ELSEVIER

European Journal of Pharmacology 313 (1996) 257–263

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Estrogen increases the expression of uterine protein kinase C isozymes in a tissue specific manner

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Received 22 February 1996; revised 21 June 1996; accepted 28 June 1996

Abstract

The pattern of protein kinase C isozyme expression in uterine smooth muscle and ventricular cardiac muscle was examined in ovariectomized rats pretreated with estradiol-17β alone or with estradiol-17β and progesterone. Protein kinase C isozyme expression was examined in membrane and cytosolic subcellular fractions by immunoblot analysis using antisera specific for α, γ, β1, β2, δ, ε, ζ, and θ isozymes. All isozymes were detectable in positive control brain extracts. The predominant isozymes in the myometrium were δ and β2 while in the ventricle, β2 and ζ were the dominant forms. In unstimulated tissues, all isozymes except PKC-δ, were predominantly found in the cytosolic compartment. Both estrogen and progesterone increased membrane-associated isozyme expression 35–125% in uterine muscle. Neither estrogen nor progesterone treatment significantly affected protein kinase C expression in cardiac muscle. These data suggest that estradiol, which increases uterine muscle hypertrophy and contractility, may exert these effects by increasing membrane-associated protein kinase C expression in a tissue-specific manner.

Keywords: Protein kinase C; Smooth muscle, uterine; Cardiac muscle; Steroid; Estradiol; Progesterone

1. Introduction

It is well established that smooth muscle contractility, including that of the uterus, is determined by the level of cytoplasmic free Ca^{2+}. Stimulation of uterine cell membrane receptors by hormones, neurotransmitters, and growth factors results in an increase in intracellular messengers affecting intracellular Ca^{2+} levels. The signaling pathway that activates selective hydrolysis of membrane inositol-containing phospholipids produces two such messengers, inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate acts to mobilize Ca^{2+} from intracellular stores and diacylglycerol activates membrane-associated protein kinase C (Berridge, 1993). In smooth muscle, Ca^{2+} mobilization and protein kinase C activation appear to function synergistically to regulate muscle contractility (Rasmussen et al., 1987).

Activation of protein kinase C results in the translocation of inactive enzyme from the cytosolic compartment to the plasma membrane where it presumably exerts its physiologic effects. Protein kinase C activation in smooth muscle has been reported to phosphorylate neurotransmitter receptors and ion channels (Bouvier et al., 1987; Galizzi et al., 1987), inhibit guanine nucleotide-binding protein (receptor signal transduction) activity (Katada et al., 1985), phosphorylate contractile filament proteins (Seto et al., 1990), and increase the Ca^{2+} sensitivity of smooth muscle contractile apparatus (Laher et al., 1989).

Molecular cloning has revealed that protein kinase C comprises a family of at least ten related Ca^{2+}/phospholipid-dependent serine/threonine protein kinases (Nishizuka, 1989). These protein kinase C isozymes differ in their mode of activation, kinetic properties, substrate specificities, and tissue or cellular distributions. Based on cofactor requirements for activation, these isozymes have been classed into conventional (α, β1, β2, γ), novel (δ, ε, η, θ), and atypical (ζ, λ) groups. Conventional protein kinase C isozymes depend on Ca^{2+}, phospholipid, and diacylglycerol for activation. Novel isozymes lack a Ca^{2+}-binding domain, and activation appears to be independent of Ca^{2+}. Activation factors for the atypical group

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PII S0014-2999(96)00525-0

appear not to require either diacylglycerol or Ca^{2+} (Nakanishi et al., 1993).

The gonadal steroids, estrogen and progesterone, exert profound effects on uterine smooth muscle. Discrete changes in both plasma and uterine levels of these hormones have been implicated with a wide variety of physiologic events affecting uterine contractility during pregnancy and labor (Thorburn and Challis, 1979). In a previous study, we reported that estrogen and progesterone regulate inositol phospholipid hydrolysis and presumably protein kinase C activation through the release of diacylglycerol in uterine smooth muscle (Ruzycky and Crankshaw, 1988). Recently, estrogen has been reported to increase levels of protein kinase C-δ in rabbit corpus luteum (Maizels et al., 1992). Little is currently known about the identity, abundance or functional specificity of protein kinase C isozymes in uterine smooth muscle.

In the present study, we have examined the expression and cellular distribution of protein kinase C isozymes in ovariectomized animals exposed to defined endocrine environments similar to those found during pregnancy (progesterone-dominated) and labor (estrogen-dominated). In addition, we have studied whether estrogen and progesterone exert tissue selective effects on protein kinase C isozyme expression by comparing enzyme levels in uterine smooth muscle and cardiac muscle.

2. Materials and methods

2.1. Animals

Virgin female Sprague-Dawley rats weighing 175–200 g were purchased ovariectomized from Taconic (Germantown, NY, USA). After a recovery period of at least 10 days post surgery, animals were treated with estrogen or estrogen and progesterone. Animals referred to as 'estrogen-dominated' were injected once every day with estradiol-17β (200 μg/kg s.c.) for 4 days, while 'progesterone-dominated' animals received estradiol-17β and progesterone (200 μg/kg and 20 mg/kg, respectively) for an additional 4 days (Ruzycky et al., 1987). Control animals did not receive any injections.

Twenty-four hours following the last hormone injection, animals were killed by decapitation. Both uterine horns were rapidly excised (0.5 cm from both the ovarian ligation and the uterine bifurcation) and placed in a physiologic salt solution at 37°C of the following composition (mM): NaCl, 118; KCl, 4.7; $MgCl_2$, 1.1, $CaCl_2$, 1.8; $NaHCO_3$, 25; KH_2PO_4, 1.0; and dextrose, 5.6. The uterine horns were carefully cleaned of surrounding fat and connective tissues and opened longitudinally along the mesenteric border. The lumenal surface of the uterus was gently rubbed with a cotton swab to remove the endometrium. Cardiac tissue was similarly prepared. Atria were crudely dissected from ventricles and discarded.

2.2. Preparation of membrane and cytosolic fractions

Tissues collected from at least 10 animals per treatment group were pooled for preparation of subcellular fractions. Membrane fractions were prepared according to procedures previously described (Ruzycky et al., 1987) with the following modification. Tissues were homogenized in a buffer containing 50 mM Tris pH 7.4, 1 mM EDTA, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 20 μg/ml phenylmethylsulfonyl fluoride. Membrane pellets were resuspended in 50 mM Tris pH 7.4 and stored at −80°C until use. The cytosolic supernatant was further concentrated using a Centriprep concentrator with a 3000 molecular weight cutoff (Amicon, Beverly, MA) to a protein concentration of at least 10 mg/ml and stored at −80°C until use.

2.3. Immunodetection of protein kinase C

Paired membrane and cytosolic fractions obtained from the same subcellular preparation (35 μg/lane) were subjected to electrophoresis on sodium dodecyl sulfate-7.5% polyacrylamide minigels (Laemmli, 1970). Proteins were transferred electrophoretically onto nitrocellulose membranes for 24 min at 85 V. Nitrocellulose membranes (Nitrobind; Microseparations, Westborough, MA) were incubated with 10% goat serum/0.1% Tween for 1 h at room temperature to saturate non-specific binding sites. Nitrocellulose membranes were then incubated with isozyme-specific antiserum for 3 h. Isozyme-specific antibodies were used at the following dilutions: polyclonal anti-PKCα, 1:5000; polyclonal anti-PKCγ, 1:15000; polyclonal anti-PKCβ1, 1:6000; polyclonal anti-PKCβ2, 1:6000; polyclonal anti-PKCδ, 1:500000; polyclonal anti-PKCε, 1:2850; polyclonal anti-PKCζ, 1:8000; polyclonal anti-PKCθ, 1:1000; monoclonal anti-PKCα, 1:1500; monoclonal anti-PKCγ, 1:1500. After three washes with 0.1% Tween, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit (0.20 μl/ml) or anti-mouse antibodies (0.20 μl/ml). Protein bands of interest were colorimetrically detected using a high sensitivity Enzygraphic Web (IBI, New Haven, CT, USA). Upon optimal reaction with the Enzygraphic Web, blots were immediately photographed using Polaroid type 55 positive/negative film. Immunoreactive bands were quantitated from photographic negatives using a GS300 scanning densitometer (Hoefer, San Francisco, CA, USA) interfaced to a personal computer.

2.4. Materials

Rabbit polyclonal antibodies (IgG fractions) directed against isozyme-specific carboxy terminal peptide sequences of protein kinase C-α, -γ, -β1, -β2, -δ, -ε, and -ζ were purchased from Research and Diagnostic Antibodies (Berkeley, CA, USA). Polyclonal ant-PKC-θ was a gener-

ous gift of Dr Amnon Altman (Baier et al., 1993). Mouse monoclonal antibodies directed against protein kinase C-α, and -γ were purchased from Seikagaku America (St. Petersburg, FL, USA). Horseradish peroxidase-conjugated antiserum was purchased from BioRad Laboratories (Richmond, CA, USA). Other reagents were from standard commercial sources.

2.5. Data and statistical analysis

All data are expressed as sample means ± S.E.M. relative to values obtained in control samples. Significant differences between sample means were analyzed by the two-tailed paired Student's *t*-test at the $P < 0.05$ level.

3. Results

3.1. Protein kinase C isoform expression in rat uterus

Membrane and cytosolic subcellular fractions of uterine muscle and heart ventricle were blotted and probed for the presence of protein kinase C-α, -β1, -β2, -δ, -ε, -ζ, and -θ. Polyclonal antibodies generated against synthetic peptides corresponding to unique sequences of individual protein kinase C isozymes (Table 1) were used to identify isoform expression in rat uterine tissue and heart ventricles. Protein samples from two different pooled preparations (containing tissues derived from five to eight animals) were assayed on three to six separate occasions. Specific isoform expression was confirmed on Western blots by comparison to immunoreactive staining of a sample of protein kinase C extracts derived from rat brain. The results of Western blot analyses of rat uterine subcellular fractions are presented in Fig. 1.

To investigate the relative abundance of all detected protein kinase C isozymes, tissue subcellular fractions were individually incubated with isozyme specific primary antibodies, washed, and then concurrently incubated with secondary antibody. The relative tissue abundance of protein kinase C isozymes (defined as the sum of immunoreactivity detected in membrane and cytosolic fractions) in rat uterine and heart tissue is presented in Table 2. These results show that the protein kinase C isozyme abundance profile is different between tissues, with the predominant species in the heart ventricle being protein kinase C-β2 and -ζ. The uterus exhibited protein kinase C-δ and -β2 as the most abundant species. In both tissues, the two predominant isozymes accounted for greater than 50% of detected isozymes.

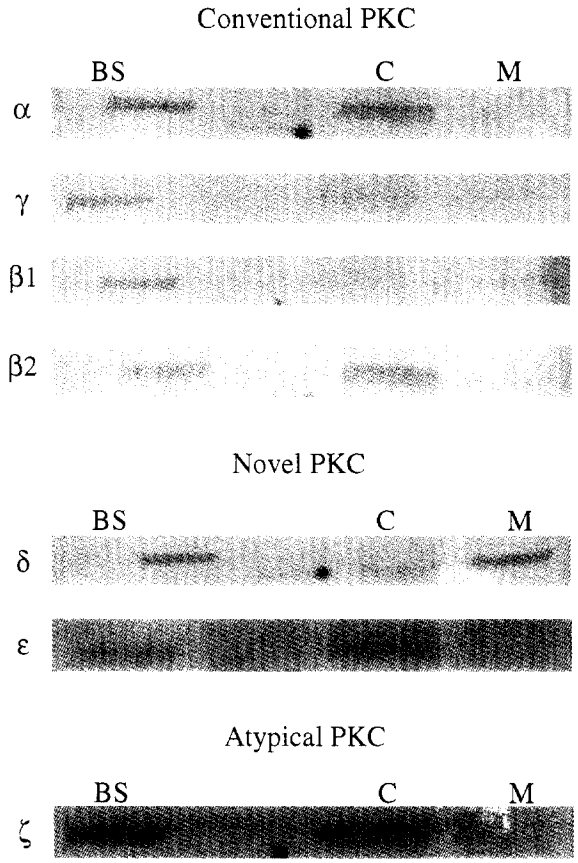

Fig. 1. Western blots of protein kinase C isozymes in rat uterine smooth muscle membrane (M) and cytosolic subcellular fractions (C). BS, denotes brain standard used as positive control.

Table 1
Protein kinase C isozyme antigen sequences

Isozyme	Source	Sequence location	Amino acid
α	Rat	313–326	WAGNZKVISPSEDRRQC
γ	Rat	306–318-Cys	NYPLELYERVRTGC
β1	Rat	661–671	SYTNPEFVINV
β2	Rat	660–673	SFVNSEFLKPEVFS
δ	Rat	662–673	SFVNPKYEQFLE
ε	Rat	728–737	FSYFGEDLMP
ζ	Rat	480–492	YINPLLSAEESV
θ	Human	311–398	IFREGPVEIGLPCSIKNEARPPC LPTPGKREPQGISWESPLDEVD KMCHLPEPELNKERPSLQIKLK IEDFILHKMLGKGSFGKVFLA

3.2. Subcellular distribution of protein kinase C isoforms

Physiologic activation of protein kinase C is thought to involve translocation of inactive enzyme from the cytosolic compartment to the plasma membrane. To investigate the subcellular distribution of protein kinase C isozymes as an indirect index of inherently activated enzyme (i.e. membrane-bound enzyme), equivalent amounts of protein from paired subcellular fractions derived from the same preparation were immunoblotted. Protein kinase C isozyme immunoreactivity in membrane and cytosolic subcellular fractions is summarized in Table 3. Protein kinase C isozyme specific immunoreactivity was detected in both cytosolic and membrane fractions of rat uterus and heart.

Table 2
Relative tissue abundance of detected protein kinase C isozymes in rat uterine and heart ventricle tissue collected from ovariectomized rats

Tissue	PKC-α	PKC-γ	PKC-β1	PKC-β2	PKC-δ	PKC-ε	PKC-ζ
Mol. wt. (kDa)	76	76	78	77	83	77	74
Uterus	6.0 ± 0.7	8.5 ± 1.0	3.9 ± 0.4	18.2 ± 4.6	46.3 ± 0.9	6.5 ± 1.9	10.6 ± 1.9
Ventricle	12.5 ± 0.5	6.0 ± 0.6	4.5 ± 0.5	37.1 ± 1.7	9.6 ± 2.0	10.9 ± 0.8	22.0 ± 0.7

Subcellular fractions were individually incubated with isozyme-specific antibodies separately. Following washes, individual nitrocellulose membrane strips were combined for incubation with secondary antibody and subsequent colorimetric development for immunoreactivity detection. Total tissue protein kinase C content was defined as total protein kinase C isozyme immunoreactivity detected in both membrane and cytosolic subcellular fractions.

In general, the amount of protein kinase C isozyme immunoreactivity was approximately two fold greater in ventricle than in uterine subcellular fractions when compared on a per microgram protein basis (data not shown). Protein kinase C-θ could not be detected in cytosolic or membrane fractions from either tissue or in control brain samples. In the absence of any exogenous activators, classical protein kinase C isozymes (α, γ, and β) were found to be present in both membrane and cytosolic fractions of rat ventricle, with approximately 60–70% of enzyme in the cytosolic compartment. Similar immunoreactivity was observed using monoclonal antibodies directed against protein kinase C-α and -γ isozymes (data not shown). Similarly the atypical ζ isozyme and the novel ε isozyme exhibited a 50–70% distribution in the cytosolic compartment. This was in contrast to the subcellular distribution found for the novel protein kinase C-δ which was predominantly found in the membrane fraction with only 40% of total detected enzyme in the cytosol. The subcellular distribution of protein kinase C isozymes was found to be qualitatively similar between uterine and cardiac tissue.

3.3. Effect of estrogen and progesterone on protein kinase C isozyme expression

To examine the effect of estrogen and progesterone on the expression and subcellular distribution of protein kinase C isozymes, paired membrane and cytosolic fractions derived from the same preparation were immunoblotted. For each isozyme, samples of equivalent protein amount (35 μg) derived from control, estrogen-, and progesterone-dominated animals were compared on the same immunoblots. Protein kinase C isozyme content in samples from control tissue was assigned the value of 1.00 and used as a standard for comparison of isozyme levels in subcellular fractions from hormonally treated animals.

As depicted in Fig. 2, treatment of ovariectomized animals with 17β-estradiol resulted in distinctly different patterns of protein kinase C isozyme expression in rat uterine and cardiac tissue. In rat ventricle fractions, membrane-associated and total protein kinase C isozyme expression was not significantly changed as a result of estrogen treatment in vivo (Fig. 2B). In contrast, estrogen treatment was associated with significantly increased expression of PKC isozymes from all three groups (Fig. 2A).

Table 3
Subcellular distribution of detected protein kinase C isozymes in rat uterine and heart ventricle tissue

Tissue	Subcellular distribution (percentage in membrane)						
	PKC-α	PKC-γ	PKC-β1	PKC-β2	PKC-δ	PKC-ε	PKC-ζ
Uterus	11 ± 1	n.d.	n.d.	9 ± 3	68 ± 7	15 ± 3	14 ± 1
Ventricle	30 ± 5	42 ± 6	33 ± 7	23 ± 1	67 ± 9	45 ± 5	28 ± 3

Paired subcellular fractions collected from ovariectomized rats (35 μg protein) were subjected to SDS-PAGE and Western blot analysis. Data are presented in terms of percentage of protein kinase C isozyme detected in membrane fraction relative to total tissue immunoreactivity (as described in Table 2). n.d., not done.

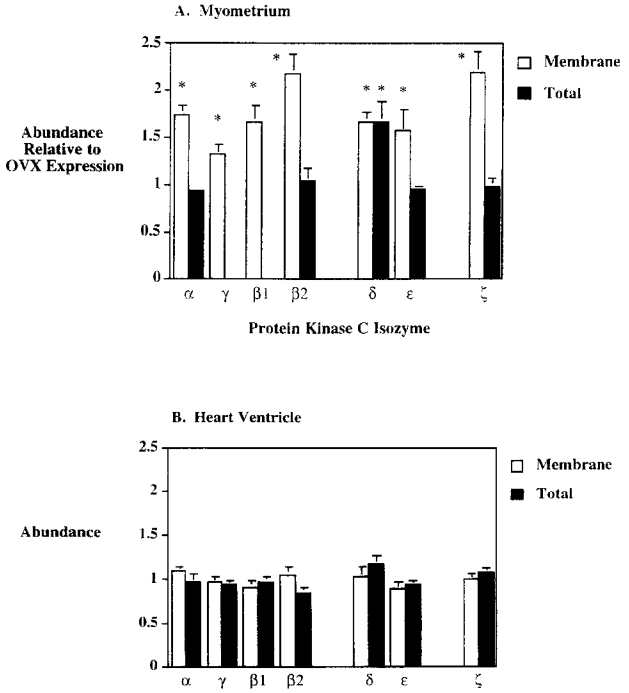

Fig. 2. Effect of in vivo 17β-estradiol treatment on uterine (A) and cardiac (B) expression of protein kinase C isozymes in subcellular fractions. Ovariectomized animals were treated for 4 days with 17β-estradiol as described in the text. Arbitrary densitometric units of immunoreactivity (expressed relative to levels detected in untreated animals) are depicted. The means ± S.E.M. of three to five experiments are presented. Total expression refers to the sum of immunoreactivity in membrane and cytosolic subcellular fractions. Asterisks denote significant differences from expression in the absence of hormone treatment ($P < 0.05$).

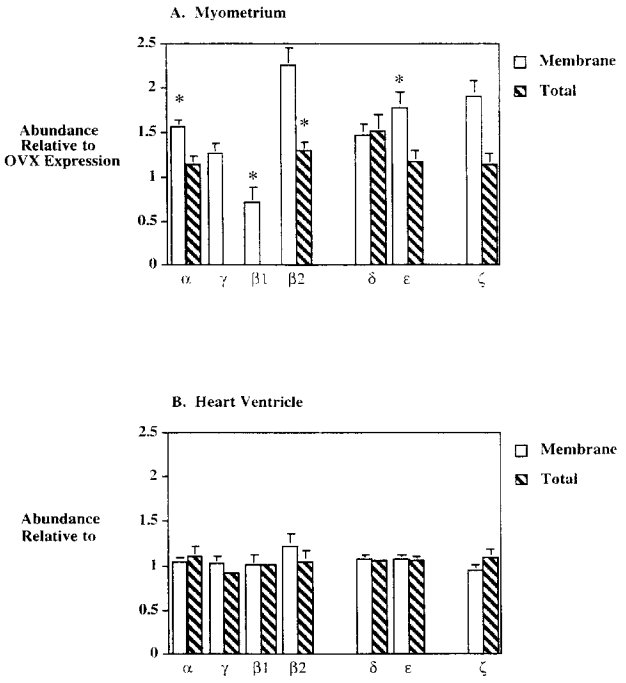

Fig. 3. Effect of in vivo progesterone treatment on uterine (A) and cardiac (B) expression of protein kinase C isozymes in subcellular fractions. Ovariectomized animals were treated for 4 days with 17β-estradiol followed by 4 days with 17β-estradiol and progesterone as described in the text. Arbitrary densitometric units of immunoreactivity (expressed relative to levels detected in untreated animals) are depicted. The means ±S.E.M. of three to five experiments are presented. Total expression refers to the sum of immunoreactivity in membrane and cytosolic subcellular fractions. Asterisks denote significant differences from expression in the presence of estrogen treatment alone ($P < 0.05$).

As total myometrial protein kinase C isozyme expression was generally unaffected by estrogen treatment (with the exception of the δ isozyme), the additional expression of isozymes was limited to a preferential distribution in the myometrial membrane compartment. The increase in membrane-associated protein kinase C isozymes varied from 35% for the γ isozyme to 125% for β2 and ζ isozymes.

Similarly, progesterone treatment was associated with distinct tissue effects on the expression of protein kinase C isozymes (Fig. 3). As observed with estrogen treatment, progesterone did not significantly affect the expression or distribution of protein kinase C isozymes in the rat ventricle (Fig. 3B). In general, progesterone treatment was associated with more modest changes in protein kinase C isozyme expression. The most striking effect of progesterone treatment was the significant decrease in the expression of a minor protein kinase C isozyme, β1, to levels below that observed in the absence of any gonadal steroids (ovariectomized animals). Progesterone treatment was also associated with modest but significant increases in membrane-associated protein kinase C-α and -ε levels, whereas total protein kinase C-β2 and -δ levels were also increased due to additional expression of this isozyme in the cytosolic compartment.

4. Discussion

In this study, we have examined the effects of estrogen- and progesterone-dominating states on the expression and distribution of protein kinase C isozymes in uterine smooth and cardiac muscles. It has long been recognized that these gonadal steroids exert profound changes on the uterus, including increased cellular hypertrophy and muscle contractility (Thorburn and Challis, 1979). Similarly, these hormones are becoming increasingly recognized as important regulators of cardiovascular function in women during pregnancy and prior to menopause (White et al., 1995).

Protein kinase C has been implicated in signaling pathways regulating the expression of muscle hypertrophy (Chien et al., 1991) and contractility (Rasmussen et al., 1987). Many hypertrophic and contractile agonists stimulate inositol phospholipid hydrolysis leading to intracellular production of diacylglycerol, the endogenous activator of protein kinase C. Similarly, direct pharmacologic activation of protein kinase C by phorbol esters leads to development of muscle hypertrophy (Chien et al., 1991) and increased muscle contractility (Ruzycky and Morgan, 1989). Moreover, isozyme specific redistribution of protein kinase C has been reported in vascular hypertrophy in vivo (Liou and Morgan, 1994).

Isozymes of protein kinase C differ in their tissue expression, respective modes and kinetics of activation, intracellular proximity to activators and phosphorylation targets, apparent substrate specificity, and their sensitivity to down-regulation and proteolysis (Nishizuka, 1995). Although translocation of protein kinase C isozymes to the membrane compartment correlates with increased agonist-induced levels of diacylglycerol and enzyme activity (Baldassare et al., 1992), the role of the various isozymes of protein kinase C in the regulation of distinct cellular processes is not well understood.

In an effort to assess the physiology of protein kinase C in uterine smooth muscle, the relative abundance and subcellular distribution of the various isozymes was analyzed. Differences in titer and hybridization efficiencies of respective isozyme-specific antibodies make precise quantitation of relative levels of individual isozymes technically difficult. However, by employing primary antibody concentrations which exhibited similar immunoreactivity and simultaneous exposure of parallel samples of paired subcellular fractions to secondary antibody and developing reagents, a crude determination of tissue protein kinase C isozyme relative abundance was made. Under such conditions, the predominant protein kinase C species were the δ and β2 isozymes, constituting roughly 45% and 18%, respectively, of uterine smooth muscle protein kinase C. In contrast, the major protein kinase C isozymes in rat ventricular muscle were β2 (37% of total) and ζ (22% of total). It was not entirely surprising that the θ isozyme was not detected in either tissue as this isoform has only been reported in hematopoietic cells (Baier et al., 1993).

That these particular protein kinase species were most abundant in uterine smooth muscle may provide a window on the major intracellular targets of protein kinase C. Protein kinase C-β2 is known to associate with actin microfilaments (Tanaka et al., 1991; Goodnight et al., 1995) suggesting that this isozyme may be important in the regulation of uterine contractility. In addition, protein kinase C-β activates the arachidonic acid cascade (Duyster et al., 1993) leading to the production of prostaglandins, which are potent stimulators of uterine contractions. In another steroid responsive tissue, the ovary, protein kinase C-δ is associated with the activation of the mitogen-activated protein kinase cascade (Yamaguchi et al., 1995). As the uterus is known to undergo significant hypertrophy during pregnancy, this isozyme may be important in mediating myometrial protein synthesis associated with cellular hypertrophy. Previously, Rybin and Steinberg (1994) reported developmental changes (fetal and neonatal versus adult) in protein kinase C isozymes in the rat ventricular myocardium.

Pharmacologic hormone replacement regimens in ovariectomized animals were used to provide a parallel to endocrine environments similar to those found during pregnancy (progesterone-dominating) and parturition (estrogen-dominating). Using these regimens, steroid-mediated uterine hypertrophy was observed as previously described (Ruzycky and Crankshaw, 1988). In both conditions, total uterine protein kinase C isozyme expression did not change significantly. However, most protein kinase C isozymes studied were preferentially redistributed to the myometrial membrane compartment. This effect of the steroid hormones was tissue-specific as similar effects in cardiac ventricular muscle were not observed.

The increase in the membrane localization of protein kinase C isozymes in the absence of any direct uterine stimulants may be indicative of an estrogen-mediated remodeling of the myometrium with increased basal protein kinase C activity. In the pituitary (Drouva et al., 1990) and mammary glands (Holladay and Bollander, 1986), chronic estrogen treatment has been shown to increase protein kinase C activity. As mentioned earlier, translocation of protein kinase C to the membrane compartment is associated with increased protein kinase C enzymatic activity. These findings are consistent with previous studies of agonist-mediated inositol phospholipid metabolism and contractility suggesting increased activation of protein kinase C (Ruzycky et al., 1987).

The association of myometrial hypertrophy with an increased estrogen-dependent activation of the mitogen-activated protein kinase cascade (Ruzycky, 1996) may explain the large increase in membrane-associated protein kinase C-ζ levels with estrogen treatment. Protein kinase C-δ, -ε and -ζ appear to be involved in hypertrophic responses, differing in their activation by mitogenic stimuli. Protein kinase C-δ is selectively activated by epidermal growth factor in fibroblasts (Ohno et al., 1994) whereas protein kinase C-ε is activated by the membrane lipid, phosphatidylinositol 3,4,5-trisphosphate (Nakanishi et al., 1993). However, protein kinase C-ζ interacts with the mitogenic switch protein, ras (Diaz-Meco et al., 1994). Under identical estrogen conditions, myometrial ras expression and mitogen-activated protein kinase activity are elevated (Ruzycky, 1996) suggesting that the uterine hypertrophic response signals through protein kinase C-ζ. The relative importance of protein kinase C-δ and -ε in the myometrial hypertrophic response are currently unknown and should not be discounted. In rat ovary cells, protein kinase C-δ expression is the predominant protein kinase C isozyme increased by estrogen (Maizels et al., 1992).

In conclusion, the present studies have established that gonadal steroids increase the expression of protein kinase C isozymes in rat uterine smooth muscle in a tissue-specific manner. The increase in enzyme expression results in a specific increase in membrane partitioning of protein kinase C isozymes which may indicate increased basal isozyme enzyme activity in these steroid-dominating environments. Membrane elevations in these isozymes is consistent with previous findings of increased hypertrophic signaling responses and increased uterine contractility.

Acknowledgements

We thank Dr Denise Cooper for providing brain protein kinase C samples. This work was supported by a grant from the Magee Womens Research Foundation.

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