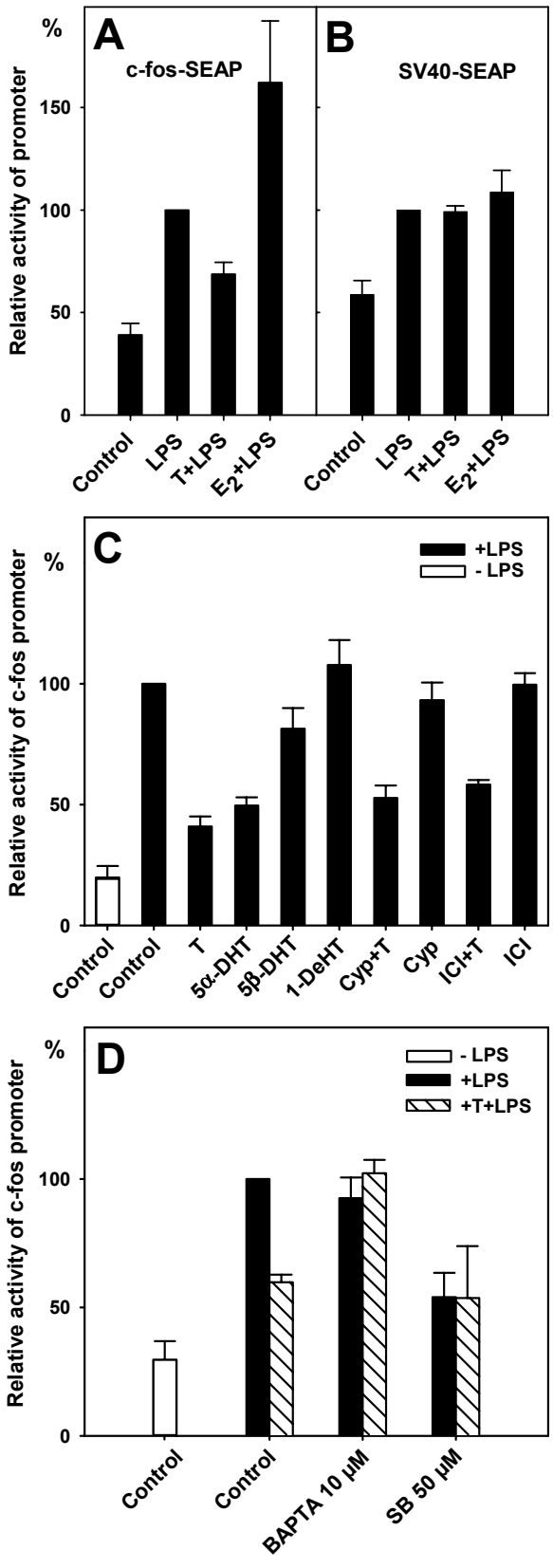


Figure 18. Effects of T on LPS-activation of *c-fos* promoter. RAW 264.7 macrophages were cultured in IMDM medium containing 5% stripped FCS for 18 h, then transiently transfected with *c-fos* promoter-SEAP (**A**) or SV40-SEAP (**B**) for 24 h before stimulation with 1 µg/ml LPS in combination with 10 nM T or 1 nM E_2 for 3 h. The *c-fos* promoter activity of each group was determined relative to the activity of vehicle-treated control with setting the LPS group as 100%. The results shown are means ± SEM of at least three independent experiments performed in triplicate. (**C**) T-attenuation of the LPS-activated *c-fos* promoter. Cells were stimulated for 3 h with 1 µg/ml LPS in combination with 10 nM of T, 5α-DHT, 5β-DHT, 1-DeHT. Before stimulation the cells were preincubated for 1 h with 1 µM cyproterone (Cyp) and 1 µM ICI 182,780 (ICI), respectively. The *c-fos* promoter activity from each group was determined and expressed relatively to the *c-fos* promoter activity induced by LPS which was set as 100%. The results shown are means ± SEM from at least three independent experiments performed in triplicate. (**D**) Cells were first preincubated with 10 µM BAPTA for 10 min or 50 µM SB 203580 (SB) for 30 min, then stimulated with 1 µg/ml LPS or LPS plus 10 nM T for 3 h. Results are presented as in (**C**).

Indeed, T caused a significant decrease of NO-production in LPS-stimulated RAW-fos13 cells (Fig. 19A). This decrease was not sensitive to the iAR-blocker cyproterone (Fig. 19A). The suppressive effect of T can be prevented by BAPTA under conditions blocking the T-induced rise in $[Ca^{2+}]_i$ (Fig. 19B). Moreover, the LPS-induced NO-production can be reduced by about 60% with 50 µM SB 203580 (Fig. 19B). This is consistent with previous reports that LPS-stimulated NO-production of macrophages is mediated through p38 MAPK (Chen and Wang, 1999). When LPS-induced NO-production is down-regulated by SB 203580, the remaining NO-production is not longer regulated by T (Fig. 19B). This indicates that T exerts its attenuative effect on LPS-stimulated NO-production largely through p38 MAPK. In addition, this depressive T-effect on NO-production can be blocked by U-73122, PTX and GF203109x (data not shown).

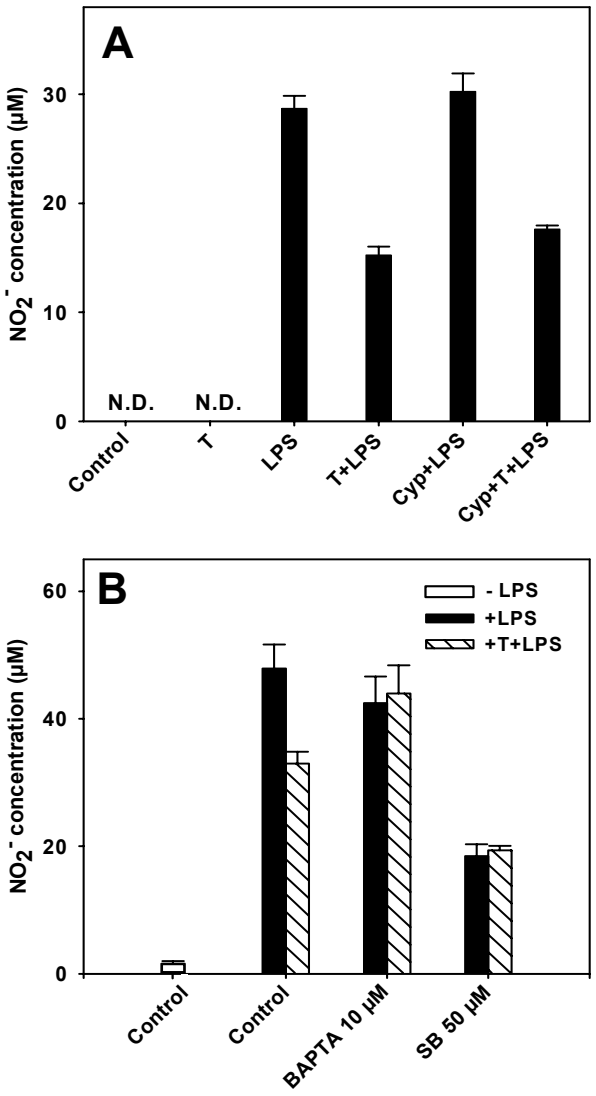


Figure 19. Influence of T on LPS-induced NO-production. (**A**) RAW-fos13 cells were cultured in IMDM medium containing 5% stripped FCS for 18 h, then stimulated for 24 h with 10 nM T and 1 µg/ml LPS. The cells were pretreated with 1 µM cyproterone (Cyp) for 1 h before addition of T and LPS. N.D. = Not detectable. The results shown are means ± SEM from three independent experiments performed in triplicate. (**B**) RAW 264.7 cells were preincubated with 10 µM BAPTA for 10 min or 50 µM SB 203580 (SB) for 30 min before addition of 1 µg/ml LPS or LPS plus 10 nM T. NO_2^- was determined 24 h later as in (**A**).

3.3.6 Up-regulation of serum signaling by T

In order to substantiate the specificity of T signaling on the down-regulation of LPS signaling, we also examined the effect of T on the signaling pathway induced by fetal calf serum (FCS). The RAW-fos13 cells were serum-starved for 18 h, then stimulated with 10% (v/v) charcoal-stripped FCS for 3 h. This resulted in an about 5-fold increase in the activity of *c-fos* promoter (Fig. 20A). However, FCS-stimulation in the presence of 10 nM T caused an even higher increase in promoter activity by about 9-10-fold. This T effect was specific since 1-DeHT had no effect at all. Moreover, the iAR-blocker cyproterone could not prevent this T-induced up-regulation of the FCS-stimulated *c-fos* promoter activation (Fig. 20A). The augmentative effect of T was due to the T-induced increase in $[Ca^{2+}]_i$ since it could be completely prevented by BAPTA pretreatment. (Fig. 20B). Moreover, the PLC inhibitor

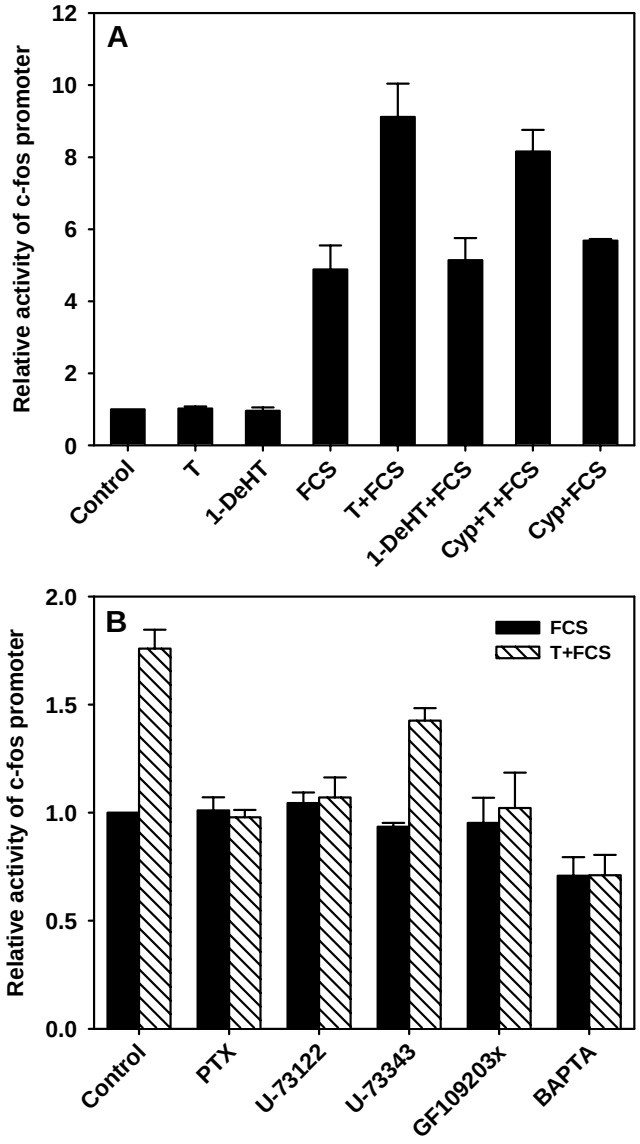


Figure 20. Effects of T on FCS-induced *c-fos* promoter activation. (**A**) RAW-fos13 cells were serum-starved for 18 h and then stimulated with 10 nM T, 10 nM 1-DeHT, 10% (v/v) FCS, and 1 µM cyproterone (Cyp) for 3 h. The *c-fos* promoter activity of each group was determined relative to vehicle-treated control. The results shown are means ± SEM from at least three independent experiments performed in triplicate. (**B**) T-induced augmentation of FCS-stimulated *c-fos* promoter activation. Cells were stimulated for 3 h with 10% (v/v) FCS, or FCS plus 10 nM T. Inhibitors were used as indicated in Fig. 13 except GF109203x, which was used at 1 µM and the pretreatment lasted for 10 min before FCS and T stimulation. The *c-fos* promoter activity from each group was expressed relatively to the *c-fos* promoter activity induced by FCS.

U-73122, but not the ineffective compound U-73343, as well as the PKC-inhibitor GF109203X inhibited the up-regulatory T effect. Also, PTX prevented the stimulatory T effect on FCS-induced activation of *c-fos* promoter (Fig. 20B).

Finally, we investigated the effect of T on the activation of p38 MAPK, ERK1/2 and JNK/SAPK induced by FCS. T did not affect p38 and JNK/SAPK activation (Fig. 21), whereas it had a significant stimulatory effect on ERK1/2. This activation occurs maximally after 60 min and is still observable after 180 min FCS-stimulation (Fig. 21).

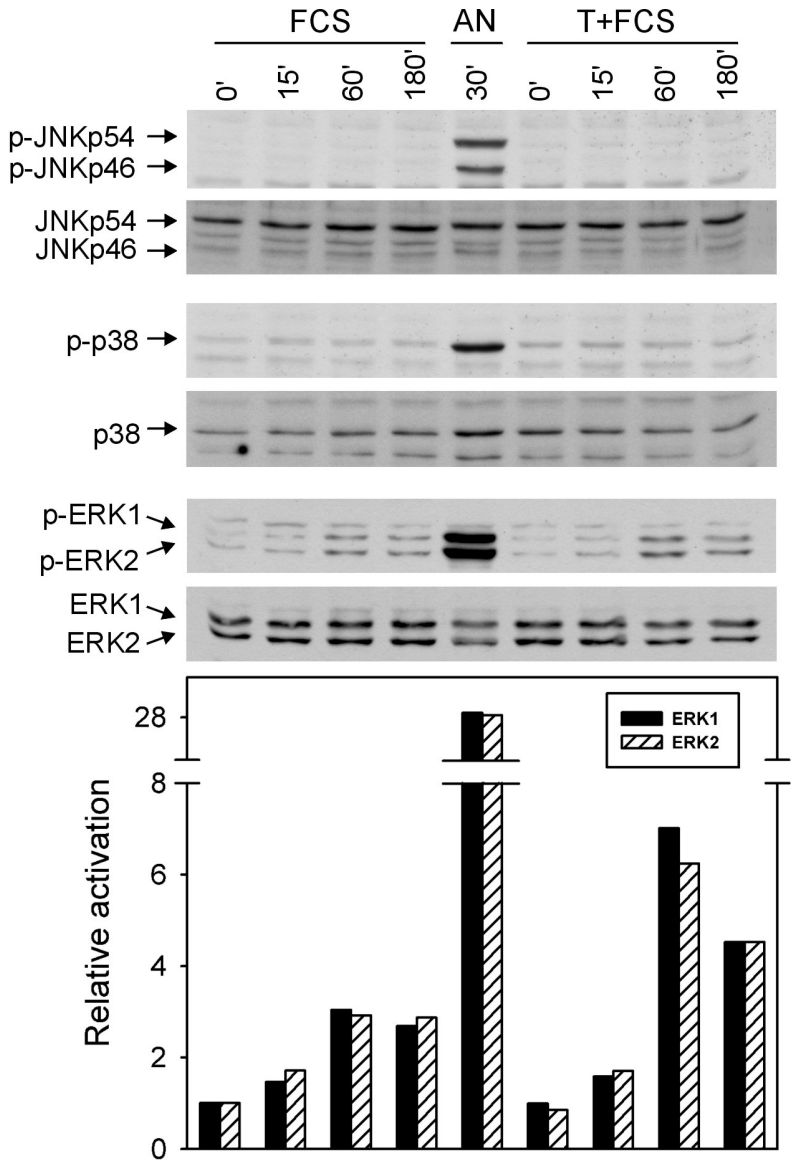


Figure 21. Effects of T on activation of MAPKs by FCS.T augments activation of ERK1/2 induced by FCS. Cells were stimulated with 10% (v/v) FCS and 10 nM T plus FCS for different periods. Cell lysates were prepared, and total proteins were subjected to immuno-detection. Results are presented and labeled as described in Fig. 16.

3.3.7 No influence of T on binding activity of CRE, SRE and SIE within *c-fos* promoter

The result that serum stimulated *c-fos* promoter activation could be enhanced by T co-stimulation prompted us to check the effects of T on the binding activity of different elements within *c-fos* promoter.

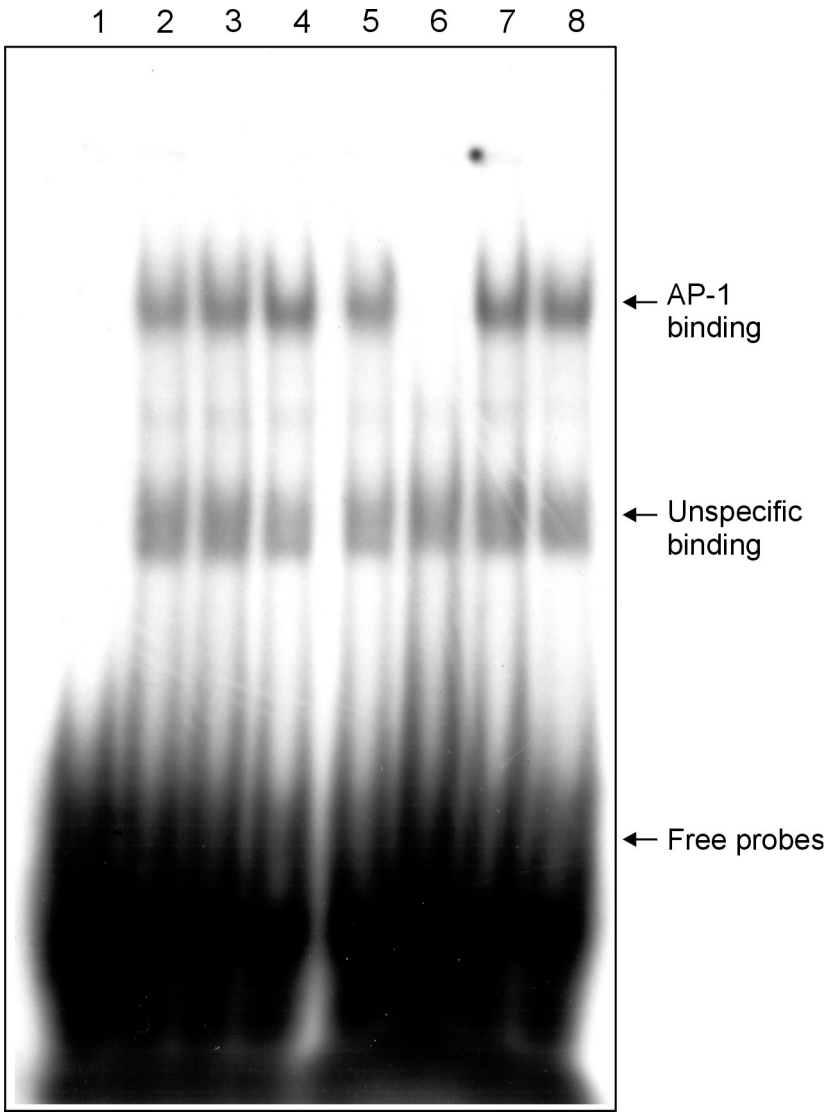


Figure 22. No direct effect of T, E_2 and FCS on AP-1 binding activity. Nuclear extracts from RAW-fos13 macrophages stimulated for 1 h with T, E_2 and charcoal-stripped FCS were extracted, stored at –70°C until analysis. EMSA gels were run following the binding reaction with ^{32}P labeled AP-1 oligonucleotide and subjected to autoradiography. Samples shown are (1) free AP-1 probes, (2) vesicle-treated control, (3) 10 nM T, (4) 1 nM E_2, (5) 10% (v/v) FCS, (6) same as (5) but only a 100-fold excess of unlabeled oligonucleotide was added in the binding reaction. (7) 10 nM T and FCS, (8) 1 nM E_2 and FCS.

There are three major cis-elements: cAMP/Ca^{2+} response element (CRE), serum response element (SRE), and sis-inducible enhancer (SIE) in *c-fos* promoter region (Karin, 1995; Hill and Treisman, 1995). Since CRE belongs to the AP-1 family we first tested the effect of T on binding activity of AP-1 oligonucleotide with consensus sequence. Although the specific binding can be confirmed by the competition experiment using 100-fold unlabeled oligonucleotide, no significant alteration in the binding activity could be observed in cell nuclear preparations from T treated RAW-fos13 cells, compared with that from controls (Fig. 22).

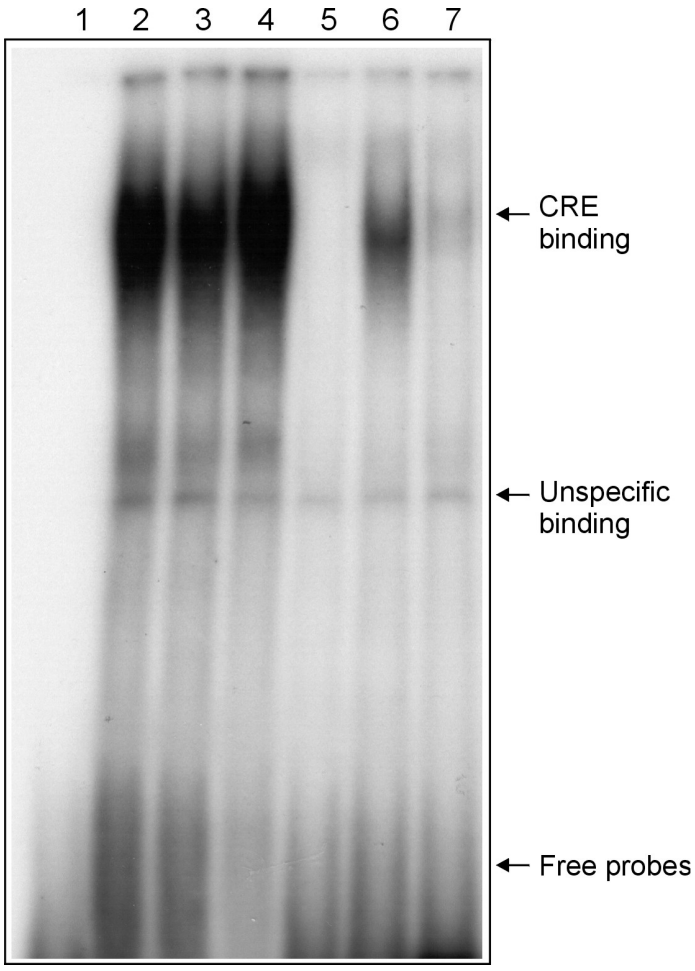


Figure 23. No influence of T and FCS on CRE binding activity. RAW-fos13 macrophages were stimulated with T and charcoal-stripped FCS for 1 h, then the nuclear extracts were prepared and stored at -70°C until EMSA analysis. The gel was run following the binding reaction with CRE oligonucleotide labeled using ^{32}P and subjected to autoradiography. Samples shown are (1) free CRE probes, (2) vesicle-treated control, (3) 10% (v/v) FCS, (4) 10 nM T and FCS, (5) nuclear extracts from FCS treated cells incubated with a 100-fold excess of unlabeled oligonucleotide in the binding reaction. (6) same as (5) but with 100-fold excess of unlabeled mutant oligonucleotide, (7) same as (5) but with 100-fold excess of CRE oligonucleotides of consesus sequence.

There is also no significant change in the binding activity of AP-1 between (1) FCS and control; (2) FCS plus T and FCS alone (Fig. 22). This might because the consensus AP-1 sequence used here is not specific enough, therefore the tests were performed again with CRE, SRE and SIE sequences derived directly from the mouse *c-fos* promoter. However, the similar negative results were obtained just as in the experiments with AP-1. Fig. 23 shows a typical result from CRE binding. The data for SRE and SIE are not shown.

3.4 Effects of E_2 on RAW-fos13 Macrophages

3.4.1 Expression of iER and mER

Macrophages have been reported to express both nuclear ER (Srivastava *et al.*, 1999) and mER (Benten *et al.*, 2001). In this study, the presence of ER and mER in RAW-fos13 cells is confirmed. As revealed by RT-PCR, RAW-fos13 macrophages express ERα, but not ERβ (Fig. 24A). However, the expression of ERα can be also detected, but only in permeabilized cells, by flow cytometry using the antibody ERα (H-180) binding to the N-terminal steroid binding domain of ERα (Fig. 24B) and the antibody ERα (MC-20) probing the C-terminal part of ERα (data not shown). The majority of ERα is localized in the cytoplasm of cells as revealed by CLSM (Fig. 24C). However, these two anti-ERα antibodies do not react with any ERα on the surface of intact RAW-fos13 cells. Nevertheless, the RAW-fos13 cells exhibit binding sites for E_2 on their surface. When intact RAW-fos13 cells were incubated with the plasma-membrane impermeable E_2-BSA-FITC for 1 min, an increased fluorescence intensity was revealed by flow cytometry (Fig. 25A). CLSM localizes this bound fluorescence exclusively on the cell surface (Fig. 25B). By contrast, the cells do not bind any BSA-FITC under the same experimental conditions (Fig. 25A and B). Moreover, the red fluorescence of ConA-rhodamine was localized on the surface of intact cells and was colocalized with E_2-BSA-FITC (Fig. 25B). When incubation time is longer, the E_2-BSA-FITC becomes sequestrated as detailed before (Benten *et al.*, 2001).

3.4.2 E_2-induced rise in $[Ca^{2+}]_i$

At the physiological concentration of 1 nM, E_2, but not 17α-E_2, induces a rise in $[Ca^{2+}]_i$ as determined in Fura-2-loaded RAW-fos13 cells by Ca^{2+} fluorescence spectroscopy (Fig. 26A).

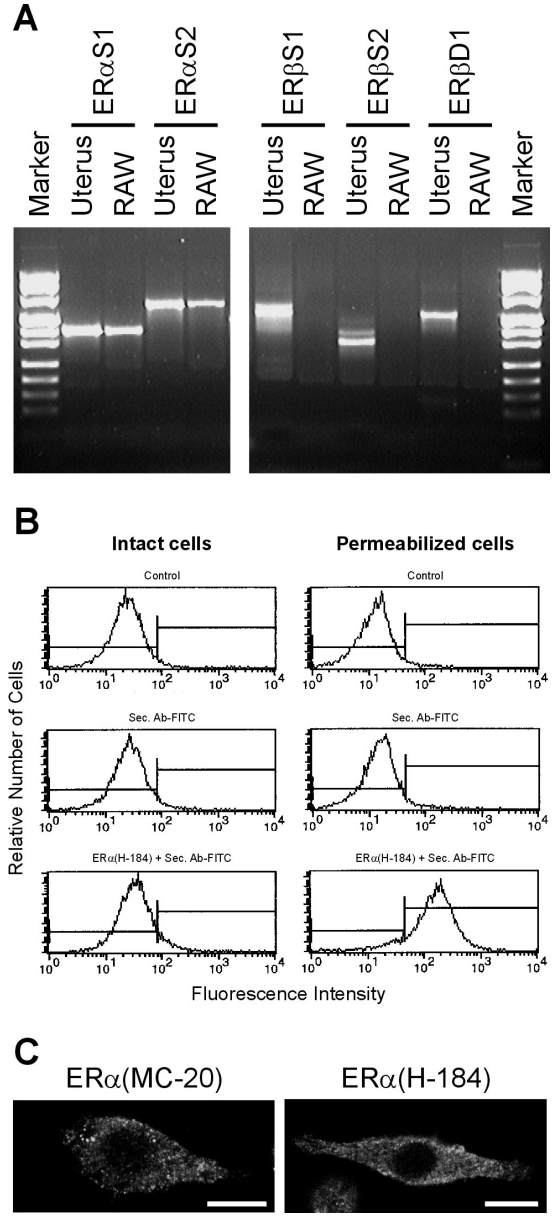


Figure 24. Detection of iERα and iERβ in RAW-fos13 cells. (**A**) RT-PCR was performed with the primer pairs ERαS1 and ERα2, spanning 385 bp and 608 bp of the steroid binding domain of ERα, respectively. Both primer pairs ERβS1 and ERβS2 amplified two iERβ-fragments of the steroid binding domain of 578 bp and 542 bp as well as 382 bp and 328 bp, respectively. The primer pair ERβD1 spanning 507 bp of the DNA binding domain of iERβ. The fragments were separated in 2% agarose gels. (**B**) Flow cytometry of intact and permeabilized RAW-fos13 cells labeled with the anti-iERα antibody ERα(H-184) or with only secondary antibody coupled to FITC (Sec. Ab-FITC). (**C**) Using CLSM, the anti-iERα antibodies ERα(MC-20) and ERα(H-184) localize ERα in the cytoplasm of permeablized RAW-fos13 cells. Bars represent 10 µm.

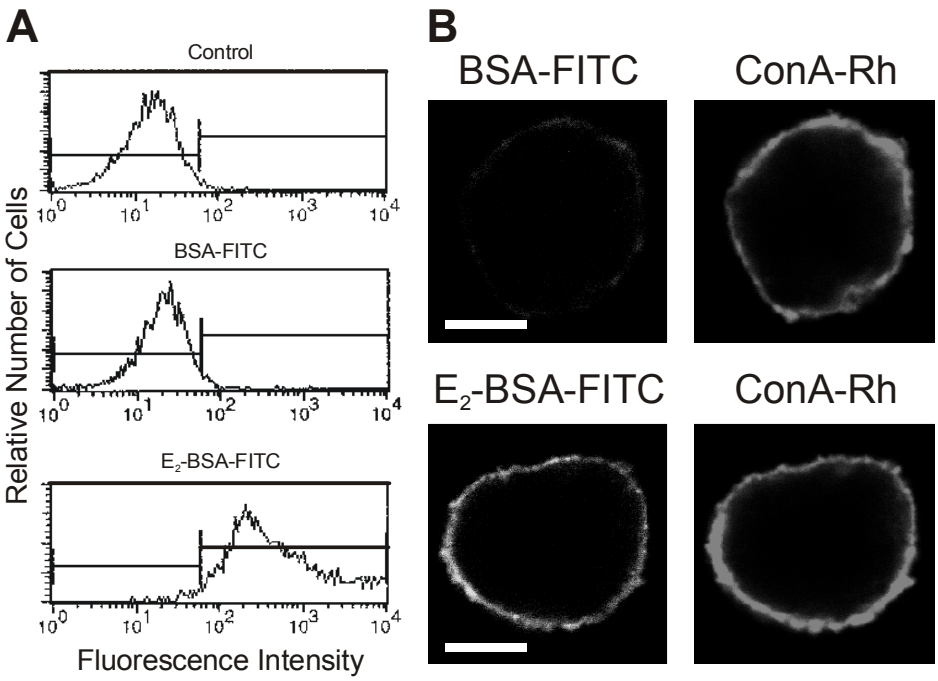


Figure 25. Existence of mER in RAW-fos13 cells. (**A**) Flow cytometry of intact RAW-fos13 cells labeled with E_2-BSA-FITC and BSA-FITC. (**B**) CLSM localizes E_2-BSA-FITC but not BSA-FITC on the surface of RAW-fos13 cells. The red fluorescence of ConA-Rh can be also localized on the cell surface. Bars represent 10 µm.

The amount of E_2-raised Ca^{2+} varies from experiment to experiment between about 50-90 nM Ca^{2+}. About the same increase in $[Ca^{2+}]_i$ can be induced with the plasma membrane-impermeable E_2-BSA, whereas BSA alone is ineffective to affect $[Ca^{2+}]_i$ (Fig. 26B). Moreover, the E_2-induced rise in $[Ca^{2+}]_i$ cannot be prevented upon pre-incubation of the cells with different iER-blockers such as ICI 182,780, tamoxifen and raloxifene (Fig. 26C and D). The rise in $[Ca^{2+}]_i$ can be due to influx of extracellular Ca^{2+} and/or to release of Ca^{2+} from intracellular Ca^{2+} stores such as endoplasmic reticulum. When extracellular Ca^{2+} is removed by 2 mM EGTA, E_2 is still able to induce a slight increase in $[Ca^{2+}]_i$ (Fig. 26E). In addition, the PLC inhibitor U-73122 prevents most of the E_2-induced rise in $[Ca^{2+}]_i$, whereas the ineffective analog compound U-73343 does not abrogate the E_2-induced increase in $[Ca^{2+}]_i$ (Fig. 26F). This indicates that E_2 induces both influx of extracellullar Ca^{2+} and release of Ca^{2+} from intracellular Ca^{2+} stores in RAW-fos13 cells. Furthermore, preincubation of cells with PTX abolishes most of the E_2-induced rise in Ca^{2+}, which suggests participation of G-proteins in the E_2-induced rise in

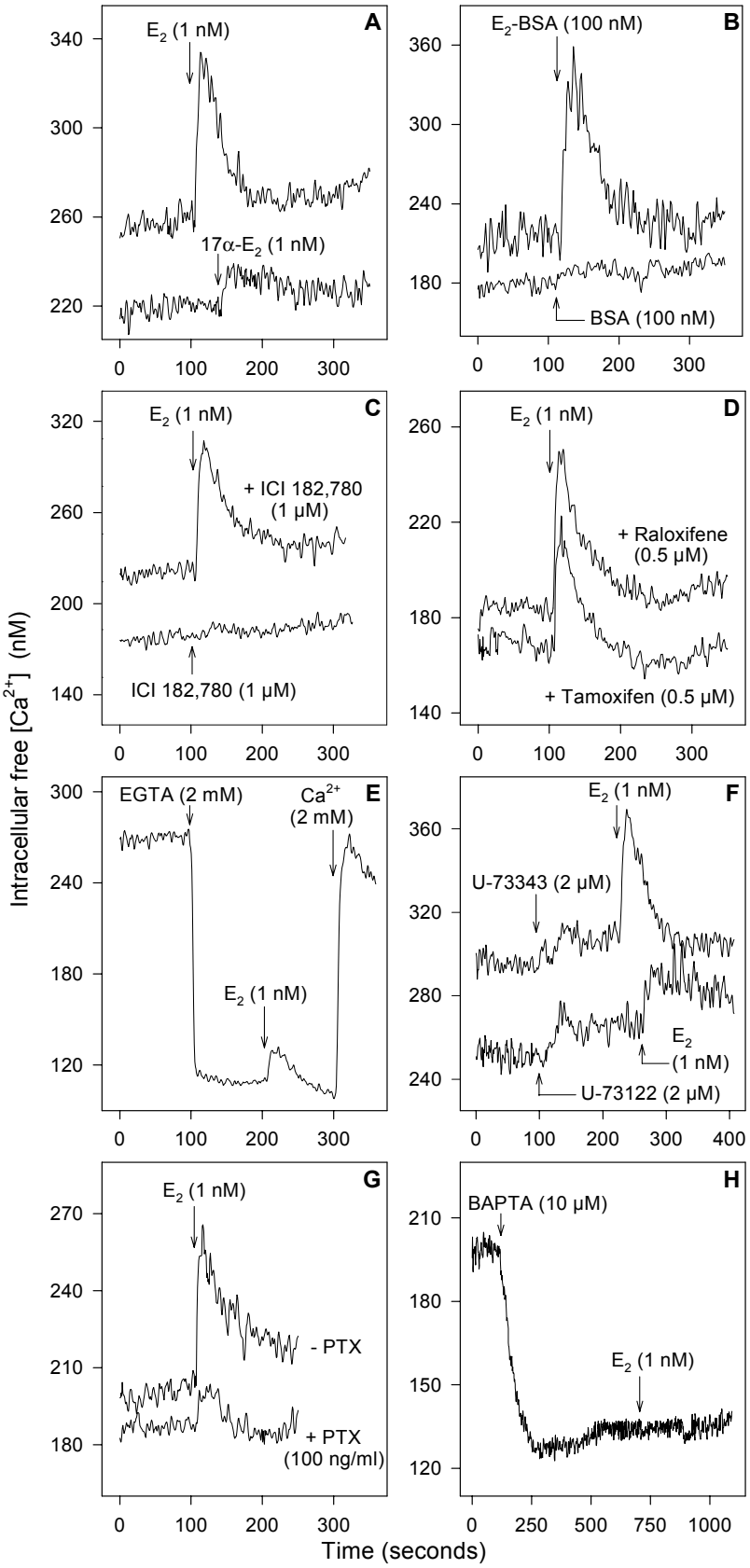


Figure 26. E_2-induced rise in $[Ca^{2+}]_i$ of RAW-fos13 cells. (**A**) E_2 triggers an immediate increase in $[Ca^{2+}]_i$ while 17α-E_2 is largely ineffective. (**B**) Cells stimulated with E_2-BSA or BSA. (**C**) Cells preincubated with ICI 182,780 for 1 h before E_2 application, ICI 182,780 alone does not affect $[Ca^{2+}]_i$. (**D**) Cells pretreated with raloxifene or tamoxifen for 1 h before addition of E_2. (**E**) Cells incubated with EGTA for 100 s before addition of E_2. (F) Cells pretreated with PLC inhibitor U-73122 or the inactive compound U-73343 for 2 min before E_2 stimulation. (**G**) Cells preincubated with or without PTX for 16 h before E_2 application. (**H**) Cells pretreated with BAPTA for 10 min before E_2 addition. Arrows show addition of reagents with indicated concentrations to RAW-fos13 cell suspensions. Representative experiments are shown and the results were verified in at least four independent experiments.

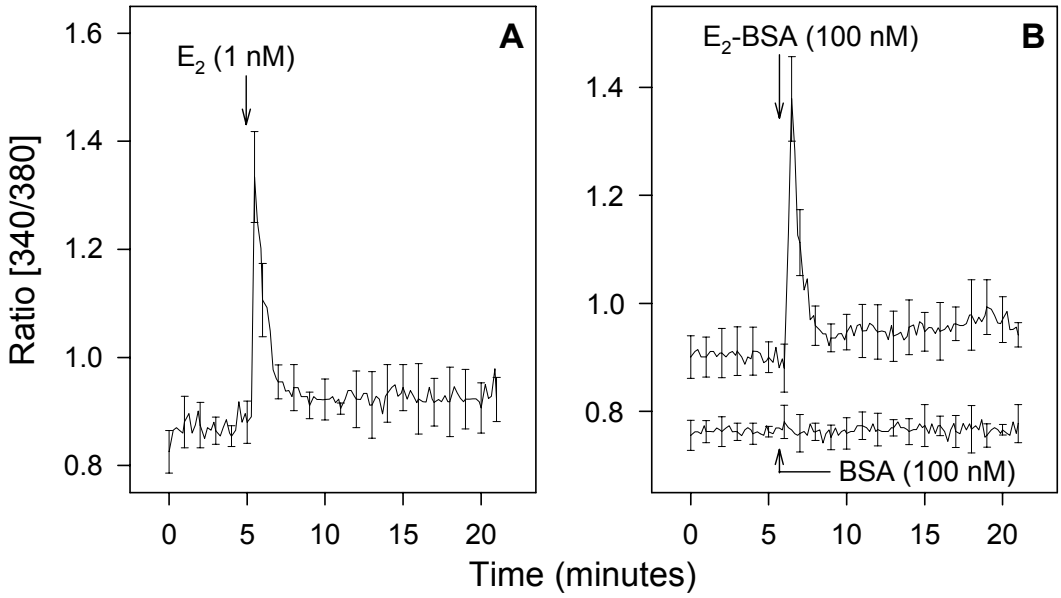


Figure 27. E_2 and E_2-BSA-induced calcium response measured by single cell calcium imaging. (**A**) E_2 elicits an increase in $[Ca^{2+}]_i$ (n=4, "n" indicates number of cells). (**B**) Cells were stimulated with E_2-BSA (n=4) and BSA (n=5), respectively. Arrows indicate addition of the substance. Representative experiments are shown and the results were verified in at least two independent experiments.

$[Ca^{2+}]_i$ (Fig. 26G). Also, E_2 and E_2-BSA-induced Ca^{2+} response has been confirmed with single cell calcium imaging (Fig. 27).

3.4.3 Inability of E_2 to activate MAPK and *c-fos* promoter

It has been reported that E_2 can activate directly MAPKs (Migliaccio *et al.*, 1996; Improta-Brears *et al.*, 1999; Razandi *et al.*, 2000; de Jager *et al.*, 2001; Duan *et al.*, 2001). We have therefore examined the effect of E_2 on the activation of the three MAPK families ERK1/2, p38, and JNK/SAPK. When RAW-fos13 cells were incubated with 1 nM E_2 for various periods up to 180 min, none of the three MAPKs becomes activated as revealed by immunodetection (Fig. 28). Moreover, E_2 has been reported to induce expression of the Ca^{2+} sensitive immediate early gene *c-fos* (Duan *et al.*, 2001). However, 1 nM E_2 is not able to induce any significant activation of the *c-fos* promoter in RAW-fos13 cells (Fig. 29A).

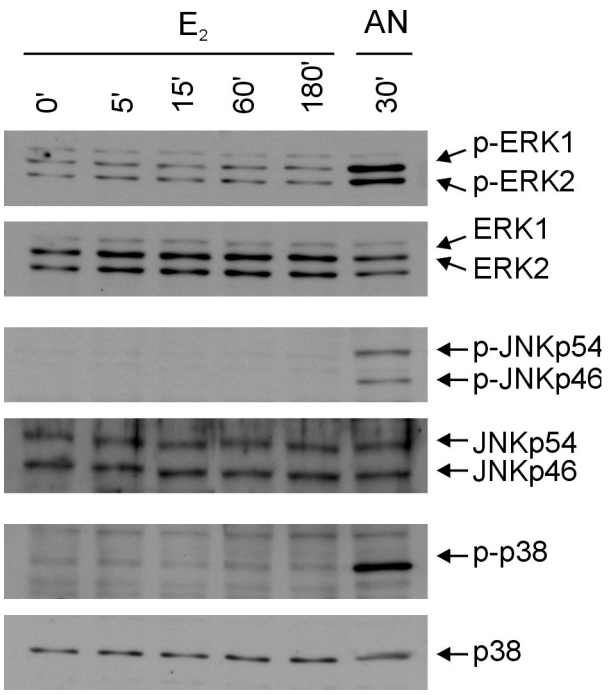


Figure 28. No direct activation of MAPK by E_2. RAW-fos13 cells were treated with 1 nM E_2 for different periods. Protein extracts were subjected to immuno-determination as described in Materials and methods. The upper panels for p-ERK1/2, p-JNK/SAPK, or p-p38 MAPK detected by anti-phospho-antibodies represent the phosphorylated forms, whereas the lower panels total ERK1/2, JNK/SAPK, or p38 MAPK as detected with anti-total MAPK antibodies. Representative blots are shown. The results were verified in at least two independent experiments. A 30 min stimulation with 10 µg/ml anisomycin (AN) was used as positive control.

3.4.4 E_2-induced up-regulation of LPS signaling

LPS is known to activate macrophages including induction of the immediate early gene *c-fos* (Introna *et al.*, 1986; Collart *et al.*, 1987). In accordance, we can show that LPS also stimulates the *c-fos* promoter by about 4-fold in RAW-fos13 cells (Fig. 29A). E_2 even augments this LPS-effect, whereas 17α-E_2 is ineffective (Fig. 29B). The up-regulatory E_2-effect cannot be prevented by the iER-inhibitors ICI 182,780, tamoxifen, and raloxifene (Fig. 29B). E_2 exerts its augmentation of LPS-stimulation on *c-fos* promoter through rise of $[Ca^{2+}]_i$. Indeed, BAPTA when used under conditions blocking intracellular free Ca^{2+} accumulation (Fig. 26H) also prevents the E_2-induced augmentation of LPS-stimulated *c-fos* promoter (Fig. 29C). There is also no increase of LPS-stimulation by E_2, when Ca^{2+} mobilization is inhibited with the PLC inhibitor U-73122, in contrast to the ineffective compound U-73343 (Fig. 29C). Moreover, the inhibitor GF109203x of the Ca^{2+} sensitive PKC prevents the E_2-induced up-regulation of LPS-activated *c-fos* promoter (Fig. 29C).

Since PTX blocks this E_2-activation, G-proteins are obviously involved in the E_2-induced augmentation of *c-fos* promoter activity (Fig. 29C).

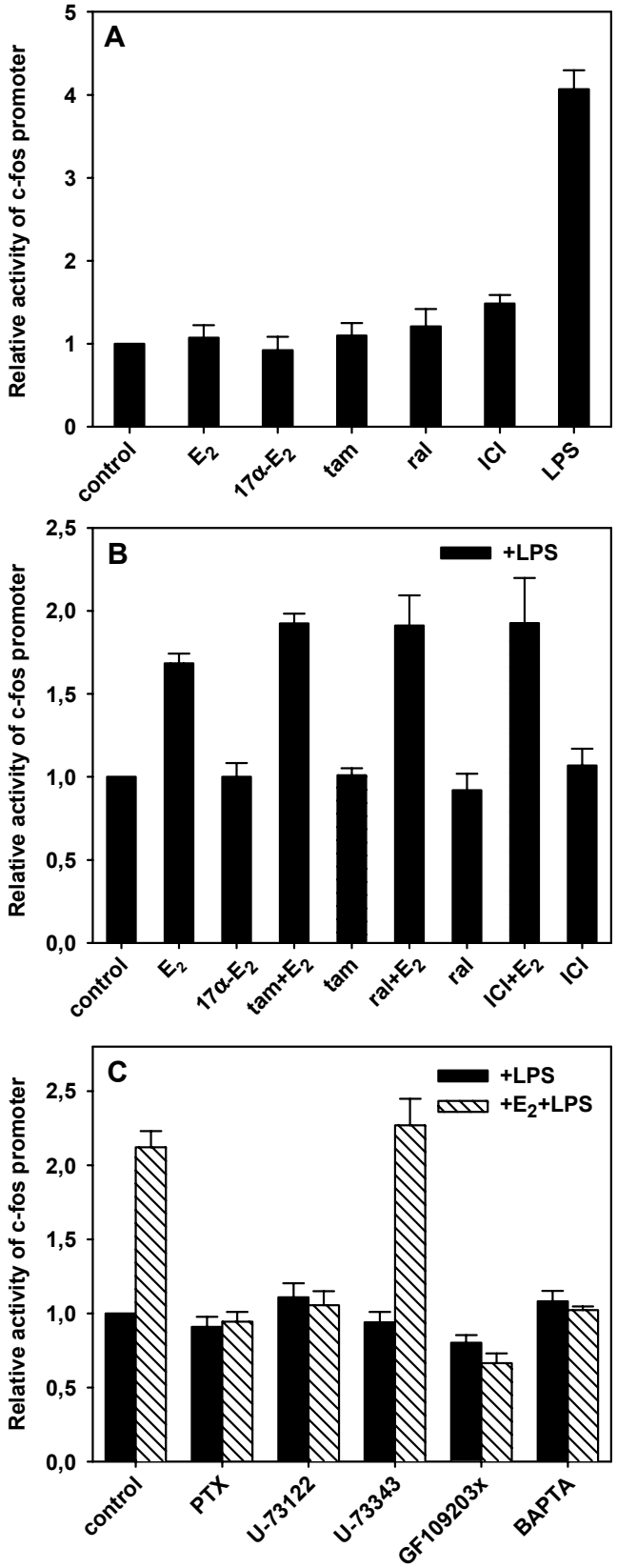


Figure 29. Effects of E_2 on activation of *c-fos* promoter induced by LPS. (**A**) No direct effect of E_2 on *c-fos* promoter activity. RAW-fos13 cells were cultured in IMDM containing 5% charcoal-stripped FCS for 18 h, then treated with 1 nM E_2, 1 nM 17α-E_2, 0.5 µM tamoxifen (tam), 0.5 µM raloxifene (ral), 1µM ICI 182,780 (ICI), or 1 µg/ml LPS for 3 h. The *c-fos* promoter activity of each group was determined relative to the activity of vehicle-treated control. Values are means ± SEM of at least three independent experiments performed in triplicate. (**B**) E_2-augmented activation of *c-fos* promoter by LPS. Cells were stimulated with 1 µg/ml LPS for 3 h (control), and in combination with 1 nM of E_2, 17α-E_2, or 0.5 µM tamoxifen (tam), 0.5 µM raloxifene (ral), and 1µM ICI 182,780 (ICI), respectively. The *c-fos* promoter activity was expressed relatively to the *c-fos* promoter activity induced by LPS alone. (**C**) Cells were stimulated with 1 µg/ml LPS alone for 3 h or in combination with 1 nM E_2 (control). In the other groups, cells were pretreated with 10 µM BAPTA for 10 min, 2 µM U-73122 for 2 min, 2 µM U-73343 for 2 min, 100 ng/ml PTX for 16 h, and 1 µM GF109203x for 10 min, respectively, before stimulation with LPS and E_2.

Activation of macrophages by LPS is also associated with activation of the three MAPK families ERK1/2, p38, and JNK/SAPK (Hambleton *et al.*, 1996; Feng *et al.*, 1999). Indeed, when RAW-fos13 cells are stimulated with LPS for various periods up to 180 min, all of the three MAPK families are mostly activated at 15 min, and, thereafter, activation is declined, but still detectable after 180 min (Fig. 30). E_2 selectively interferes with activation of MAPKs induced by LPS. It does not affect the activities of ERK1/2 and JNK/SAPK, but only the activation of p38 becomes up-regulated at 15 min (Fig. 30).

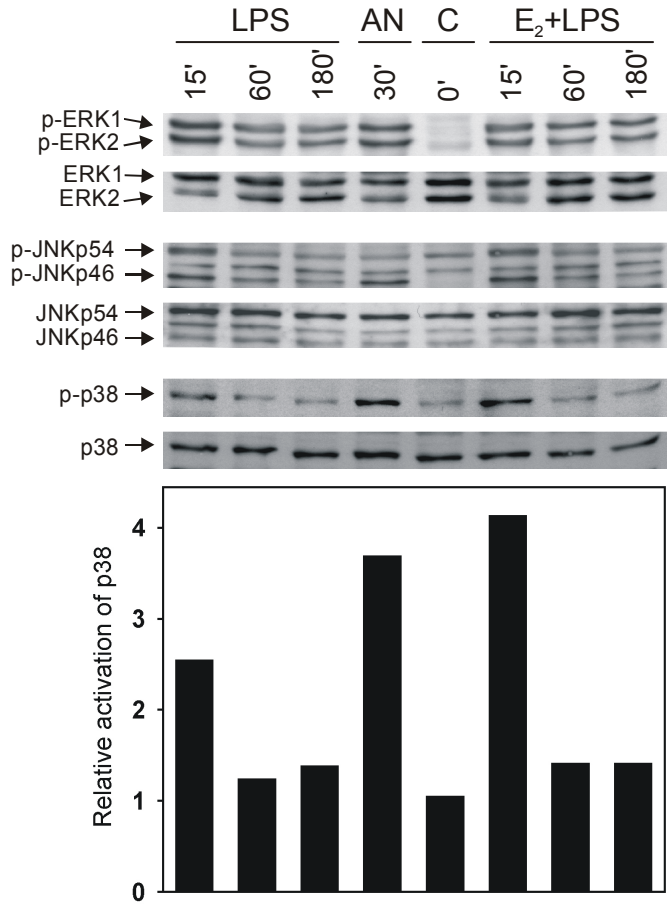


Figure 30. Effects of E_2 on activation of three different MAPKs induced by LPS. RAW-fos13 cells were stimulated with 1 µg/ml LPS and in combination with 1 nM E_2 for different periods, and protein extracts were subjected to immuno-determination. The upper panels for p-ERK1/2, p-JNKp46/p54, or p-p38 MAPK represent the phosphorylated forms, and the lower panels the corresponding total kinases. The relative activation of p38 MAPK was expressed in terms of the ratios of the phosphorylated forms to total kinase protein with setting the ratio of the negative unstimulated control (C) as one. As a positive control for MAPK activation the cells were stimulated with 10 µg/ml anisomycin (AN) for 30 min. Representative blots are shown and the results were verified in at least two independent experiments.

Activation of p38 MAPK is known to be involved in the control of NO-production of macrophages (Chen *et al.*, 1999). We have therefore examined the effect of E_2 on LPS-stimulated NO-production. In RAW-fos13 cells, stimulation with LPS for 24 h causes a dramatic increase in NO-production to approximately 30 µM nitrite (Fig. 31A). Moreover, co-incubation with E_2 results even in an increase of NO-production by about 50%. This stimulatory E_2 effect cannot be inhibited by tamoxifen and raloxifene (Fig. 31A). However,

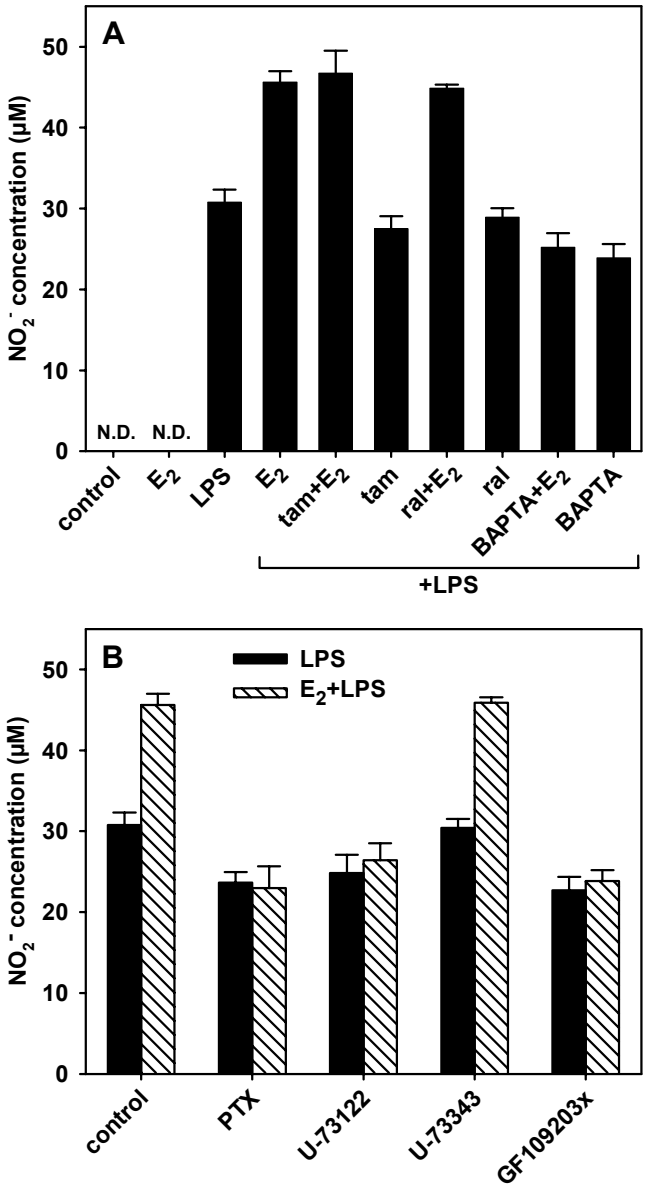


Figure 31. Influence of E_2 on LPS-induced NO-production of RAW-fos13 cells. (**A**) Cells were cultured in IMDM containing 5% stripped FCS for 18 h, then stimulated for 24 h with 1 nM E_2, 1 µg/ml LPS, with or without 1 h preincubation of 0.5 µM tamoxifen (tam), 0.5 µM raloxifene (ral), or 10 min preincubation with 10 µM BAPTA. N.D. = Not detectable. The results shown are means ± SEM from at least three independent experiments performed in triplicate. (**B**) Cells were stimulated with 1 µg/ml LPS or LPS plus 1 nM E_2. Before stimulation, the cells were pretreated with U-73122, U-73343, PTX, GF109203x, as described in **Fig. 29.**

the E_2 effect can be abrogated by preincubation of cells with the intracellular Ca^{2+} chelator BAPTA (Fig. 31A). The view that Ca^{2+} is important for this E_2 up-regulatory activity is further supported by the fact that the PLC-inhibitor U-73122 blocks the E_2-stimulated LPS-activation of NO-production, but not the ineffective analog compound U-73343 (Fig. 31B).

Moreover, the inhibitor GF 109203x of the Ca^{2+}-sensitive PKC prevents the E_2-induced up-regulation of LPS-stimulated NO-production just as PTX (Fig. 31B).

3.4.5 E_2-induced down-regulation of FCS signaling

In contrast to its effects on LPS-signaling, we found the opposite effect of E_2 on FCS-induced signaling. FCS stimulates activation of the *c-fos* promoter by about 5-fold, whereas

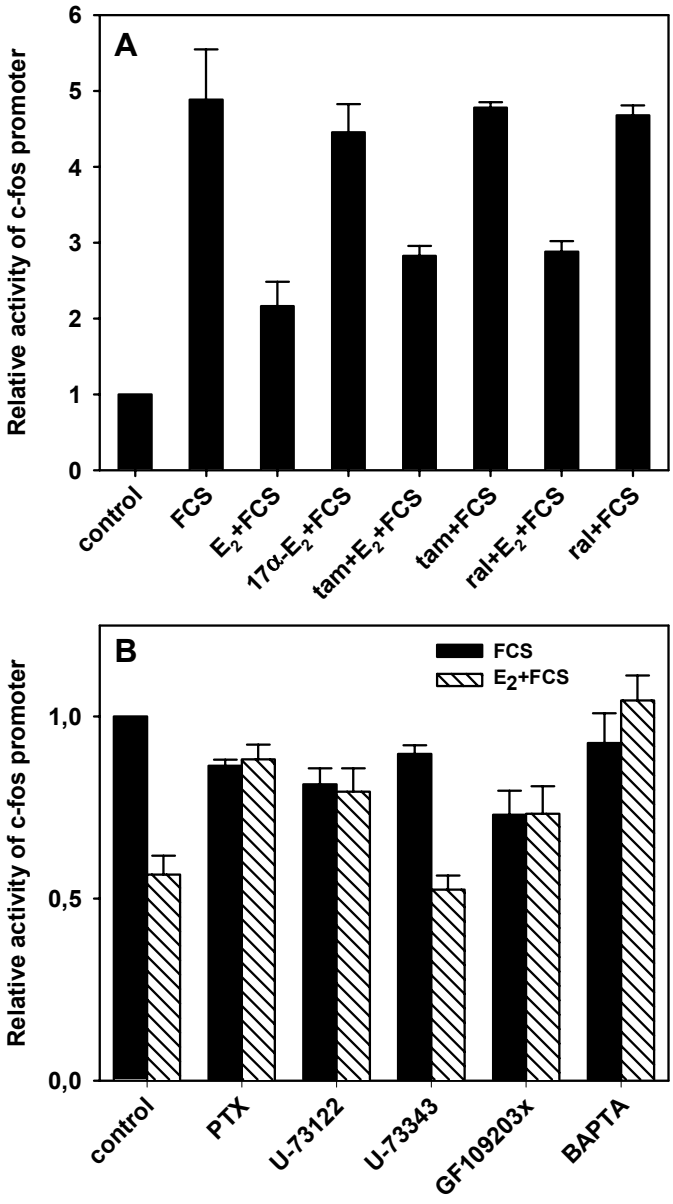


Figure 32. E_2-attenuated activation of *c-fos* promoter induced by FCS. (**A**) RAW-fos13 cells were serum-starved for 18 h and then stimulated for 3 h with 10% (v/v) FCS, or in combination with 1 nM E_2, 1 nM 17α-E_2, 0.5 µM tamoxifen (tam) and 0.5 µM raloxifene (ral). The *c-fos* promoter activity of each group was determined relatively to the vehicle-treated control. The values represent means ± SEM from at least three independent experiments performed in triplicate. (**B**) Cells were stimulated for 3 h with 10% (v/v) FCS or FCS plus 1 nM E_2. Inhibitors were used as indicated in Fig. 4. The *c-fos* promoter activity from each group was expressed relatively to the *c-fos* promoter activity induced by FCS.

E_2, but not 17α-E_2, down-regulates FCS-stimulated activity of *c-fos* promoter to about 2-3 fold (Fig. 32A). This down-regulation cannot be prevented by tamoxifen or raloxifene (Fig. 32A). The down-regulatory effect of E_2 is mediated through mobilization of intracellular Ca^{2+}, as substantiated by experiments using BAPTA, U-73122, U-73343, and GF109203x

(Fig. 32B). Also, PTX prevents the E_2-induced down-regulation of FCS-stimulated *c-fos* promoter activation (Fig. 32B). FCS-stimulation of RAW-fos13 cells activates ERK1/2, but not p38 and JNK/SAPK (Fig. 33). The presence of E_2 during FCS stimulation down-regulates ERK1/2 activation to a similar extent as it does to *c-fos* promoter activity (Fig. 32A).

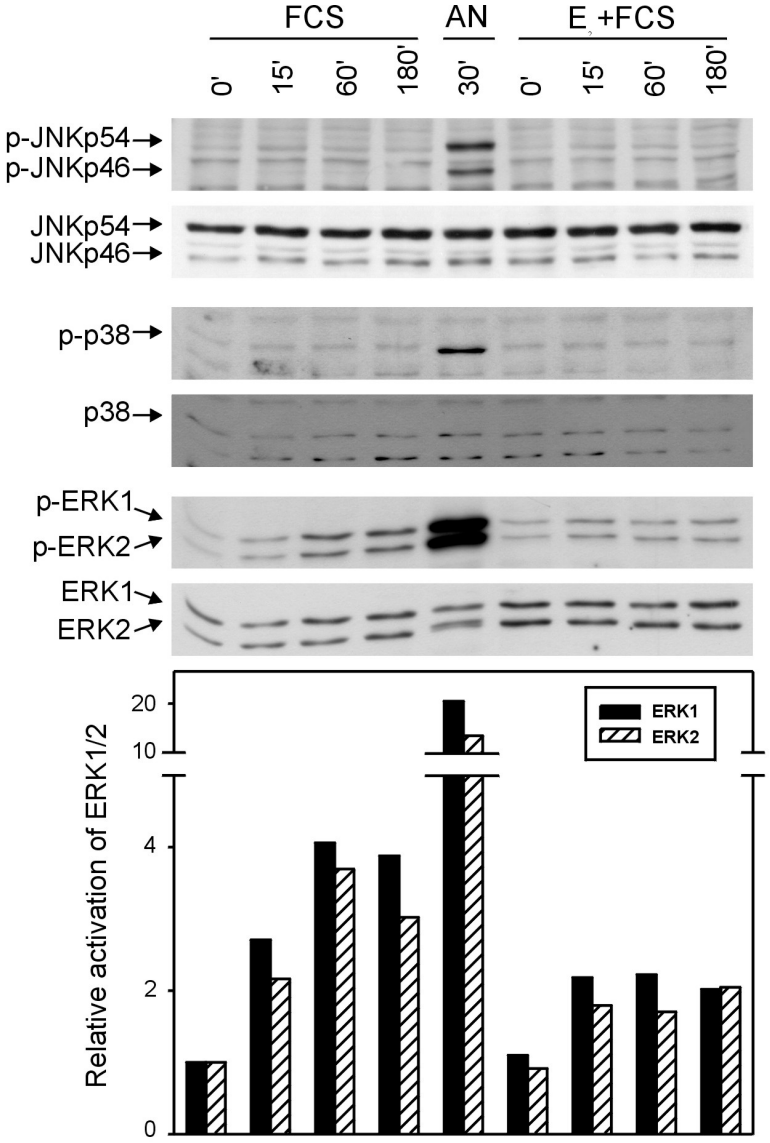


Figure 33. Effects of E_2 on activation of MAPKs induced by FCS. Cells were stimulated with 10% (v/v) FCS and 1 nM E_2 plus FCS for different periods, with 10 µg/ml anisomycin (AN) as a positive control of MAPK activation. Cell lysates were subjected to immunodetection and the relative activation of ERK1 and ERK2 from each group was expressed relatively to the activation level at 0 min of FCS stimulation. Representative blots are shown, and the results were verified in at least two independent experiments.

4 DISCUSSION

4.1 T- and E_2-induced Nongenomic Signaling in Macrophages

Increasing evidence has been accumulated that steroids can act not only through genomic but also through nongenomic pathways (Brann *et al.*, 1995; Falkenstein *et al.*, 2000). The genomic pathway usually involves transcriptional regulation of gene expression, while the rapid nongenomic effects of steroids often become evident as increases of $[Ca^{2+}]_i$, cAMP, or activation of PKC, etc (Falkenstein *et al.*, 2000). The male sex hormone T, for example, has been reported to induce a rapid increase in $[Ca^{2+}]_i$ in male rat osteoblasts (Lieberherr and Grosse, 1994). Previous work from our group also showed that T induces a rise in $[Ca^{2+}]_i$ within seconds in mouse splenic T lymphocytes (Benten *et al.*, 1997; Benten *et al.*, 1999). In this study, another type of immune cells, the macrophages was investigated. In both IC-21 and RAW-fos13 macrophages, the physiological concentrations of 10 nM T can induce a rapid rise of $[Ca^{2+}]_i$. This increase of $[Ca^{2+}]_i$ is predominantly due to release of Ca^{2+} from intracellular calcium stores rather than the influx of the extracellular Ca^{2+} as revealed by the Ca^{2+} chelator EGTA and the PLC inhibitor U-73122. This is different from that of osteoblasts and T lymphocytes. In osteoblasts T induces both Ca^{2+} release from intracellular calcium store and influx of extracellular Ca^{2+}, while in T lymphocytes, the $[Ca^{2+}]_i$ increase is resulted solely from the Ca^{2+} influx. These data suggests that the mechanism of T-induced nongenomic Ca^{2+} signaling may differ among various cell-types.

It has been reported that nongenomic actions of steroids can be mediated through the classical intracellular steroid receptors, or some membrane or membrane-bound receptors. In human prostate cancer cell line LNCaP, the androgen dimethylnortestosterone or 5α-DHT raised $[Ca^{2+}]_i$ can be inhibited by preincubation of the cells with the iAR-blocker hydroxyflutamide, suggesting the involvement of iAR (Steinsapir *et al.*, 1991). However, for both IC-21 and RAW-fos13 macrophages, the T-induced nongenomic Ca^{2+} signaling is not mediated through iARs since both cell lines do not express any iAR, as revealed by RT-PCR, flow cytometry, Western-blot, and CLSM. In line with this result, the iAR-blocker cyproterone can not prevent the T-induced rise in $[Ca^{2+}]_i$. Also, we can exclude a possible action of T, after aromatization to 17β-estradiol, through the iER, because the T-induced

Ca^{2+} response was also not sensitive to the iER-blockers raloxifene, tamoxifen and ICI 182,780.

Our data demonstrate that T exerts its nongenomic iAR- and iER-independent effects through putative mAR. This has been supported by two pieces of evidence: (i) an increase in $[Ca^{2+}]_i$ is also inducible with the plasma membrane-impermeable ligand T-BSA; (ii) the specific binding sites for T can be localized on the surface of both intact IC-21 and RAW-fos13 cells with T-BSA conjugated to FITC. The T-raised $[Ca^{2+}]_i$ can be completely blocked by preincubation of the cells with the G-protein inhibitor PTX, or the PLC inhibitor U-73122, but not with the inactive compound U-73343. This proposed that T-induced nongenomic Ca^{2+} signaling maybe mediated through a membrane receptor belonging to the class of G-protein-coupled receptors (GPCR).

It is known that a wide variety of GPCRs, e.g. the prototypic β2-andrenergic receptor, become sequestrated after ligand binding, which is considered important for regulation of signaling, recycling and responsiveness for GPCRs (Yu *et al.*, 1993; Pippig *et al.*, 1995; Koenig and Edwardson, 1997). In accordance, IC-21 and RAW-fos13 cells have shown sequestration a few minutes after binding of T as revealed by labeling with T-BSA-FITC. The internalization process is not a simple fluid endocytosis or a constitutive endocytotic pathway, but rather is ligand-specific. This has been proved by the fact that T and T-BSA but not 1-DeHT and E_2 competitively inhibit internalization of surface-bound T-BSA-FITC. In addition, the internalization of GPCR for T is selective, i.e. only distinct plasma membrane domains are internalized excluding surface markers such as F4/80 and ConA-rhodamine. Generally, GPCRs internalize via the clathrin-coated vesicle-mediated endocytotic pathway (Doxsey *et al.*, 1987; Robinson *et al.*, 1996; Zhang *et al.*, 1996), although entry maybe mediated through caveolae (Chun *et al.*, 1994; Kiss and Geuze, 1997). However, our data suggest that GPCR for T does not proceed along such pathways. Indeed, the punctate fluorescence of internalized T-BSA-FITC in IC-21 and RAW-fos13 macrophages is associated with neither clathrin- nor caveolin nor acidic vesicles. Obviously, the ligand-triggered entry of GPCR for T into IC-21 and RAW-fos13 cells is mediated by a clathrin- and caveolin- independent internalization pathway. On the other hand, the internalization process of GPCR for T in IC-21 and RAW-fos13 macrophages resembles the process of numerous other GPCRs as it is critically dependent on temperature, ATP, and cytoskeletal elements (Morrison *et al.*, 1996; Koenig *et al.*, 1997). Nongenomic T signaling through mAR has also been described in rat osteoblasts and mouse spleen T lymphocytes, but they

are different from those in IC-21 and RAW-fos13 macrophages. First, different Ca^{2+} sources were used for the generation of Ca^{2+} rise (as described above). Second, the mAR in T lymphocytes (Benten *et al.*, 1999) and rat osteoblasts (Lieberherr and Grosse, 1994) can not be internalized upon ligand binding, although the latter has also been proposed to be GPCR for T in the cell membrane (Lieberherr and Grosse, 1994).

Though still unknown in molecular terms, the mARs of the macrophages are likely to mediate actions specific for different androgens, since only T and 5α-DHT can induce about the same rise in $[Ca^{2+}]_i$, whereas 5β-DHT and 1-DeHT are ineffective to evoke changes in $[Ca^{2+}]_i$. As we known, 5α-DHT is a physiological effective derivative of T *in vivo*, while 5β-DHT and 1-DeHT are less active forms (Sheridan, 1991). Therefore, our study suggests that the ability of androgen to induce Ca^{2+} response might be functionally related to its physiological activity *in vivo*.

As a control for the specificity of the T effects, the female sex hormone E_2 has also been investigated in addition to the androgens above. Nongenomic E_2 signaling also manifests itself as an E_2-induced rapid rise of $[Ca^{2+}]_i$ in RAW-fos13 macrophages, as it has been recently found in murine IC-21 macrophages (Benten *et al.*, 2001). The experiments with extracellular Ca^{2+}-chelator EGTA and PLC-inhibitor U-73122 indicate that the E_2-induced Ca^{2+} rise is due to both influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular Ca^{2+} stores. This is in agreement with previous data also showing E_2-induced Ca^{2+} rise due to both influx of extracellular Ca^{2+} and intracellular Ca^{2+} mobilization in IC-21 macrophages (Benten *et al.*, 2001), rat osteoblasts (Lieberherr *et al.*, 1993), murine splenic T lymphocytes (Benten *et al.*, 1998), and pig granulosa cells (Lieberherr *et al.*, 1999). However, there are also data showing that E_2 induces only influx of extracellular Ca^{2+} in LNCaP human prostate cancer cells (Audy *et al.*, 1996) and human spermatozoa (Luconi *et al.*, 1999) or only intracellular Ca^{2+} mobilization in chicken granulosa cells (Morley *et al.*, 1992) and human peripheral monocytes (Stefano *et al.*, 1999).

It is noteworthy that in the same IC-21 and RAW-fos13 macrophages the E_2-induced Ca^{2+} response is different from that of T, because: (1) T induces only Ca^{2+} release from the intracellular calcium stores, while E_2 induces both release from intracellular Ca^{2+} stores and influx of extracellular Ca^{2+}. (2) The optimal concentration for T to induce the Ca^{2+} response is 10 nM while for E_2 is 1 nM. The E_2-induced Ca^{2+} response is specific, since 17α-E_2, which has a similar structure to E_2, is ineffective to elicit Ca^{2+} response in both IC-21 (Benten *et al.*, 2001) and RAW-fos13 macrophages. This proposed again that the

nongenomic Ca^{2+} signaling of steroids is not an unspecific universal effect but rather steroid-specific.

E_2-induced nongenomic Ca^{2+} signaling has been described to be dependent on the classical nuclear ER as well as to be independent of ER (Audy *et al.*, 1996; Benten *et al.*, 2001). For instance, in the human breast cancer cell line MCF-7, the E_2-induced rapid increase in $[Ca^{2+}]_i$ can be blocked by co-stimulation of the cells with iER-blocker ICI 182,780, suggesting the involvement of iER in this process (Improta-Brears *et al.*, 1999). In contrast to this data, the E_2-induced Ca^{2+} signaling in macrophages is not mediated through iER, but rather through mER. This view is supported by the following findings. First, the E_2-induced rise in $[Ca^{2+}]_i$ cannot be prevented by the ER-blockers ICI 182,780, tamoxifen, and raloxifene. Second, E_2-BSA, which cannot across the cell membrane and which binds to neither ERα nor ERβ (Stevis *et al.*, 1999), is also capable of rapidly raising $[Ca^{2+}]_i$, just as free E_2. Third, although the RAW-fos13 cells express ERα, they exhibit distinct binding sites for E_2-BSA-FITC on their surfaces. These surface binding sites are not accessible for anti-ERα antibodies on intact cells as revealed by flow cytometry and CLSM with two different anti-ERα antibodies. Fourth, it has been reported that transfection of CHO cells with ERα and ERβ cDNA resulted in the expression of both ERs on plasma membranes and the responsiveness of the cells to E_2 via the activation of G-proteins and PLC. This E_2 signaling could be inhibited by ER-blocker ICI 182,780 (Razandi *et al.*, 1999). In contrast to this data, in RAW-fos13 macrophages the E_2-induced increase of $[Ca^{2+}]_i$ via PLC is insensitive to ICI 182,780. This strongly supports the view that the E_2-induced nongenomic Ca^{2+} signaling in RAW-fos13 cells does not rely on the classical ER linked somehow to the plasma membrane. Fifth, inhibition of the E_2-induced Ca^{2+} signaling by PTX indicates that the mER is not identical to the classical nuclear ER as described so far, but rather belongs to the class of GPCRs. In accordance, E_2 induced nongenomic effects has also been reported to involve GPCRs in different tissues such as central nerve system and reproductive tissues (Kelly and Wagner, 1999).

As mAR and numerous other GPCRs, the mER is also agonist-sequestrable as recently characterized in mouse IC-21 macrophages (Benten *et al.*, 2001). Briefly, the mERs in RAW-fos13 cells can be sequestrated upon E_2 binding as indicated by E_2-BSA-FITC labeling. This internalization process is ligand-specific, since only E_2 and E_2-BSA but not 17α-E_2, T and T-BSA can competitively inhibit this process. Like mAR described before, mER internalization is selective, i.e. only distinct plasma membrane domains are

internalized, and these domains exclude, for example, the macrophage specific surface molecules F4/80. The mER sequestration is also mediated through a clathrin- and caveolin-independent pathway since there is no colocalization of vesicles containing E_2-BSA-FITC with caveolin, clathrin, and transferrin. This is in accrodance with the internalization of mAR in the same macrophages. However, there are also differences between mAR and mER, although both are proposed to be GPCRs. First, mAR mediates the T-induced Ca^{2+} rise solely through the mobilization of intracellular Ca^{2+}, while E_2 induced Ca^{2+} rise via mER is due to both mobilization of intracellular Ca^{2+} and influx of extracellular Ca^{2+}. Second, the internalization of mAR involves the cytoskeletal elements tubulin and microfilaments, while for mER the internalization is independent of this cytoskeletal elements as revealed by nocodazole and cytochalasin inhibition experiments (Benten *et al.*, 2001). It remains to be seen as to whether mAR and mER are two different receptors or there is only one receptor with different binding sites for E_2 and T coupled to different signaling pathways.

In addition to Ca^{2+} response, another frequently described nongenomic action of steroids is activation of MAPK. For androgens, it has been reported that stimulation of LNCaP cells with 5α-DHT leads to a rapid and reversible activation of ERK1/2 (Peterziel *et al.*, 1999). T induces activation of MAPKs in diverse cells such as osteoblasts (Kousteni *et al.*, 2001). However, in both IC-21 and RAW-fos13 cells no significant activation of ERK1/2 can be observed upon T stimulation. This discrepancy may rely on the fact that LNCaP cells and osteoblasts are AR-expressing cells, while the RAW-fos13 macrophages used here are AR-free. Therefore it is likely that the direct rapid activation of ERK1/2 requires iAR. In this context, the same authors also reported that the wild-type AR-free PC3 cells, in contrast to LNCaP cells, failed to show the rapid ERK1/2 activation following 5α-DHT treatment. While after transfection with an AR-expression vector, the PC3 cells began to show the ERK1/2 activation. For estrogens, it has been described that E_2 can induce a rapid activation of ERK1/2 in human MCF-7 breast cancer cells (Migliaccio *et al.*, 1996; Improta-Brears *et al.*, 1999), in rat cardiomyocytes (Nuedling *et al.*, 1999), in rat osteoblast-like ROS 17/2.8 cells (Endoh *et al.*, 1997), in rat cerebral cortical explants (Singh *et al.*, 1999), and in ERα-transfected CHO cells (Razandi *et al.*, 1999) through iER-dependent or iER-independent manner. By contrast, no significant activation of ERK1/2 can be observed after E_2 stimulation in RAW-fos13 cells. The reason is unknown, a possible explanation could be that E_2 induces ERK1/2 activation in a cell-type specific manner.

4.2 Nongenomic T and E_2 Signaling Regulates Genotropic Signaling

Our data described above have shown both T and E_2 can induce a rapid Ca^{2+} signaling in macrophages through specific membrane receptors. A question remains to be answered is whether the rise in $[Ca^{2+}]_i$ induced by T and E_2 has some influences on gene expression and cell function. Normally, expression of a certain gene is controled at transcriptional level through modulation of transcription factor activity. It has been reported that some transcription factors, especially the Ca^{2+}-sensitive transcription factors such as NFAT, JNK, ATF-2 and NFκB can be activated by an increase of $[Ca^{2+}]_i$ (Dolmetsch *et al.*, 1997). However, in IC-21 macrophages we were unable to show any significant activation of these transcription factors following T and E_2-induced Ca^{2+} increase. The possible reasons for the difference could be: (1) the T-induced increase of $[Ca^{2+}]_i$ in IC-21 macrophages is at the range of 50-100 nM, which is much lower than that of 200-400 nM described in Dolmetsch *et al.* (1997) and may be not high enough to activate the transcription factors; (2) the experiments by Dolmetsch *et al.* (1997) was carried out with special B lymphocytes derived from HEL-immunized mice, while IC-21 macrophages we used here was originated from C57BL/6 mice and may be less sensitive to $[Ca^{2+}]_i$ increase; (3) Western-blot may be not sensitive enough to detect the tiny activation of transcription factors induced by T and E_2. This prompted us to try more sensitive methods, for example, using a reporter gene assay to trace the early genotropic changes upon T and E_2 stimulation.

The proto-oncogene *c-fos*, as an IEG, is known to provide a link between short-term signals elicited at the membrane and long-term cellular responses. It has been reported that the strongly Ca^{2+}-sensitive *c-fos* can be activated by an increase of $[Ca^{2+}]_i$ through different mechanisms (Roche and Prentki, 1994; Johnson *et al.*, 1997; Van Haasteren *et al.*, 1999). Moreover, *c-fos* has been reported to be induced rapidly by numerous extracellular stimuli including steroids. For example, 5α-DHT induces a rapid transcription of *c-fos* in cells of the human prostate cancer cell line LNCaP within minutes (Peterziel *et al.*, 1999). E_2 induces a rapid activation of *c-fos* promoter in cells of the human breast cancer cell line MCF-7 (Duan *et al.*, 2001) and the human neuroblastoma cell line SK-N-SH (Watters *et al.*, 1997). Therefore, we supposed that T and E_2 may also be able to activate *c-fos*. In order to examine this possibility, the *c-fos* promoter-SEAP reporter construct was transiently or stably transfected into the cells and used as a molecular marker for the early genotropic activation.

Because IC-21 macrophages was difficult to transfect, the RAW264.7 macrophages was used in our study. However, to our surprise, in the experiments with RAW 264.7 and RAW-fos13 macrophages, both T and E_2 failed to induce a significant activation of the *c-fos* promoter directly. This reflects the steroid-induction of *c-fos* might be cell-type specific. In terms of gene activation, the possible explanation could be that although the Ca^{2+} signal by itself is important and necessary for the regulation of *c-fos* promoter activation, it is still not sufficient, probably some co-factors or co-activators are also required for the function. These co-factors normally can be provided by the co-stimulation of the cells with some other activators. This view has been supported by the theory of double- or mutiple signal models (Bhalla and Iyengar, 1999), for example, the maximal activation of NFκB, NFAT, JNK can be achieved only when the cells were treated simultaneously with both Ca^{2+} ionophore ionomycin and PKC activator PDBU (Dolmetsch *et al.*, 1997). For macrophages the co-activators could be provided by co-stimulation of cells with LPS or serum, therefore, the cells were stimulated with T/E_2 in context with LPS or FCS.

Thus, LPS stimulates *c-fos* promoter activation in RAW 264.7 and RAW-fos13 macrophages. This is in agreement with previous reports (Introna *et al.*, 1986; Introna *et al.*, 1987). The LPS-induced *c-fos* promoter stimulation can be dramatically attenuated by T-induced Ca^{2+} signaling. This attenuation can be completely abolished by the intracellular Ca^{2+} chelator BAPTA confirming the involvement of the T-induced increase in $[Ca^{2+}]_i$ and it is specific as supported by the following results. First, 5α-DHT is just as effective as T to induce the attenuation, whereas 5β-DHT and 1-DeHT are ineffective. Second, E_2 causes just an opposite effect as T, namely an enhanced activation of the *c-fos* promoter following LPS-stimulation. Third, T has no effect on SV40 promoter activity.

The nongenomic T calcium signaling does not only exert genotropic actions in context with LPS-signaling, but also has a specific impact on cell function. This manifests itself as a T-induced attenuation of LPS-stimulated NO-production of RAW 264.7 macrophages. In accordance, a recent report also has shown testosterone-induced attenuation of LPS-stimulated NO-production (Friedl *et al.*, 2000). Our data demonstrate that nongenomic T calcium signaling is the reason for the T-attenuated LPS-activated NO-production since the latter can be abolished by the intracellular Ca^{2+} chelator BAPTA.

The T-induced attenuation of LPS-activated *c-fos* promoter and NO-production may be suspected to reflect only a simple uniform dampening of all LPS-signaling parameters in macrophages through the increased intracellular free Ca^{2+} ions. However, this attenuation is

specific, which can be deduced from our following data. Thus, LPS activates all the three MAPK families ERK1/2, JNK/SAPK and p38, which is in accordance with previous data (Feng *et al.*, 1999; Hambleton *et al.*, 1996). T, however, selectively down-regulates only the LPS-activation of p38, but not that of ERK1/2 and JNK/SAPK. This p38 down-regulation by T is indeed due to the nongenomic T calcium signaling, because when the free Ca^{2+}-ions induced by T are captured by BAPTA, p38 is again fully stimulatable by LPS.

The specificity of down-regulation of LPS-signaling through nongenomic T signaling is further substantiated by the fact that nongenomic T signaling causes an up-regulation of FCS-signaling in the same RAW-fos13 macrophages. The latter has been embodied by the increased activation of *c-fos* promoter and ERK1/2, as revealed by promoter assay and Western-blot following FCS stimulation. This up-regulatory effect of T, just like its down-regulatory effect on LPS signaling, is also through T-induced nongenomic Ca^{2+} signaling, because all inhibitors including BAPTA, PTX and U-73122, which abolish the rise of $[Ca^{2+}]_i$, can also abrogate this T-induced up-regulation of FCS signaling, while the blockers for classical nuclear receptors could not prevent the effects.

Why using the same ligand T, we got the opposite effects on FCS and LPS signaling? Although both LPS and serum can activate *c-fos* promoter, it is believed that different signaling pathways are involved in these two processes. This can also be deduced from our data showing that different types of the MAPKs are activated and the activation is with different kinetics after LPS and FCS stimulation. LPS activates all the three MAPK families and the maximal activation for all the three is about 15 min post stimulation, while FCS activates only ERK1/2 and the maximal phosphorylation occurs at 1 h. It is generally accepted that FCS signal is mainly transmitted through RTK pathway, in which the ligand-binding induces tyrosine phosphorylation and the activation of the kinase cascade of Raf-1-MEK-ERK1/2, resulting in a growth- and proliferation- orientated signal transduction. While LPS signaling is through the activation of G-proteins, RTK and other yet unknown components, leading to an inflammation- and activation-directed signal transduction. Therefore it is likely that T plays opposite roles in growth/proliferation and inflammation/activation of macrophages.

Besides T, the same experiments have also been performed in parallel with E_2. The E_2-triggered nongenomic Ca^{2+} signaling independent of the iER can also specifically regulate LPS and FCS gentropic signaling in RAW-fos13 macrophages. As we have described before, E_2 alone, as T, is not able to directly induce any activation of the *c-fos* promoter, or

one of three MAPK families p38, ERK1/2, JNK/SAPK. Nonetheless, all these parameters are responsive to E_2, which becomes apparent when the cells are activated by LPS or FCS. In contrast to T, E_2 down-regulates LPS-stimulated *c-fos* promoter activation, whereas it up-regulates FCS-induced *c-fos* promoter activation in RAW-fos13 macrophages. These E_2-effects are not mediated through ERα, but rather through nongenomic Ca^{2+} signaling. This view is supported by our findings that (i) these E_2-effects can not be inhibited by the iER-blockers ICI 182,780, tamoxifen and raloxifene, (ii) they can be completely abolished by the intracellular Ca^{2+} chelator-BAPTA which blocks the rise of $[Ca^{2+}]_i$ generated by E_2, (iii) they can be prevented by inhibitors of PLC and by PTX which both inhibit E_2-induced rise in $[Ca^{2+}]_i$. The respective down- and up-regulation of genotropic FCS- and LPS-signaling by E_2-induced rise in $[Ca^{2+}]_i$ are not simply a general dampening or stimulation of all cell activities, but rather are highly specific. Indeed, though LPS activates all of the three MAPK families p38, ERK1/2, and SAPK/JNK, only the p38 MAPK is up-regulated by the E_2-induced Ca^{2+} signaling, the other two are not influenced as revealed by Western-blot. Finally, the specific up-regulatory effect of E_2-induced Ca^{2+} signaling on LPS-stimulated p38 and *c-fos* promoter has correlated with an increased NO-production of macrophage. Again, this up-regulatory effect of E_2 is independent of iERα but rather mediated through E_2-induced rise in $[Ca^{2+}]_i$, because the intracellular Ca^{2+} chelator BAPTA and all the inhibitors including G-protein inhibitor PTX, PLC inhibitor U-73122, but not the inactive analogue U-73343, which block the rise of $[Ca^{2+}]_i$ can also abolish the effects of E_2.

There are several common points in T- and E_2-induced nongenomic signaling. First, both sex hormones induce a rise in $[Ca^{2+}]_i$. Second, both hormones function not through the classical nuclear receptors, but rather through specific membrane receptors belonging to the class of GPCR. Third, both T and E_2 nongenomic signaling involve internalization of the surface receptors. Fourth, both T and E_2 stimulation alone has no effect on the activation of *c-fos* promoter and MAPKs, both are effective only in context with LPS and FCS stimulation. All these common features between T and E_2 may represent the general aspects of sex hormone-induced nongenomic signaling. However, there are also differences between these two steroid-induced signaling. First, the different Ca^{2+} sources are used for the increase of $[Ca^{2+}]_i$ in T and E_2-induced signaling. Second, T and E_2 have different optimal concentrations for the Ca^{2+} response. Third, different internalization processes have been processed upon T and E_2 binding. Fourth, moreover, the seemingly same rises in $[Ca^{2+}]_i$ have opposite impacts on LPS and FCS-induced genotropic signaling in macrophages. That is, T

decreases, while E_2 increases the activation of *c-fos* promoter and NO-production following LPS stimulation, while in FCS-stimulated signaling the situation is reversed, i.e. T augments, while E_2 attenuates this process. These differences may reflect the steroid specificity in signal transduction of macrophages and the mechanisms underlying these differences remain to be clarified.

Our data demonstrate that T/E_2-induced nongenomic Ca^{2+} signaling through surface receptors has a specific influence on LPS and FCS-induced genotropic signaling. A general signal transduction cascade could be that the rise of $[Ca^{2+}]_i$ may regulate some kinases, esp. the Ca^{2+}-sensitive kinases, through which it modulates the activation of the related transcription factors and controls gene transcription ultimately. At this time it is still too early to define the exact point of the interaction between nongenomic T/E_2 signaling and LPS/FCS-induced genotropic signaling. Although the direct interaction of T/E_2 with the LPS and FCS surface receptors could not be ruled out, it is not likely the case. Because when the increase of $[Ca^{2+}]_i$ was prevented by BAPTA, T/E_2 still have the chance to interact with the LPS and FCS surface receptors, but they could not influence the LPS and FCS signaling anymore. Therefore, it seems that the interactions occur somewhere else downstream the Ca^{2+} signal elicited by T/E_2, but upstream the MAPK p38 or ERK1/2. The central role of p38 MAPK is further substantiated by our finding at least, in the case of T, that the p38 inhibitor SB 203580 diminishes the LPS-activation of *c-fos* promoter activation and NO-production and it abolishes the responsiveness to T of the remaining LPS-effect. Collectively, our data indicate that there is a cross-talk of the T-induced nongenomic Ca^{2+} signaling with the LPS-signaling pathway: the T-induced rise in $[Ca^{2+}]_i$ attenuates the LPS-stimulation of p38, which further downstream attenuates both the LPS-activated *c-fos* promoter and NO-production.

Furthermore, how does the T/E_2-induced increases of $[Ca^{2+}]_i$ influence MAPK p38 and ERK1/2? The possible interaction point(s) might be at or upstream the PKC point(s) because pretreatment of the cells with the PKC inhibitor GF109203x could completely block the T/E_2-induced effects. Since the activation of some types of PKC is Ca^{2+}-dependent, it is possible for T/E_2 to influence the activation of PKC. The altered PKC activation may result in change of the MAPK activation (Liebmann, 2001), which in turn may lead to the alteration of transactivity of transcription factors modulating the expression of *c-fos*. (The possible regulation of *c-fos* promoter by T/E_2-altered MAPK activation will be discussed in detail later).

Since the signaling events are not simply transmitted in a linear way, but an interactive networked way (Bhalla and Iyengar, 1999), it will not be surprising to find that the interactions of T/E_2 and LPS/FCS occur at more than one point on the signal transduction pathways. For instance, besides the possible regulation of MAPK by PKC, it has also been reported that GPCR can regulate the activation of MAPK directly (Lopez-Ilasaca, 1998; Gutkind, 2000; Naor *et al.*, 2000). As we demonstrated before, the mAR and mER in RAW-fos13 macrophages belong to GPCR, so T/E_2-induced up- or down-regulation of ERK1/2 or p38 MAPK may directly result from the GPCR-MAPK interactions, too.

At the *c-fos* promoter level, it is generally accepted that the modulation of *c-fos* promoter activation is mediated through regulation of certain response elements in its promoter (Hill and Treisman, 1995; Susini *et al.*, 2000). Therefore the question is which parts (elements) of *c-fos* promoter are involved in T/E_2-induced up- and down-regulation of LPS and FCS signaling? It has been reported that the serum response element (SRE) at -325 to -296 of *c-fos* promoter was E_2- responsive and the E_2-induced *c-fos* promoter activation in MCF-7 cells is mediated through the increased binding of SRE (Duan *et al.*, 2001). However, in RAW-fos13 macrophages, no significant change in the binding activity of all the three major response elements CRE, SRE, SIE can be observed following E_2- and T -stimulation. Under the same experimental conditions FCS and LPS also could not result in any significant change in binding activity of the three elements. This is in accordance with the previous reports that in some cell types the SRE and AP-1 like response elements in *c-fos* promoter are constitutively occupied *in vivo* (Herrera *et al.*, 1989; Konig, 1991) and that therefore epidermal growth factor regulates *c-fos* promoter activity not through the change of the binding activity of SRE (Herrera *et al.*, 1989). It has been demonstrated that activation of some response elements e.g. CRE does not result in a change of the binding of the proteins, but induces phosphorylation of the already bound proteins (Hagiwara *et al.*, 1992; Karin, 1995; Gupta *et al.*, 1999). It is therefore most likely that T/E_2 regulate *c-fos* promoter activation not through the change of the binding activity but rather through chemical modification of the already bound transcription factors. Further experiments using special antibodies such as anti-phospho antibodies against the possible bound transcription factors are required to confirm this hypothesis. On the other hand, the intensive study on *c-fos* promoter, e.g. series of clone deletion from *c-fos* promoter should be helpful to figure out the possible *c-fos* promoter elements involved in T/E_2 regulation.

T- and E_2-induced up- and down-regulation of *c-fos* promoter activation following FCS stimulation may be mediated through the T- and E_2-altered ERK1/2 activation after FCS treatment. Because activated ERK1/2 is known to phosphorylate and regulate the activation of the transcription factors e.g. Elk-1, which is one of the TCF interacting with SRF and bind to the SRE in the *c-fos* promoter. Therefore it is quite reasonable to regard that T-increased ERK1/2 activation causes enhanced Elk-1 activation leading to up-regulation of *c-fos* promoter activation. Similarly, E_2-induced decreased ERK1/2 activation may down-regulate the *c-fos* promoter activation through the same pathway. However, further experiments concerning the effects of T/E_2 on the activation of Elk-1 are required to examine these hypotheses.

On the other hand, our study suggests that the down- and up-regulation of *c-fos* promoter activation by T/E_2 following LPS stimulation may be mediated through the T/E_2-altered p38 MAPK activation. This has been supported by the results, at least in the case of T, that the p38 MAPK inhibitor SB 203580 can abolish the T-effect on *c-fos* promoter activation. It has been reported that the activated p38 MAPK phosphorylates and regulates the activation of some transcription factors, e.g. ATF-2. The latter is known to belong to the AP-1 family and can form homo- or heterodimers with c-Fos, c-Jun, Jun-B and CREB etc. to bind to the CRE on the *c-fos* promoter. The decreased p38 MAPK activation may cause decreased ATF-2 activation and diminished CRE activation leading to the down-regulation of *c-fos* promoter activation, through which the T-attenuated activation of p38 MAPK may result in down-regulation of *c-fos* promoter activation. Likewise, following LPS stimulation of macrophages the augmented activation of p38 MAPK by E_2 may lead to the up-regulation of *c-fos* promoter activation.

Additionally, the T-induced decreased NO production of macrophages following LPS stimulation also results from, or predominately from T-decreased p38 MAPK activation. This has been proven by the data that SB 203580 can completely abrogates T-effect on LPS-induced NO-production. Indeed, there are also AP-1 elements in the promoter of iNOS gene, which are responsible for the high levels of NO production following LPS-stimulation. For example, T-attenuated activation of p38 MAPK may result in decreased ATF-2 activation and subsequently down-regulation of iNOS promoter activation leading to decreased NO production of macrophages following LPS stimulation. Conversely E_2-induced increased NO production following LPS treatment may result from, or partly result from E_2-enhanced p38

MAPK activation. Further experiments detecting the effects of T/E_2 on activation of ATF-2 may be required to examine these possibilities.

Our data for the first time provide a linkage between nongenomic T/E_2 Ca^{2+} signaling and genotropic signaling induced by LPS and FCS in macrophages. Normally, nongenomic signaling induced by steroids are characterized as short time duration, and always regarded as less effective on gene expression and cell functioning. However, the nongenomic Ca^{2+} signaling elicited by steroids is still possible to influence gene expression because some Ca^{2+}-sensitive IEG such as *c-fos* can be easily regulated by the increase of $[Ca^{2+}]_i$. This view has been supported by our data that T and E_2 induce up- and down-regulation of *c-fos* activation in LPS and FCS signaling. Is it a paradox that "nongenomic" signaling regulates "genomic" signaling? In fact, the concept "nongenomic" is used only to emphasis the direct independence of transcription and gene expression and does not refer to the absolute independence of the transcription and translation of genes. The change in the levels of second messagers in steroid nongenomic signaling, in turn, will influence the corresponding kinase cascades, transcription factors and finally expression of genes and cell function.

4.3 Physiological Significance of Nongenomic T- and E_2- Signaling

The regulatory effects of nongenomic T and E_2 signaling on macrophage genotropic signaling we described above may have important physiological significance *in vivo*. At present, it is still not clear if these effects can be generally applied to different types of cells or only restricted to macrophages. Even if these effects are only restricted to macrophages, the potential significance for immunity should not be ignored.

It has been reported that modulations of functional activities in differentiated macrophages are accompanied by early and transient increase or decrease in *c-fos* gene transcription (Collart *et al.*, 1987). Therefore, these T- and E_2-induced alterations of *c-fos* promoter activation might be the initial events to induce the more sustained modulation of other gene expressions that trigger more profound changes in macrophages. For instance, the T/E_2-altered NO-production in macrophages may be the result of T/E_2-changed *c-fos* gene activation. As known, the product of *c-fos* gene activation is c-Fos protein. It acts as transcription factor to form homo- or hetero-dimers with c-Jun, Jun-D, Fos-B, ATF-2, etc., then bind with AP-1 response elements on the promoter of target genes to regulate their transcription (Karin, 1995; Karin *et al.*, 1997). It is noteworthy that the iNOS promoter,

which is responsible for LPS-induced NO-production, contains AP-1 response elements and can be regulated by c-Fos. Therefore, it is quite reasonable to regard that T/E_2-induced decreased/increased NO-production may result from the T-down/up-regulated *c-fos* activation in macrophages. However, this hypothesis remains to be examined further.

Our result that T decreases NO-production of macrophages following LPS stimulation is consistent with previous data showing that T treatment decreases iNOS mRNA expression and NO-production in murine RAW 264.7 macrophages (Friedl *et al.*, 2000). Macrophages are known to be one of the major defense instruments of mammalian hosts and a lot of cytotoxic effects exerted by macrophages have been shown to involve sustained NO production (Hibbs, Jr. *et al.*, 1988a; Morris, Jr. and Billiar, 1994; Nathan and Xie, 1994; Xie and Nathan, 1994). Therefore T-decreased NO-production following LPS stimulation might represent an impaired defensive capability of macrophages during pathogen invasion and may be related to the increased male incidence in infectious diseases. In this respect, the attenuated NO-production through iAR-independent nongenomic T signaling may be of important relevance for previous findings showing that T dramatically deteriorates the capability of mice to eliminate blood stages of *Plasmodium chabaudi* malaria (Wunderlich *et al.*, 1988; Wunderlich *et al.*, 1991). T does not only prevent the development of protective immunity against *Plasmodium chabaudi* infections but even impairs the efficacy of protective vaccination against *P. chabaudi* malaria (Wunderlich *et al.*, 1993). This immunosuppressive effect of T, is mediated neither through iAR nor, after aromatization of T to E_2, through iER (Benten *et al.*, 1992; Benten *et al.*, 1993).

On the other hand, this study for the first time shows that nongenomic E_2 signaling induces an increased NO-production in macrophages following LPS stimulation, this might also have some relevance to the increased incidence of autoimmune diseases in females. Because NO is known to be an important non-specific cytotoxic effector (Hibbs, Jr. *et al.*, 1988b), E_2-induced augmented or probably an over-production of NO might cause the self-injure of tissues and play a role in the occurrence of the autoimmune diseases.

5 SUMMARY

According to the prevailing view, steroid hormones exclusively function through a genomic fashion mediated by the intracellular cognate steroid receptors. However, an increasing number of reports show that steroids can also act through nongenomic pathways mediated not only by intracellular steroid receptors but also specific unconventional membrane receptors. This study is aimed at investigating the existence of membrane receptors for testosterone (T) and 17β-estradiol (E_2) in mouse macrophages of the cell line RAW 264.7 and IC-21 as well as their roles in nongenomic pathways, gene expression and cell functioning.

Both macrophage cell lines lack intracellular androgen receptor (iAR) and intracellular estrogen receptor (iER) β, but contain iERα. Fura-2 loaded macrophages respond to T and E_2 with rapid rises in intracellular free calcium concentration ($[Ca^{2+}]_i$). These rises in $[Ca^{2+}]_i$ cannot be inhibited by cyproterone, a blocker for classical iAR, as well as tamoxifene, raloxifen and ICI 182,780, blockers for classical iER, but rather are mediated through unconventional membrane androgen receptors (mAR) and membrane estrogen receptors (mER), respectively. Pharmacological approaches using the G-protein blocker pertussis toxin (PTX) and the phospholipase C (PLC) inhibitor U-73122 suggest that these receptors belong to that class of membrane receptors which are coupled to PLC via PTX sensitive G-proteins. Although still not characterized in molecular terms, these membrane receptors can be localized as specific surface binding sites for T-BSA-FITC and E_2-BSA-FITC by confocal laser scanning microscopy and flow cytometry. Both mAR and mER are characterized by their agonist-sequestrability in macrophages.

In order to examine a possible role of the T- and E_2-induced rise in $[Ca^{2+}]_i$ on gene expression, a *c-fos* promoter reporter gene construct was transiently or stably transfected into RAW 264.7 macrophages. The increases in $[Ca^{2+}]_i$ induced by T and E_2 cannot significantly activate the *c-fos* promoter in macrophages directly. Also, no significant activation of the three MAPK families ERK1/2, JNK/SAPK and p38 can be observed following T- and E_2-stimulation alone. However, both T- and E_2-induced rises in $[Ca^{2+}]_i$ have specific effects on gene expression in context with lipopolysaccharide (LPS)-induced genotropic signaling: T specifically down-regulates LPS-induced activation of *c-fos* promoter, p38 MAPK and NO-production and, conversely, E_2 up-regulates LPS-induced activation of *c-fos* promoter, p38 MAPK and NO-production. In fetal calf serum (FCS)-induced genotropic signaling, the situation is reversed, i.e. T augments and E_2 suppresses the activation of *c-fos* promoter and ERK1/2.

This study demonstrates, for the first time, a cross-talk between the sex hormone-induced nongenomic Ca^{2+} signaling and the genotropic signaling induced by LPS and FCS in macrophages. The down- and up-regulatory effects of T and E_2 on LPS signaling of macrophages through nongenomic Ca^{2+} signaling might play a role in the increased female incidence for autoimmune diseases and the increased susceptibility of males to infectious diseases.

6 ZUSAMMENFASSUNG

Gemäß der vorherrschenden Lehrmeinung wirken Steroidhormone ausschließlich durch genomische Signalwege, die durch intrazelluläre Steroidhormonrezeptoren vermittelt werden. Aber es gibt eine wachsende Zahl an Befunden, die zeigen, daß Steroide auch auf nichtgenomische Signalwege wirken können, und dies nicht nur mittels intrazellulärer Steroidhormonrezeptoren, sondern auch über spezifische unkonventionelle Membranrezeptoren. Die vorliegende Studie wurde durchgeführt, um sowohl die Existenz von Membranrezeptoren für Testosteron (T) und 17β-Estradiol (E_2) in Maus-Makrophagen der Zellinien RAW 264.7 und IC-21 als auch die Rolle dieser Hormone in nichtgenomischen Signalwegen, der Genexpression und der Zellfunktion zu untersuchen.

Beide Makrophagen-Zellinien exprimieren keinen intrazellulären Androgenrezeptor (iAR) und keinen intrazellulären Estrogenrezptor (iER) β, wohl aber iERα. Fura-2-beladene Makrophagen antworten auf T und E_2 mit einem schnellen Anstieg der freien intrazellulären Kalzium-Konzentration ($[Ca^{2+}]_i$). Diese Erhöhung der $[Ca^{2+}]_i$ kann weder durch Cyproteron, einem Blocker für klassische iAR, noch durch Tamoxifen, Raloxifen und ICI 182,780, Blocker für klassische iER, unterbunden werden, sondern werden durch unkonventionelle Membran-Androgenrezeptoren (mAR) und Membran-Estrogenrezeptoren (mER) vermittelt. Pharmakologische Untersuchungen mit dem G-Protein-Blocker Pertussistoxin (PTX) und dem Phospholipase C (PLC)-Inhibitor U-73122 legen nahe, daß es sich bei diesen Rezeptoren um Membranrezeptoren handelt, welche über PTX-sensitive G-Proteine an PLC gekoppelt sind. Wenn auch die molekulare Struktur dieser Membranrezeptoren noch nicht aufgeklärt ist, so können sie doch als spezifische Oberflächenbindungsstellen für T-BSA-FITC und E_2-BSA-FITC durch konfokale Laserscanning-Mikroskopie und Durchflußcytometrie dargestellt werden. Für beide, mAR und mER, ist ihre Liganden-spezifische Sequestrierung in Makrophagen charakteristisch.

Um eine mögliche Rolle des T- und E_2-induzierten Anstiegs der $[Ca^{2+}]_i$ auf die Genexpression zu untersuchen, wurde ein *c-fos*-Promotor-Reportergen-Konstrukt transient oder stabil direkt in RAW 264.7 Makrophagen transfiziert. Die T- oder E_2-induzierte Erhöhung der $[Ca^{2+}]_i$ konnte jedoch alleine den *c-fos*-Promotor in Makrophagen nicht signifikant aktivieren. Ebenso war keine spezifische Aktivierung der drei MAPK-Familien ERK1/2, JNK/SAPK und p38 nach alleiniger Stimulation mit T und E_2 zu beobachten. Allerdings haben die T- und E_2-induzierten Anstiege der $[Ca^{2+}]_i$ eine spezifische Wirkung auf die Genexpression im Kontext mit einer Lipopolysaccharid (LPS)-induzierten Aktivierung von genotropischen Signalwegen: T reguliert spezifisch die LPS-induzierte Aktivierung des *c-fos*-Promotors, der p38-MAPK und der NO-Produktion herunter, während E_2 die LPS-induzierte Aktivierung des *c-fos*-Promotors, der p38-MAPK und der NO-Produktion hochreguliert. Beim Kälberserum (FCS)-induzierten genotropischen Signalweg ist die Situation genau umgekehrt, T verstärkt und E_2 supprimiert z. B. die Aktivierung von *c-fos*-Promotor und ERK1/2.

Die vorliegende Studie zeigt zum ersten Mal eine Wechselwirkung zwischen Geschlechtshormon-induziertem nichtgenomischem Ca^{2+}-Signalweg und dem LPS- und FCS-induzierten genotropischen Signalweg in Makrophagen. Die hoch- und runterregulatorischen Effekte von T und E_2 auf den LPS-Signalweg spielen möglicherweise eine Rolle bei der erhöhten Inzidenz von Autoimmunerkrankungen bei Frauen und der erhöhten Suszeptibilität von Männern für Infektionskrankheiten.

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8 ABBREVIATIONS

AP-1	activating protein-1
APS	ammonium peroxodisulfate
AR	androgen receptor
ATF-2	activating transcription factor-2
BAPTA/AM	1,2-*bis*-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxy-methyl) ester
BCA	bicinchoninic acid
bp	base pair (s)
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5', -monophosphate
CLSM	confocal laser scanning microscopy
cpm	counts per minute
CRE	cAMP response element
CSCS	charcoal stripped calf serum
DABCO	1,4-diazabicyclo-[2.2.2]octane
DAG	diacylglycerol
DBD	DNA-binding domain
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E_2	17β-estradiol
E_2-BSA-FITC	β-estradiol 6-(o-carboxymethyl)oxime:BSA-fluorescein isothiocyanate conjugate
ECL	enhanced chemiluminscence
EDTA	ethylene diaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FACS	fluorescent activated cell scanner
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
HRE	hormone response element
IEG	immediate early gene
iNOS	inducible NO synthase
IP_3	inositol 1, 4, 5-triphosphate
JNK	Jun N-terminal kinase

LBD	ligand-binding domain
LPB	LPS-binding protein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
min	minute (s)
MR	mineralocorticoid receptor
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGE_2	prostaglandin E_2
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PR	progesterone receptor
PTK	protein tyrosine kinase
PTX	potusiss toxin
RLU	relative light units
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
SEAP	secreted alkaline phosphatase
s	second (s)
SEM	standard error of mean
SIE	sis-inducible element (enhancer)
SRE	serum response element
T	testosterone
T-BSA-FITC	testosterone3-(o-carboxymethyl)oxime:BSA-fluorescein isothiocya-nate conjugate
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TRE	o-tetradecanoylphorbol 13-acetate (TPA) response element
Tris	Tris-(hydroxylmethyl) aminomethane
U	unit (s)
U-73122	1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)-amino)hexyl)-1H-pyrrole-2,5-dione
U-73343	1-(6-((17β-3metoxyestra-1,3,5(10)-trien-17-yl)-amino)-hexyl)-2,5-pyrrolidine-2,5-dione
UV	ultra violet light

9 DANKSAGUNGEN

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