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Nuclear prostaglandin receptors

Review Article

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Summary

Prostaglandins and thromboxane are ubiquitous compounds and play important roles in cardiovascular homeostasis, inflammation, reproduction, respiration, mitogenesis and gene transcription and so on. These actions of prostanoids are presumed to be mediated by plasma membrane receptors belonging to the superfamily of G protein-coupled receptors. However, several lines of evidence suggest prostanoids may also act at the nuclear level. Nuclei contain cyclooxygenases and other intermediates required for prostanoid synthesis and receptor-mediated responses. This review focuses closely on various signal transduction cascades that exist in the nuclear membranes, including the presence of other nuclear G protein-coupled receptor, and discusses the discovery of functional nuclear prostaglandin E_2 receptor. These data add new dimensions to the functions and signaling mediated by prostaglandin receptors.

I. Introduction

Prostaglandins (PG) and thromboxane, collectively named prostanoids, are products of arachidonic acid metabolism. Prostanoids produce numerous physiologic and pathophysiologic effects, regulating cellular processes in nearly every tissue. These compounds act as local hormones, acting in the vicinity of their site of production and function in an autocrine and/or paracrine manner to maintain local homeostasis. Prostanoids are widely distributed and can be formed by nearly every tissue and cell type; the same prostanoid has the ability to provoke different responses in various tissues (Campbell and Halushka, 1996).

There are five physiologically important prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. PGE_2 in particular, has a wide spectrum of physiological and pharmacological actions in diverse tissues which include effects on the immune, endocrine, cardiovascular, renal and reproductive systems as well as the contraction and relaxation of smooth muscle (Campbell and Halushka, 1996; Negishi et al., 1993a). PGE_2 is one of the most abundant prostanoids in the brain (Leffler and Busija, 1985) and plays an important role in many cerebral functions particularly in the newborn (Leffler and Busija, 1987; Chemtob et al., 1996). PGE_2 also influences mitogenesis (Hashimoto et al., 1997; Glantschnig et al., 1996), promotes growth and metastasis of tumors (Paoletti et al., 1989; Fulton et al., 1991) and modulates the transcription of many genes (Danesch et al., 1994; Gashler and Sukhatme, 1995; Umayahara et al., 1997; Paliogianni and Boumpas, 1996; Minghetti et al., 1997).

II. PGE_2 receptor subtypes and signaling

Prostanoids exert their effects through GTP-binding protein (G protein)-coupled, rhodopsin-type receptors. The receptors for PGE_2 are termed EP, which include EP_1, EP_2, EP_3 and EP_4 subtypes (Coleman et al., 1994). High specific PGE_2 binding has been observed in the brain, kidney, uterus, liver, thymus and the adrenal medulla (Robertson, 1986). PGE_2 has versatile and opposing actions due to multiple EP receptor subtypes and the coupling of EP receptor isoforms to a variety of signal transduction pathways (**Table 1**). Molecular cloning of these receptor cDNAs has revealed that the EP_3 receptor isoforms are generated by alternative mRNA splicing (Narumiya, 1996).

Table 1 Classification of prostanoid EP receptors and their signal transduction.

Ligand	Type	Subtype	Isoform	G protein	Signal Transduction
PGE_2	EP	EP_1		G_q (?)	PI↑, Ca^{2+} ↑
		rEP_1variant			Ca^{2+} ↓ (in co-expression with EP_1)
		EP_2		G_s	cAMP ↑
		EP_4		G_s	cAMP ↑
		EP_3	EP_{3A}	G_i	cAMP ↓
			EP_{3B}	G_s	cAMP ↑
			EP_{3C}	G_s	cAMP ↑
			EP_{3D}	$G_{i/s/q}$	cAMP ↓ , cAMP↑, PI↑

Modified and summarized from Coleman et al., (1994) and Narumiya, (1996). Data obtained from receptors of various species; the EP_3 isoforms from bovine; the rEP_1 variant from rat; others from mouse. PI denotes phosphoinositol turnover.

EP_1 receptors mediate Ca^{2+} mobilization (Watabe et al., 1993; Funk et al., 1993; Okudu-Ashitaka et al., 1996) by activating phospholipase C (PLC) and increasing inositol 1,4,5-trisphosphate (IP_3) (Katoh et al., 1995; Suba and Roth, 1987). However, PGE_2 has also been shown to stimulate Ca^{2+} mobilization by activating EP_1 without altering IP_3 formation in RCCT cells (Hebert et al., 1991), and without activating phospholipase C (PLC) in myometrial cells (Asboth et al., 1996). An alternative splice variant has been identified for rat EP_1 (rEP_1-v), which completely lacks a cytoplasmic carboxy terminus (Okudu-Ashitaka et al., 1996). EP_1 attenuates the action of PGE_2 on tissues by interfering with signaling mediated by other EP receptors and constitutes an example of crosstalk between receptor subtypes. EP_2 receptors and EP_4 receptors stimulate adenylyl cyclase by coupling to G_s. EP_3 receptors inhibit adenylyl cyclase (Namba et al., 1993) but some of its functions are mediated by other second messenger pathways. Several isoforms of EP_3 receptors, produced by alternative splicing, have been found in bovine, human, rat, mouse and rabbit tissues (Narumiya, 1996). These isoforms of EP_3 receptors perform different functions but differ only in the carboxy terminal tail, which is known to influence the coupling selectivities and activities of G proteins (Pierce et al., 1995). However, a recent study suggests that this is not the only determinant and that regions in both the third intracellular loop and in the carboxyl termini of the prostanoid receptors contribute to the specificity of receptor-G protein interactions (Neuschafer-Rube et al., 1997). The constitutive activity of EP_3 receptor isoforms and their coupling to G_i (Negishi et al., 1996) as well as their response to prolonged exposure to agonist also differ (Negishi et al., 1993b). The EP_3 receptor can also couple to voltage-sensitive and -insensitive Ca^{2+} channels (Tanaka et al., 1998) and Cl^- channels (Sakai et al., 1995).

The biological actions of PGE_2 have been attributed to result from its interaction with cell surface EP receptors (Coleman et al., 1994). However, several lines of evidence suggest that prostaglandins may act intracellularly and exert a direct nuclear action. Recent studies have shown that cyclooxygenases which synthesize prostanoids are located mainly in the nuclear membrane (Spencer et al., 1998; Morita et al., 1995). Phospholipase A_2 (PLA_2), which releases arachidonic acid is activated at nuclear membranes (Schievella et al., 1995). In addition, a transporter which mediates the influx of prostanoid has been identified (Schuster, 1998). It is thus possible that PGE_2 may exert some of its effects via intracellular EP receptors as has been proposed by several workers (Goetzl et al., 1995; Morita et al., 1995; Smith, 1997). This article reviews the literature suggesting the nuclear action of prostanoids and the discovery of functional G protein-coupled receptors, such as the prostanoid EP receptors, at the nuclear membrane.

III. Mitogenic effects of PGE_2 and modulation of gene transcription

Substantial evidence suggests that PGE_2 affects gene transcription and regulates growth and cell proliferation. Endogenous PGE_2 is important in regulating the growth of epithelial cells (Konger et al., 1998). PGE_2 exhibits mitogenic activities in bone cells and stimulates DNA synthesis (Glantschnig et al., 1996). PGE_2 is also involved in the growth and metastasis of tumors (Paoletti et al., 1989; Fulton et al., 1991). The inhibition of prostaglandin synthesis has been shown to result in growth retardation of tumors in experimental animals (Lupulescu, 1978). The importance of PGE_2 in neoplastic development is also suggested by the association of a decreased risk of colon

cancer and the use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin (Williams et al., 1997). It is of interest to note that PGE_2 inhibited the anti-proliferative effects of aspirin on growth factor-stimulated DNA synthesis (Castano et al., 1997).

Cellular immediate-early genes such as c-*fos* and *erg*-1 serve as nuclear couplers of early cytoplasmic events to long term alterations in gene expression and these genes are closely related to proliferation and/or differentiation (Gashler and Sukhatame, 1995). PGE_2 regulates c-*fos* and *erg*-1 gene expression (Simonson et al., 1994; Danesch et al., 1994; Glantschnig et al., 1996). PGEs are also immunomodulatory agents and PGE_2 inhibits nuclear transcription of interleukin 2 (IL-2) by decreasing the binding of transcription factors AP-1 and NF-AT. Agents that elevate cAMP also inhibit transcription of IL-2 gene; however, the IL-2 promoter lacks a cAMP response element (CRE) and therefore the mechanism remains to be shown (Paliogianni and Boumpas, 1996). The transcription factor CCAAT/Enhancer-binding protein δ (C/EBP δ) is a PGE_2 activated transcription regulator of the insulin-like growth factor-1 gene; however, C/EBP δ is not phosphorylated by protein kinase A (PKA) and the mechanism of activation is unclear (Umayahara et al., 1997). Other genes modulated by PGE_2 include the inducible nitric oxide synthase (iNOS) (Milano et al., 1995; Minghetti et al., 1997) and the constitutive endothelial nitric oxide synthase (eNOS) via EP_3 receptors (Dumont et al., 1998; 1999).

The precise mechanism by which PGE_2 affects gene expression is not fully known. Recently PGE_2 was shown to inhibit lipogenic gene expression through a pertussis toxin (PTX)-sensitive G protein signaling cascade (Mater et al., 1998). Other studies showed that PGE_2 signals through a novel cAMP response element binding protein/CRE pathway, which appears to be independent of cAMP generation (Audoly et al., 1999). The cAMP (Paliogianni and Boumpas, 1996; Audoly et al., 1999), protein kinase A (PKA) (Glantschnig et al., 1996; Umayahara et al., 1997), or protein kinase C (PKC) (Glantschnig et al., 1996) pathways are not obligatory for mediating the effects of PGE_2. Recent studies have identified several aspects to EP_3 receptor function distal to the G protein. An EP_3 receptor-dependent activation of MAP kinase, which is followed by its translocation into the nucleus, has been shown by Burkey and Regan (1995). The involvement of the small GTPase Rho in the EP_3 receptor-mediated stress fiber formation in kidney cells (Hasegawa et al., 1997) and neurite retraction (Katoh et al., 1996) has been identified. The latter occurs through a pathway, distinct from adenylyl cyclase inhibition or protein kinase C activation (Katoh et al., 1996). More recent studies have demonstrated that the stimulation of EP_3 receptors induces the translocation of the transcription factor NFκB to the nucleus (Meyer-Kirchrath et al., 1998). The discovery of nuclear EP receptors might now provide possible explanations for some of these observations.

IV. Nuclear phospholipase A_2 (PLA_2) and cyclooxygenase enzymes

The presence in the nuclear region of the machinery for prostanoid synthesis favors the possibility of their nuclear actions. Prostaglandin synthesis is initiated by activation of PLA_2. PLA_2 is one of the growing family of enzymes, which catalyze the hydrolysis of phospholipids at the *sn*-2 position, liberating free fatty acids including arachidonic acid, the precursor of platelet -activating factor, and other lysophospholipids (Campbell and Halushka, 1996). This arachidonate release step is the major site of regulation of prostanoid biosynthesis and activation of PLA_2 is the rate-limiting step in this process.

PLA_2 exist in both calcium-dependent and -independent isoforms. Among the various groups of PLA_2, the importance of cytosolic PLA_2 ($cPLA_2$) in mediating the generation of prostanoids has been suggested (Mukarami et al., 1997). The most convincing data have come from studies using transgenic mice deficient in $cPLA_2$; these studies demonstrate that PLA_2 is essential for both the calcium- and lipopolysaccharide-induced PGE_2 production (Bonventre et al., 1997). $cPLA_2$ is a ubiquitously distributed enzyme which requires Ca^{2+} in nanomolar range for its activity and is activated via increases in intracellular Ca^{2+} (Kramer and Sharp, 1997). The N-terminal calcium-dependent lipid binding (CaLB) domain is responsibile for Ca^{2+}-dependent translocation of $cPLA_2$ from the cytosol to the perinuclear and endoplasmic reticular membranes. Translocation to nuclear membranes results in loss of arachidonic acid from this site verifying functional activation of $cPLA_2$ at the nuclear membrane (Schievella et al., 1995; Peters-Golden et al., 1996).

The extracellular or secretory PLA_2 ($sPLA_2$) may be involved in arachidonic release and prostaglandin production (Reddy and Herschman, 1996). $sPLA_2$ enzymes require millimolar concentrations of Ca^{2+} to exert their enzymatic action. Thus $sPLA_2$ is activated continuously by the levels of Ca^{2+} found in the extracellular environment and can mediate transcellular prostanoid biosynthesis since they are secreted extracellularly and can bind to cell surfaces of neighbouring cells (Mukarami et al., 1999). Data derived from $sPLA_2$ -deficient mice reveal this enzyme does not play a crucial developmental role (Kennedy et al., 1995).

The initial step in the synthesis of prostanoids from arachidonic acid is mediated by cyclooxygenase (COX, also called prostaglandin H synthase or prostaglandin enderoperoxide synthase) (Campbell and Halushka, 1996). COX converts arachidonic acid to PGH_2, which is then acted upon by discrete prostaglandin synthases to yield different prostanoids (Smith, 1997).

COX enzymes are membrane-bound hemoproteins and include two isozymes, a constitutive form (COX-1) and an inducible form (COX-2); these isoforms function independently (Smith, 1997; Vane et al., 1998). Aspirin and other currently available NSAIDs inhibit both enzymes. At the subcellular level, both enzymes are located on the luminal surface of the endoplasmic reticulum and in inner and outer nuclear membranes (Morita et al., 1995; Spencer et al., 1998). COX-2 has also been shown to translocate to the nucleus in response to growth factors (Coffey et al., 1997). To date, a nuclear localization sequence (NLS) for COX has not been identified; it has been proposed that these proteins reach the nuclear membrane via lateral diffusion following synthesis in the endoplasmic reticulum (Spencer et al., 1998).

The two COX isoforms play distinct roles in regulating arachidonic metabolism (Smith, 1997; Smith et al., 1997), and have distinctive roles in human biology and during development. COX-1 is constitutively and almost ubiquitously expressed and is responsible for the low prostaglandin synthesis required for cell homeostasis. COX-2 is an inducible enzyme, which is *de novo* synthesized in response to a wide range of extracellular and intracellular stimuli (cytokines, growth factors and tumor promoters) in the course of inflammation or other cellular stresses; it contributes to the generation of prostanoids at sites of inflammation and at certain stages of cell proliferation and differentiation. The induction of COX-2 expression and prostaglandin formation has been associated with the activation of mitogen-activated protein kinase (MAPK) and c-Jun-N-terminal kinase (JNK) pathways (Guan et al., 1998). Oxidant stress may also induce COX-2 since it can be activated by intracellular peroxides (Shitashige et al., 1998) whereas much higher peroxide levels are needed to activate COX-1 (Kulmacz and Wang, 1995). The brain is one of the few organs where COX-2 is constitutively expressed; it is expressed exclusively in neurons (Kaufmann et al., 1996) and is the primary isoform in the brains of the neonate (Peri et al., 1995).

Prostanoids synthesized by COX-2 are essential for the survival of fetuses since the majority of offspring born to homozygous COX-2 knockout mice do not survive (Morham et al., 1995); whereas COX-1-null mice survive well (Langenbach et al, 1995). COX-2-null mice exhibit renal abnormalities, cardiac fibrosis and corpora lutea defects implicating the importance of this enzyme in the development of diverse organ systems (Morham et al., 1995). COX-2 expression has been shown to be protective against apoptosis (Von Knethen and Brune, 1997) and oxidant-induced injury of cardiomyocytes (Adderley and FitzGerald, 1999). However, elevated COX-2 expression has been associated with carcinogenesis (Kutchera et al., 1996; Tucker et al., 1999).

Cyclooxygenases utilizes different pools of arachidonic acid for synthesizing prostanoids; low concentrations of arachidonic acid are utilized predominantly by COX-2 whereas high concentrations are utilized preferentially by COX-1 (Reddy and Herschman, 1996; Shitashige et al., 1998). Two kinetically different prostanoid generating pathways, the immediate and delayed phases have been elucidated, implying the recruitment of different sets of biosynthetic enzymes, expression and activation which are tightly regulated by distinct transmembrane signalings (Mukarami et al., 1997; Naraba et al., 1998). The immediate phase of prostanoid biosynthesis occurs within several minutes of stimulation and is elicited by agonists that mobilize intracellular Ca^{2+}; it is characterized by a burst release of arachidonic acid, and is mediated predominantly by COX-1. In the delayed phase, COX-2 dependent prostanoid biosynthesis proceeds over several hours in parallel with the induction of COX-2 expression following growth or proinflammatory stimuli (Reddy and Herschmann, 1997). Moreover, preferential coupling between particular PLA_2 and COX has been suggested (Reddy and Herschman, 1997; Murakami et al., 1999). Thus, the perinuclear COX-2 plays a role in the prolonged generation of prostanoids that may act at nuclear sites and would be expected to modify nuclear events associated with cell differentiation and replication (Goetzl et al., 1995).

V. Transport of prostanoids across membranes

Prostanoids are charged anions at physiological pH and diffuse poorly across biological membranes. Unlike hormones, these substances are not stored and they function as autocoids, binding to specific receptors on the same or nearby cells, signaling a wide variety of physiological functions. The plasma half-life of prostanoids is short (< 3 min) and their signal must be terminated locally since a single prostanoid molecule can signal diverse biological events, depending on the cell type.

A prostaglandin transporter (PGT), which plays a primary role in mediating prostanoid transport and metabolic clearance, has been recently identified (Schuster, 1998). The PGT plays a role in the uptake of newly released prostanoids thus acting as a carrier across the plasma membrane before intracellular oxidation (Kanai et al., 1995; Lu et al., 1996). Moreover, PGT can facilitate intracellular actions of circulating as well as intracellularly produced prostanoids. The PGT mRNA is broadly expressed in diverse tissues, but its expression is most abundant in the lung, liver, brain and kidney. The PGT preferentially transports PGE_2, PGE_1, $PGF_{2\alpha}$, PGD_2, with high affinity and to a lesser extent, TXB_2 and PGI_2 (Lu et al., 1996).

VI. Signal transduction pathways at the nucleus

It has generally been assumed that the signal transduction cascades are initiated at the plasma membrane leading to the stimulation of activities in target organelles such as the nucleus, and that the nuclear envelope played a passive role in this cascade. However, it is becoming increasingly clear that the nuclear envelope plays a major role in signaling cascades as summarized in the following discussion.

A. Nuclear G proteins and G protein-coupled receptors

Receptors coupled to heterotrimeric G proteins comprise the largest known family of cell surface receptors (Gudermann et al., 1997). The significance of G proteins as a crucial link between plasma membrane receptors and intracellular events is well known and has been extensively studied (see reviews, Clapham, 1996; Hamm, 1998; Lefkowitz, 1998). Several recent studies have provided evidence for a similar role of both heterotrimeric and low molecular weight G proteins in nuclear signaling.

Rubins et al. (1990) identified G proteins in the nuclear envelope of isolated rat liver nuclei. This finding was later confirmed by Takei et al. (1994) who showed that these nuclear G proteins were PTX-sensitive and involved in a pathway of nuclear protein transport. The presence of the small G protein RhoA in isolated nuclei has also been demonstrated (Balboa and Insel, 1995). Saffitz et al. (1994) showed the localization of $G_{s\alpha}$ in the nucleus of S49 lymphoma cells by immunoelectron microscopy. Nuclear translocation of $G_{i\alpha}$ and its association with nuclear chromatin has been shown to occur in response to insulin as well as serum and epidermal growth factors (Crouch, 1991). It has been recently reported that the heterotrimeric G_i protein translocates to the nucleus in response to growth factors where it regulates mitosis (Crouch and Simson, 1997).

Until recently, there were a limited number of studies on functional G protein-coupled receptors that have been localized at the nucleus. The presence of muscarinic acetylcholine receptors was demonstrated by radioligand binding studies in isolated nuclei from rabbit corneal and Chinese hamster ovary cells (Lind and Cavanagh, 1993; 1995). A similar approach was used by two groups of researchers (Booz et al., 1992; Tang et al., 1992) to identify AT_1 subtype of angiotensin II receptors in isolated rat liver nuclei. Recently, the AT_1 receptor has also been detected in nuclei of rat cardiomyocytes by immunocytochemistry techniques (Fu et al., 1998). Stimulation of nuclear angiotensin II receptors induces transcription of renin and angiotensinogen mRNA (Eggena et al., 1993). Lu et al. (1998) demonstrated that the AT_1 receptor in brain neurons contains a nuclear localization signal (NLS) and is translocated to the nucleus only upon stimlution with angiotensin II; this was the first example of nuclear targeting of a G protien-coupled receptor upon binding of ligand. However, angiotensin II failed to cause nuclear translocation of AT_1 in vascular smooth muscle cells or in astroglial cells (Lu et al., 1998).

It has been suggested that the bradykinin B_1 receptors and the endothelin ET_A and ET_B receptors may also exist at the nuclear membrane and may modulate nuclear Ca^{2+} concentration (Bkaily et al., 1997a, b; Naik et al., 1998). Other G protein-coupled receptors have been detected in the perinuclear region, usually as a consequence of agonist-induced internalization, such as reported for somatostatin (Krisch et al., 1998), substance P (Grady et al., 1995), neurotensin (Faure et al., 1995; Castel et al., 1992) and other neuropeptides (Beaudet et al., 1998). Recent studies have also identified functional opioid receptors in isolated myocardial nuclei and these modulate opioid peptide gene transcription via PKC (Ventura et al., 1998).

B. Nuclear calcium and inositol cycles

Ca^{2+} signals in the nucleus control a variety of nuclear functions including gene transcription, DNA synthesis and repair, nuclear envelope breakdown or its reconstruction, protein transport and apoptosis (Malviya and Rogue, 1998). Ca^{2+} does not passively diffuse across the nuclear envelope, suggesting that regulatory mechanisms exist to modulate intranuclear Ca^{2+} levels (Nicotera et al., 1989). Nuclear Ca^{2+} signals are generated not only by Ca^{2+} transport into and out of the nucleoplasmic space, but also into and out of the nuclear envelope, which serves as the pool for nuclear Ca^{2+}. Nuclear and cytosolic Ca^{2+} signals are differentially regulated and are independent of each other because of the presence of nucleocytoplasmic barrier to Ca^{2+} movement (Malviya and Rogue, 1998; Badminton et al., 1998).

Increases in nuclear Ca^{2+} activate gene transcription by a mechanism that is distinct from gene regulation by cytoplasmic calcium signals. For example the expression of c-*fos* is differentially regulated upon increasing cytosolic or nucleoplasmic Ca^{2+} (Hardingham et al., 1997). Nuclear Ca^{2+} concentration specifically controls Ca^{2+}-activated gene expression mediated by the cyclic-AMP-response element (CRE) and the CRE-binding protein, CREB, which function as a nuclear Ca^{2+}-responsive transcription factor. There is crosstalk between cytosolic and nuclear Ca^{2+} pools. The concentration of Ca^{2+} in the nucleus is lower (Al-Mohanna et al., 1994) or higher (Przywara et al., 1991; Waybill et al., 1991) than that in the cytosol depending upon the cell system studied, the method used, or the physiological state.

In the cytosol, Ca^{2+} signals are produced by the release of Ca^{2+} from intracellular storage sites, mainly the endoplasmic reticulum; this mediated by IP_3 and inositol

1,3,4,5-tetrakisphosphate (IP_4) (Berridge, 1993). The IP_3 receptor acts as an IP_3-gated Ca^{2+} channel; it is not known if the IP_4 receptor functions as a Ca^{2+} channel (Humbert et al., 1996). Ca^{2+} mobilization can also be mediated by another second messenger, cyclic adenosine diphosphate ribose (cADP ribose) via ryanodine receptors, which are also an intracellular Ca^{2+} channels and present in the endoplasmic reticulum.

The nucleus contains the necessary machinery for IP_3 production and possesses distinct phosphoinositide cycles (Divecha and Irvine, 1995). Nuclear phosphoinositide cycles act independently from that of plasma membrane in that its activation takes place when the cytoplasmic cycle is not affected in response to external stimuli (Martelli et al., 1992). IP_3 is generated by the breakdown of phosphatidylinositol 4,5-biphosphate (PIP_2). Two interconnected pools of PIP_2 have been identified in the nucleus, one in the nuclear membrane and the other located within the nucleus (Malviya and Rogue, 1998). Other components of the nuclear inositide cycles, including phospholipase C (β1 and δ4 isoforms) which hydrolyze PIP_2 to generate IP_3 and diacylglycerol, are present within the nucleus (see below).

Functional IP_3 and ryanodine receptors are located on the inner nuclear membrane; the outer nuclear membrane, which constitutes a continuum with the endoplasmic reticulum, is the site of the location of IP_4 receptors and Ca^{2+} pump ATPase (Humbert et al., 1996). The nuclear IP_3 receptors are not identical with the microsomal IP_3 receptors (Matter et al., 1993) and play a role in the meiotic process in the mouse oocyte (Pesty et al., 1998). Upon binding to their receptors on the inner nuclear membrane, IP_3 or cADP ribose open the Ca^{2+}-release channels allowing selective release of Ca^{2+} into the nucleoplasm (Humbert et al., 1996; Gerasimenko et al., 1996). The nuclear IP_3 receptor is phosphorylated by nuclear PKC, which is activated by diacylglycerol (Matter et al., 1993). The nuclear Ca^{2+}-ATPase is an ATP-mediated nuclear Ca^{2+}-transporter, which is responsible for filling the nuclear calcium pool and is functionally distinct from the endoplasmic reticulum Ca^{2+}-ATPase (Humbert et al., 1996).

Nuclear calcium uptake is also mediated by IP_4, which is generated from IP_3 by phosphorylation via a Ca^{2+}/calmodulin-dependent IP_3-3-kinase. High affinity nuclear IP_4 receptors have been localized on the outer nuclear membrane and these are different from other IP_4 receptors documented so far (Malviya and Rogue, 1998).

Besides calcium channels (Bkaily et al., 1997a, b; Gerasimenko et al., 1996), zinc channels have also been identified in the inner nuclear membrane (Longin et al., 1997). Ca^{2+}-activated K^+ channels are localized in the outer nuclear membrane in isolated pancreatic nuclei which are only sensitive to changes in the nuclear Ca^{2+} concentration, and are activated by a relatively high Ca^{2+} concentrations (approximately 200 μM) (Gerasimenko et al., 1996). Numerous Ca^{2+} binding proteins such as calmodulin, calreticulin and calpain have been have been identified in the nucleus (Malviya and Rogue, 1998). Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase), which control gene expression through the phosphorylation of key regulatory sites on nuclear transcription factors such as CREB, has been detected within the nucleus. These kinases may serve to decode Ca^{2+} signals to the nucleus (Heist and Schulman, 1998).

C. Nuclear protein kinase C (PKC)

PKC is a family of serine/threonine kinases that is involved in the transduction of a wide variety of cellular signals (Nishizzuka, 1992; Buchner, 1995). PKC family comprises of at least eleven isoforms that can be divided into three groups, namely the Ca^{2+}-dependent or conventional PKC, Ca^{2+}-independent isoforms or novel PKC, and the atypical PKC. The activity of the conventional PKCs that are composed of the α, β, and γ isoforms is dependent on Ca^{2+}, phospholipids and diacylglycerol, whereas the novel PKCs, δ, ε, η, and θ require diacylglycerol and phospholipids but are Ca^{2+}-independent. A third branch of the family, atypical PKCs, consisting of ζ, ι, μ and λ has also been identified and are characterized by lacking one of the two cysteine-rich zinc-finger regions present in the other isoforms; their activity is independent of Ca^{2+}, phospholipid, diacylglycerol and phorbol esters.

The presence of PKC isoforms has been described for many cellular compartments and there are many examples of translocation to other compartments including the cell nucleus, where upon stimulation, they complex with and phosphorylate specific protein substrates (Malviya and Block, 1993). In addition to the stimulus-dependent translocation of PKC into the nucleus (Leach et al., 1992; Martelli et al., 1992), a constitutive localization of PKC in the nuclear compartment has been described (Buchner, 1995). Both Ca^{2+}-dependent (α, β, and γ) and Ca^{2+}-independent isoforms (δ, ε, and η) are located in isolated nuclei from various cells and the ζ isoenzyme is associated with nuclei isolated from brain. PKC isoforms can also be differentially distributed within the subnuclear compartments.

Intracellular targeting mechanisms of PKC isoforms in the nucleus are unknown; No known NLS sequences of PKC have been identified (Buchner, 1995; Schmalz et al., 1998). It has been suggested that PKC may be directed to their intracellular sites of action through specific interactions with a growing family of docking proteins (Mochly-Rosen, 1995) such as the A kinase-anchoring protein, AKAP79 (Faux and Scott, 1997), and the RACK protein (Buchner, 1995). Specific lipid components within the nuclear membrane play a key role in PKC signaling by stimulating PKC activity at the nucleus. Nuclear

diacylglycerol can also activate nuclear PKC (Topham et al., 1998).

D. Nuclear adenylyl cyclase and protein kinase A

cAMP and cAMP-dependent protein kinase A (PKA) mediate signal transduction pathways that regulate a variety of physiological responses in cells. Compartmentalization of cAMP and PKA control specific signal functions (Yabana et al., 1995). Localization of functional adenylyl cyclase has been demonstrated in nuclear envelopes in human cardiomyocytes (Yamamoto et al., 1998). There is evidence for cAMP accumulation in the nucleus (Barsony and Marx, 1990). The nuclear localization of the catalytic subunit of PKA, which does not contain an endogenous nuclear localization signal, has also been reported (Wiley et al., 1999).

E. Nuclear phospholipase C

Phospholipase C (PLC) signaling occurs in the nucleus (Divecha and Irvine, 1995; Cocco et al., 1999). PLCβ_1 is the major isoform in the nuclei of various cells but other isoforms, PLCβ_2, PLCβ_3 and PLCβ_4 are also present. The PLCβ family is regulated by heterotrimeric G proteins. Nuclear PLCβ_1 is activated independently of its plasma membrane counterpart, and increases during cell growth and decreases during differentiation (Divecha and Irvine, 1995). The long carboxy-terminal region was shown to be necessary for the nuclear localization of PLCβ_1 (Kim et al., 1996).

F. Nuclear phospholipase D

Phospholipase D (PLD) is a ubiquitous enzyme that catalyzes the hydrolysis of cellular phospholipids, particularly phosphatidylcholine, in response to a variety of hormones, neurotransmitters and growth factors (Exton, 1997). Phosphatidic acid, the primary lipid product of PLD possesses growth factor-like properties and can act as a second messenger in certain cell types. Low molecular weight G proteins, ADP-ribosylation factor (ARF) and RhoA play key roles in agonist-induced PLD activation (Exton, 1997).

PLD is enriched in plasma membrane and cytosol but is also present with high activity in isolated nuclei (Exton, 1997; Balboa and Insel, 1995). PLD is a component of a novel nuclear signaling cascade, defined as nuclear envelope signal transduction (NEST), involving the induction of specific nuclear lipid metabolism (Baldassare et al., 1997). Mitogen induced activation of nuclear PLD is mediated by the translocation of RhoA to the nucleus; the activated PLD leads to the production in the nuclear envelope of the signaling molecule, phosphatidic acid.

VII. Nuclear EP receptors

Until recently, it was believed that the diverse effects of PGE_2 could only be mediated by plasma membrane EP receptors. However, there is no direct evidence that effects of PGE_2 on modulation of gene transcription (Danesch et al., 1994; Simonson et al., 1994; Paliogianni and Boumpas; Minghetti et al., 1997), mitogenesis (Pasricha et al., 1992; Hashimoto et al., 1997) and growth and metastasis of tumors (Paoletti et al., 1989; Fulton et al., 1991; Yano et al., 1997) results exclusively from its interaction with cell surface receptors.

The possible existence of nuclear EP receptors is suggested by the occurrence of enzymes involved in the biosynthesis of prostanoids (COX-1, COX-2, PLA_2) at the nuclear envelope (Spencer et al., 1998; Schievella et al., 1995). It has been proposed that the inducible COX-2 provides prostaglandins for nuclear eicosanoid signaling system, which participate in cellular growth, replication and differentiation (Goetzl et al., 1995; Minghetti and Levi, 1998). Thus COX-2 could play a role in prolonged generation of prostanoid which would modify nuclear events (Reddy and Herschmann, 1997). Moreover, the identification of a prostanoid transporter, which mediates the influx of prostanoid (Kanai et al., 1995), provided an efficient means of delivering circulating PGE_2 to the nuclear site. It has been demonstrated that other prostanoids, namely PGD_2 and its metabolite PGJ_2, can activate the peroxisome proliferator-activated receptors (PPARs), which are members of the nuclear receptor superfamily of ligand-dependent transcription factors; however, PPARs are not responsive to PGE_2 (Kliewer et al., 1995; Hertz et al., 1996) and therefore distinct from nuclear EP receptors identified by us (Bhattacharya et al., 1998, 1999). Also, as discussed above, nuclear membranes contain various intermediate factors involved in EP receptor-mediated signal transduction systems, such as G proteins, calcium, PLC, adenylyl cyclase, PKA and PKC.

Our recent studies have demonstrated that functional EP receptors are indeed localized in nuclear membranes in a variety of cells and tissues (Bhattacharya et al., 1998, 1999). Nuclear PGE_2 binding sites were detected by radioligand binding studies in both newborn (porcine brain) and adult (porcine myometrium and rat liver) tissues; the PGE_2 binding kinetics to nuclear and plasma membrane fractions were similar. All subtypes of EP receptors (EP_1, EP_2, $EP_{3\alpha}$ and EP_4) were detected in nuclear membranes. However in each tissue, the relative distribution of EP receptors in plasma membrane and nuclear membrane differed. Since the molecular weights and pharmacological characteristics EP receptors in plasma and nuclear membranes were similar, it would appear that the receptors in both cellular compartments are highly homologous or identical.

The presence of EP receptors in the nuclear membrane was verified by immunoelectron microscopy of primary cultures of porcine newborn cerebral

microvascular endothelial cells using specific rabbit anti-EP receptor antibodies (Zhao et al., 1995) (**Fig. 1**). Moreover, the presence of nuclear EP receptors was also detected *in vivo*, in adult rat brain cortex endothelial cells and neurons (Bhattacharya et al., 1998); both these tissues are important sources of brain prostanoids (Li et al., 1994; Parfenova et al., 1997; Mingetti and Levi, 1998).

Over-expression of human EP_1, $EP_{3\alpha}$ and EP_4 receptors cDNA in human embryonic kidney (HEK) 293 cells that do not express prostanoid receptors (Boie et al., 1997) revealed a perinuclear localization of EP receptors by indirect immunofluorescence (Bhattacharya et al., 1998; 1999); a similar distribution was observed by expressing EP_1 receptor fused to green fluorescent protein in HEK 293 cells. These data once again suggest similar identity between plasma membranes and nuclear envelope EP receptors.

Although the stimulation of nuclear EP receptors by prostaglandin analogs did not modify the generation of second messengers, cAMP or IP_3, it affected gene transcription and nuclear calcium signals (Bhattacharya et al., 1998; 1999). The stimulation of nuclear EP_1 receptors in isolated nuclei by prostaglandin analogs was found to modulate *c-fos* gene transcription and nuclear calcium transients. Stimulation of intact nuclei isolated from primary cultures of porcine brain endothelial cells with the EP_3 receptor agonist M&B 28,767 (0.1 μM) increased transcription of iNOS (**Fig. 2a**) to a greater extent than after stimulation of whole cells as determined by dot RNA hybridization.

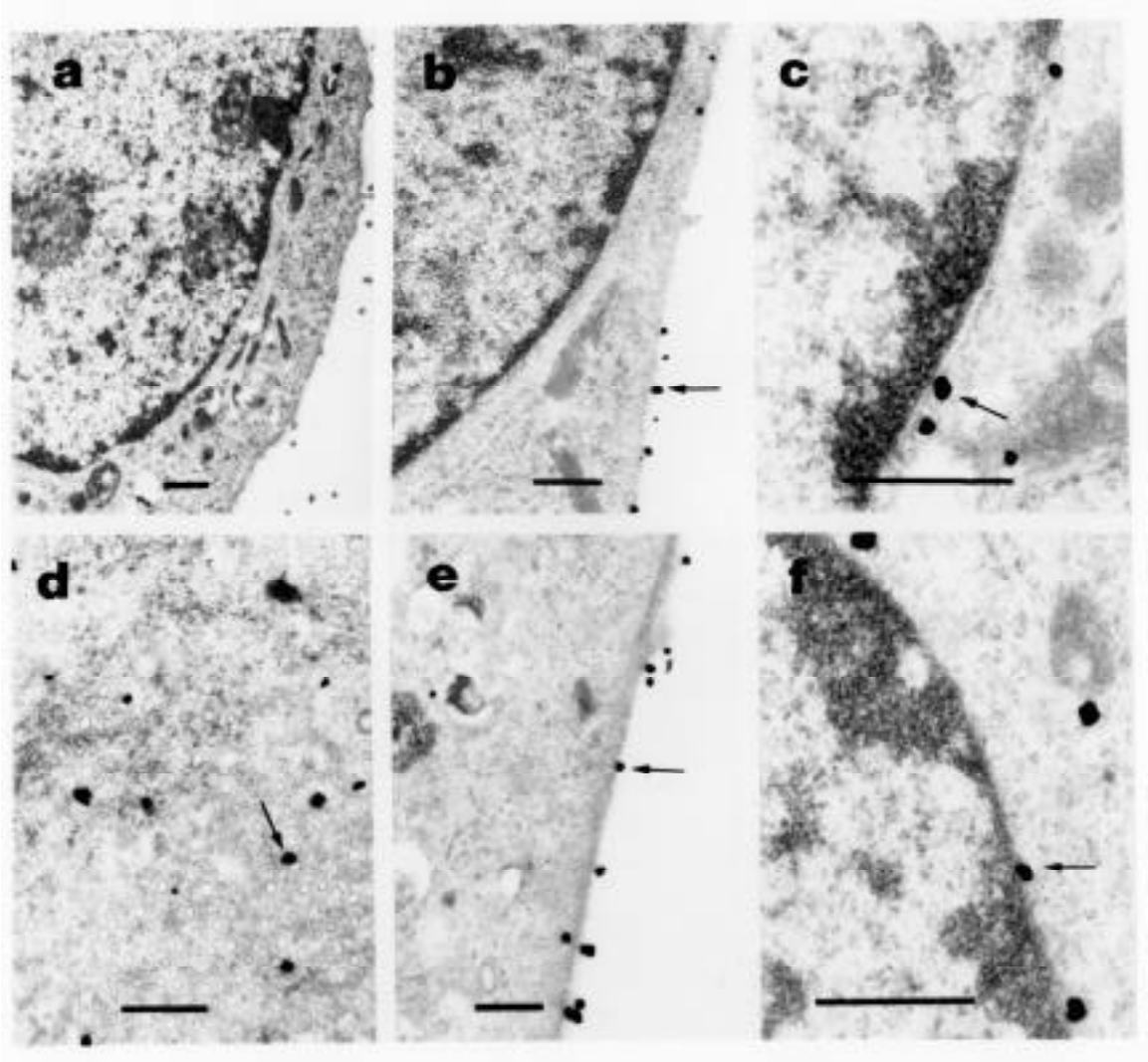

Figure 1. Immunogold localization of $EP_{3\alpha}$ and EP_4 receptors in porcine cerebral microvascular endothelial cells (primary culture from newborn brain) by electron microscopy (see arrows). (a) Anti-rabbit gold-conjugated IgG alone; note absence of immunostaining when primary antibody is omitted. $EP_{3\alpha}$ immunoreactivity on (b) plasma membrane, and (c) nuclear membrane. EP_4 immunoreactivity on (d) Golgi vesicles, (e) plasma membrane, and (f) nuclear membrane. Scale bar in each represents 0.5 μm.

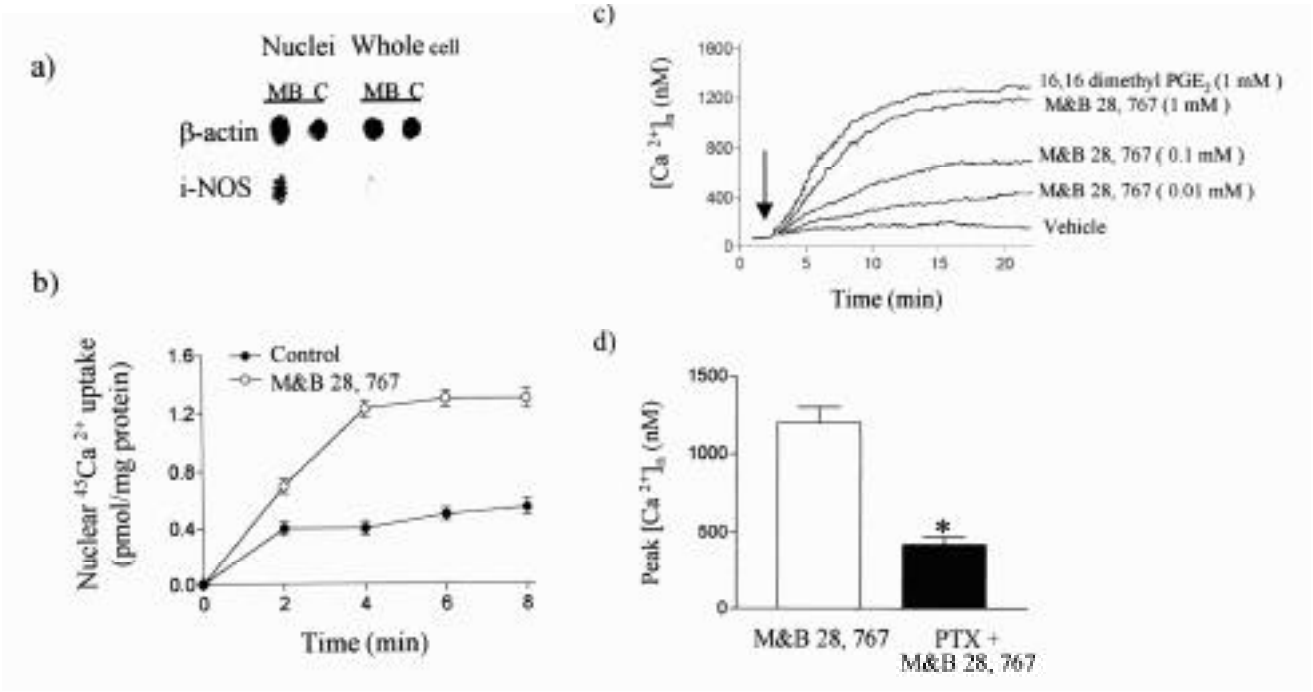

Figure 2. Effects of nuclear EP receptor stimulation on iNOS gene transcription and nuclear calcium transients. (**a**) Effects of EP_3 agonist M&B 28,767 (0.1 μM) on iNOS transcription in porcine cerebral microvascular endothelial cells (primary culture from newborn brain) as determined by dot blot hybridization of RNA; MB and C refer to M&B 28,767 and control (unstimulated) respectively. β-actin dot blot indicates equal loading. One representative dot blot of three is shown. (**b**) Effect of M&B 28,767 (1 μM) on $^{45}Ca^{2+}$ uptake by isolated liver nuclei; control refers to absence of drug. The free calcium concentration was 400 nM. The movement of $^{45}Ca^{2+}$ transient after a given time was defined as radioactivity at a given time minus the radioactivity at time 0. (**c**) Typical tracings showing effects of PGE_2 analog 16, 16-Dimethyl PGE_2 (1 μM) and EP_3 agonist M&B 28,767 (0.01-1 μM) on intranuclear calcium concentrations ($[Ca^{2+}]_n$) in isolated liver nuclei loaded with fura-2 AM; arrow shows the time of application of test agents. (**d**) Peak increases in isolated liver intranuclear calcium ($[Ca^{2+}]_n$) after addition of M&B 28,767 (1 μM) in the presence or absence of PTX preincubation (20 μg/ml, 20 min at 25 °C). Experiments (b-d) were carried out on three independent isolations of intact nuclei each one performed in duplicate.

Previous studies have reported that iNOS expression is modulated in an opposite way by endogenous and exogenous PGE_2; endogenous PGE_2 has a stimulatory effect on iNOS as opposed to the inhibitory effect of exogenous PGE_2 (Milano et al., 1995; Minghetti et al., 1997). It is thus possible that endogenous PGE_2 might act via EP nuclear receptors as speculated (Minghetti et al., 1997).

Application of EP_3 agonist M&B 28,767 to intact isolated liver nuclei also caused rapid nuclear uptake of $^{45}Ca^{2+}$ (**Fig. 2b**). In addition, M&B 28,767 produced a dose-dependent increase in rat liver nuclear calcium transients as determined by fura-2 AM, a fluorescent dye which localizes in the nuclear envelope space (Gerasimenko et al., 1996) (**Fig. 2c**). At an equivalent concentration, M&B 28,767 was nearly as effective as the non-selective EP agonist 16,16-dimethyl PGE_2 in increasing nuclear Ca^{2+} levels (**Fig. 2c**). EP_3 couples mainly to G_i or G_o (Namba et al., 1993), which are known to affect Ca^{2+} mobilization (Namba et al., 1993; Kojima et al., 1986; Huges et al., 1987; Hescheler et al., 1988); such G proteins are detected in rat liver nuclei (Takei et al., 1994). Pretreatment of isolated nuclei with PTX, an inhibitor of G_i or G_o markedly attenuated the stimulatory effect of M&B 28,767 on intranuclear calcium levels suggestive of the involvement of a PTX-sensitive G-protein in mediating the effects of nuclear EP_3 receptors (**Fig. 2d**). These findings are consistent with other studies which have demonstrated that G proteins, especially G_i (Hescheler et al., 1988; Rosenthal et al., 1988) may directly control Ca^{2+} channels independent of cAMP or IP_3 (Kojima et al., 1986; Hughes et al., 1987).

Our studies indicated that functional nuclear EP receptors were present in newborn porcine brain (Bhattacharya et al., 1998, 1999; **Figs. 1** and **2**). PGE_2 is

one of the most abundant prostanoids in the brain during the perinatal period (Leffler and Busija, 1985) and plays an important role in many cerebral functions, such as in the regulation of cerebral blood flow (see Chemtob, et al., 1996) and modulating the gene transcription of nitric oxide synthase (Dumont et al., 1998). However, in the newborn brain and cerebral microvasculature, high levels of prostaglandins have been shown to result in downregulation of plasma membrane EP receptors and associated functions (see Chemtob et al., 1996; Li et al., 1994). On the other hand, PGE_2 increases the expression of nitric oxide synthase via stimulation of EP_3 receptors in the neonate (Dumont et al., 1998). This effect was prevented in the presence of an inhibitor of the prostanoid transporter (Dumont et al., 1999), which supports a role of nuclear EP receptors in modulating NOS gene transcription.

The details of the mechanism by which nuclear EP receptors activate gene transcription of *c-fos* and iNOS need to be elucidated. Our studies revealed that selective stimulation of nuclear EP_1 receptors increased the transcription of c-*fos*; Simonson et al., (1994) also showed that PGE_2 elevated c-*fos* expression by cAMP-independent but PKC-dependent mechanisms. The increase in nuclear calcium transients following a stimulation of nuclear EP receptors might be the mechanism by which calcium regulates the transcription of many genes (Malviya and Rogue, 1998) including *c-fos* (Hardingham et al., 1997; Malviya and Rogue, 1998). The transcription of iNOS is predominantly activated by the ubiquitous transcription factor NF-κB (Xie et al., 1994) and EP_3 receptors have been shown to modulate NF-κB-dependent cellular signaling (Meyer-Kirchrath et al., 1998). Recent studies indicate that there may be constitutive expression of NF-κB in the nucleus (Hunot et al., 1997; Delfino and Walker, 1998) and this may suggest a possible mechanism by which nuclear EP_3 receptors may modulate iNOS transcription.

To date, the presence of a NLS sequence has not been reported in the literature in any of the cloned EP receptor subtypes (Narumiya, 1996). A search of the human EP receptor protein sequence database against a ProfileScan database revealed the presence of a consensus sequences with high degree of similarity to bipartite NLS signals in the C-terminals tails of human EP_1 (Funk et al., 1993) and EP_3 (Kotani et al., 1997) isoforms (amino acids 236-253 for EP_1 and 350-367 for EP_3). This places prostanoid receptors together with the AT_1 receptor, in a distinct class of G protein-coupled receptors, which function at the cell surface as well as at the nucleus, and revolutionizes our current understanding of signal transduction mediated via G protein-coupled receptors. Nevertheless, an important distinction between the AT_1 and prostanoid EP receptors is that the latter were detected at the nuclear envelope without prior stimulation with PGE_2.

Angiotensin II receptors have been detected in chromatin fragments, and it has been suggested that Angiotensin II alone or complexed to its receptor, might be involved in the regulation of gene expression through interactions with nuclear DNA (Re et al., 1984; Eggena et al., 1996). There are other reports of nuclear peptides capable of binding DNA (Castel et al., 1992). In contrast, there have been no reports of PGE_2 binding chromatin; only the prostaglandins of the J series and their receptors, the PPARs, have been detected within the nucleus (Narumiya and Fukushima, 1986; Kliewer et al., 1995; Hertz et al., 1996). Interestingly, it has been reported that putative NLS sequences have been detected in other G protein-coupled receptors such as the human platelet activating factor receptor and the human M1, M3 and M5A muscarinic acetylcholine receptors (Lu et al., 1998). However, whether or not these receptors are actually localized at the nucleus and are functional is yet to be established.

The details of the mechanism of intracellular trafficking that target EP receptors to the nucleus remain to be elucidated. Since over-expression of the EP receptor cDNA in HEK 293 (Bhattacharya et al., 1998, 1999) and Swiss 3T3 cells or transfection of EP_1-GFP fusion protein in HEK 293 cells revealed a perinuclear localization (Bhattacharya et al., 1998), it is very possible that translational or post-translational mechanism are operative in determining trafficking of the receptors to either plasma membranes or nuclear membranes. Segments of EP receptors may contain docking sites for interaction with proteins that may function to target the receptors to defined subcellular locations as reported for the T-cell protein tyrosine phosphatase (TCPTP) by Tiganis et al. (1997). TCPTP contains both an endoplasmic reticulum-targeting motif and a bipartite NLS sequence in the C-terminal but is associated exclusively with the endoplasmic reticulum, which indicates that the endoplasmic reticulum targeting event is the dominant event. A cytoplasmic protein has been shown to interact with residues in the C terminus and directs the enzyme to the endoplasmic reticulum; the binding site of this protein overlaps residues in the NLS cluster and therefore might control the accessibility of the NLS to nuclear import factors. Another protein that is located in the plasma membrane but is also targeted to the nucleus is the myelin basic protein; this too possesses putative NLS sequences (Pedraza et al., 1997).

Putative NLS sequences have so far not been detected on EP_2 or EP_4 receptors. In fact, known NLS sequences are lacking in various proteins that are targeted to the nucleus such as COX (Spencer et al., 1998), PKC (see Buchner, 1995; Schmalz et al., 1998) or the kinase Raf-1 (Lu et al., 1998). There may be some additional targeting motifs, which direct some of these proteins to the nuclear membranes. Nuclear translocation of Raf-1 may be accomplished by its binding to a carrier protein (Lu et al., 1998) and nuclear targeting of PKC may involve its interaction with docking proteins (Buchner, 1995; Faux and Scott, 1997). EP receptors may be directed to the nucleus by a piggy-back mechanism that involves

additional protein(s) containing a basic nuclear localization sequence as demonstrated for I-κBα, which is constitutively transported to the nucleus when it is not bound to NF-κB (Turpin et al., 1999).

VIII. Conclusions

The discovery of nuclear EP receptors (Bhattacharya et al., 1998, 1999) proposes new avenues for the intracellular actions of prostanoids. In addition, these studies shed new light to the complex field of signaling via G protein-coupled receptors. Further studies are needed to clarify the details of the molecular mechanisms involved in this action of prostaglandins via nuclear EP receptors. It would also be interesting to determine whether there are nuclear receptors for other prostanoids (FP, DP, TP, IP). The findings that will result from these studies will provide valuable insight in the field of prostanoid pharmacology. Given that prostanoids are widely distributed in body tissues and play a major role in various physiological and pathophysiological conditions, these studies may lead to new therapeutic strategies for a number of pathological conditions.

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