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The Role of Phospholipase $\text{C}\gamma 1$ Tyrosine Phosphorylation During Phasic Myometrial Contractions

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Abstract

Objective: Phospholipase $\text{C}\gamma 1$ ($\text{PLC}\gamma 1$) is expressed in myometrium, and is activated by tyrosine phosphorylation. These studies sought to determine the association between $\text{PLC}\gamma 1$ tyrosine phosphorylation and spontaneous uterine contractions.

Study Design: *In vitro* contraction studies were performed with spontaneously contracting rat uterine strips along with strips treated with potassium bisperoxo(1,10 phenanthroline)oxovanadate (bpV(phen), a protein tyrosine phosphatase inhibitor). Additional studies were performed using phenylarsine oxide (PAO, a $\text{PLC}\gamma$ inhibitor) and other inhibitors. Western blots were performed to determine the phosphotyrosine $\text{PLC}\gamma 1$ levels.

Results: Spontaneous contractile activity and tyrosine phosphorylation of $\text{PLC}\gamma 1$ (but not $\text{PLC}\gamma 2$) were significantly increased in response to bpV(phen); in contrast, oxytocin and thrombin produced comparable contractile activity, but did not alter phosphotyrosine- $\text{PLC}\gamma 1$. PAO and neomycin significantly decrease bpV(phen)-stimulated contractions and $\text{PLC}\gamma 1$ tyrosine phosphorylation; other inhibitors only suppressed contractions.

Conclusions: These studies support the hypothesis that spontaneous myometrial contractions are associated with tyrosine phosphorylation of $\text{PLC}\gamma 1$; both of which are further enhanced by inhibition of protein tyrosine phosphatase activity.

Keywords

Phospholipase $\text{C}\gamma 1$ ($\text{PLC}\gamma 1$); tyrosine phosphorylation; rat uterus; myometrial contractions

Introduction:

Myometrial contractile activity is mediated in part by stimulation of the phosphatidylinositol signaling pathway through activation of phospholipase C (PLC) enzyme activity¹. Several PLC isoforms have been demonstrated in myometrial tissue. The $\text{PLC}\beta$ isoforms are activated through G-protein coupled receptor stimulation by uterotonic agonist such as oxytocin and prostaglandin $\text{F}_{2\alpha}$; in contrast, the activation mechanisms for the $\text{PLC}\delta$ isoforms remain poorly defined^{1,2}. The $\text{PLC}\gamma$ isoforms are the only isoforms that contain

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Src-homology 2 (SH2) domains, thereby allowing activation by receptor and non-receptor protein tyrosine kinases^{1,2}. Western blot studies by our laboratory in 1992 were the first to demonstrate the expression of the PLC γ isoform in pregnant rat myometrial tissue³. A subsequent study using RT-PCR has allowed us to confirm PLC γ 1 and PLC γ 2 mRNA expression in pregnant rat myometrium⁴. More recently, we have used immunohistochemical techniques to confirm the expression of these two PLC isoforms in smooth muscle cells of the pregnant rat uterus⁵. Our studies using rat uterine tissue are consistent with those reported by Phaneuf et al.⁶, who utilized Western blots to confirm the expression of PLC γ 1 and PLC γ 2 protein in human myometrial cells.

The goal of the present study was to test the hypothesis that constitutive and enhanced tyrosine phosphorylation of PLC γ 1 and/or PLC γ 2 is causally related to presence and intensity of spontaneous uterine contractions. Specifically, these studies sought to utilize various inhibitors of the phosphatidylinositol signaling pathway to determine the relationship between PLC γ 1 tyrosine phosphorylation and spontaneous uterine contractions.

Materials & Methods:

Uterine tissue for these studies was obtained from proestrus/estrus Sprague-Dawley rats under a protocol approved by the Animal Care and Utilization Committee at the University of Vermont. The *in vitro* isometric contraction studies were performed at 37° C using longitudinal segments of uterine tissue (6-8 mm relaxed length) in 3 mL muscle baths containing Earle's balanced salt solution (EBSS) as previously reported in our laboratory⁷⁻⁹. Dose response studies were performed using 1-40 μ M potassium bisperoxo(1,10 phenanthroline)oxovanadate (bpV(phen)) with and without PI-signaling pathway inhibitors: 83 μ M phenylarsine oxide (PAO, a selective PLC γ inhibitor in vehicle: dimethylsulfoxide (DMSO)), 2.5 mM Neomycin (a PLC inhibitor in vehicle: deionized water), 80 μ M 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC, another PLC inhibitor in vehicle: DMSO), 60 μ M 2-aminoethoxydiphenyl borate (2-APB, a calcium entry and IP3-receptor inhibitor in vehicle: DMSO), and 50 μ M ruthenium red (RRED, a ryanodine receptor inhibitor in vehicle: deionized water). Control studies were performed using comparable volumes of vehicle alone in place of the PI-signaling pathway inhibitors. As previously reported from our laboratory⁷⁻⁹, the analog contraction data were computer digitalized, analyzed to determine total contractile area in selected 5-minute intervals, normalized for tissue cross-sectional area, and reported as percent of spontaneous contractile activity (percent control).

For the Western blots, the uterine strips were homogenized in a protease/phosphatase inhibitor 50 mM Tris buffered saline solution (containing 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 5 μ g/mL aprotinin, 3 μ g/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). After centrifugation at 800 x g to remove the cellular debris, the protein concentrations of the crude homogenates were determined by BCA protein assay. Subsequently, the proteins were resolved on 10% SDS-PAGE gels using the Biorad Mini-PROTEAN 3 Electrophoresis system (BioRad Laboratories, Hercules, CA). The resolved proteins were then electrophoretically transferred to nitrocellulose membranes utilizing the Biorad wet transfer system. The nitrocellulose membranes were blocked with 5% powdered milk, then incubated overnight in the primary antibody solution containing anti-phosphotyrosine PLC γ 1 or anti-phosphotyrosine PLC γ 2 polyclonal antibodies (Cell Signaling Technology, Danvers, MA), or with anti-PLC γ 1 monoclonal or PLC γ 2 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for total PLC γ 1 or PLC γ 2 protein. Immunodetection of the primary antibodies was performed using horseradish peroxidase-conjugated anti-mouse or rabbit IgG antiserum (Bio-Rad Laboratories). Chemiluminescence of the target bands was produced by incubating

the nitrocellulose membranes briefly in Supersignal HRP Substrate (Calbiochem/EMB Biosciences, San Diego, CA); then the nitrocellulose membranes were exposed to Hyperfilm photographic film (Amersham Biosciences, Piscataway, NJ). The relative density of the detected protein bands visible on the Hyperfilm was determined using the Kodak 1-D Digital Science Electrophoresis Documentation and Analysis System (Eastman Kodak, Rochester, NY).

Because much of the data was not normally distributed, statistical analyses were performed using the nonparametric Kruskal-Wallis ANOVA on ranks with multiple comparisons using the Student-Newman-Keuls test, or performed using the Mann-Whitney Rank Sum test where appropriate. Statistical significance occurred when $p < 0.05$.

Results:

In vitro contraction studies with bpV(phen):

Inhibition of protein tyrosine phosphatase activity using bpV(phen) at concentrations of 1-40 μM produced a marked increase in the contractile activity of nonpregnant rat uterine strips (Fig 1A). Compared to vehicle alone, bpV(phen) produced a significant increase in overall contractile activity over this concentration range ($N = 8$, $P < 0.05$), as observed in Figure 1B. The effects of treatment of uterine strips with various PI-signaling pathway inhibitors on bpV(phen) enhanced contractile activity are shown in Figures 2 and 3, and in Table I. PAO (a specific PLC- $\gamma 1$ inhibitor) at a dose of 83 μM resulted in significant inhibition of spontaneous and bpV(phen) enhanced contractile activity compared to vehicle treated control uterine strips ($p = 0.006$) (Figure 2A). Treatment with neomycin (2.5 mM) (a non-isoform selective inhibitor of PLC activity) also significantly suppressed spontaneous and bpV(phen) enhanced contractions compared to vehicle treated control strips ($p = 0.005$), as observed in Figure 2B. NCDC (another non-isoform specific PLC inhibitor) significantly suppressed spontaneous and bpV(phen) enhanced contractions, although the later was not statistically significant (Figure 3A). 2-APB (an inhibitor of calcium entry and the IP3 receptor) markedly suppressed spontaneous and bpV(phen) enhanced uterine contractions (Figure 3B and Table I). Ruthenium red (a ryanodine receptor inhibitor) suppressed spontaneous contractions (Figure 3C); however, it did not suppress the bpV(phen) enhanced increase in contractile activity compared to vehicle control treated uterine strips.

Western blots and PLC- $\gamma 1$ phosphorylation:

BpV(phen) induced a concentration and time-related increase in tyrosine phosphorylation of PLC- $\gamma 1$ in rat uterine tissue (Figure 4A). Of note, comparable concentrations of bpV(phen) did not stimulate tyrosine phosphorylation of the PLC- $\gamma 2$ isoform; whereas a 2-fold higher concentration of bpV(phen) (i.e. 40 μM) for 15 minutes produced a phosphotyrosine PLC- $\gamma 2$ band (Figure 4B). In contrast to the response to bpV(phen), classic uterotonic agonists (e.g. oxytocin (2-32 nM) and thrombin (1-20 U/mL)) at concentrations producing a robust increase in contractile activity were not associated with an apparent change in phosphotyrosine PLC- $\gamma 1$ levels (Figure 5).

Tyrosine phosphorylation of PLC- $\gamma 1$ was determined after pretreatment of the uterine strips with the previously described signaling pathway inhibitors followed by bpV(phen) treatment (see Table II). As observed in Figure 6, PAO and neomycin significantly suppressed phosphotyrosine PLC- $\gamma 1$ levels, consistent with the ability of these PLC inhibitors to significantly inhibit bpV(phen) enhanced contractile activity. Interestingly, NCDC had no significant effect on phosphotyrosine PLC- $\gamma 1$ levels despite its trend toward suppression of bpV(phen) enhanced contractile activity; these data suggest that the effects of NCDC on PLC signaling are not mediated by modulation of tyrosine phosphorylation of PLC- $\gamma 1$.

Ruthenium red and 2-APB did not suppress the tyrosine phosphorylation of PLC- γ 1 (Figure 6), consistent with the fact that the intracellular effects of these inhibitor are mediated by mechanisms unrelated to the PLC proteins.

Comments:

Based on the significant inhibitory effect of PAO on the contractile activity of rat uterine strips, spontaneous myometrial contractions appear to be associated with constitutive tyrosine phosphorylation of PLC γ 1. This hypothesis is further supported by the observation that PLC γ 1 tyrosine phosphorylation and contractile activity are significantly enhanced in response to bpV(phen), a protein tyrosine phosphatase inhibitor. Suppression of PLC γ 1 tyrosine phosphorylation using neomycin also resulted in a marked decrease in spontaneous and bpV(phen) enhanced uterine contractions. In contrast, inhibitors of the PI-signaling pathway that have no direct effect on PLC γ 1 were not associated with a significant reduction in PLC γ 1 tyrosine phosphorylation. Therefore, these data support the hypothesis that tyrosine phosphorylation of PLC γ 1 has a strong (possibly causative) relationship with spontaneous myometrial contractions in the proestrus/estrus rat uterus.

Our laboratory has previously reported that PLC γ 1 and PLC γ 2 are expressed at the mRNA and protein levels in pregnant rat myometrium, along with immunohistochemical studies confirming the expression on of these two PLC isoforms in smooth muscle layer of the pregnant rat uterus ^{4, 5}. Our studies are consistent with those reported by Phaneuf et al. ⁶, who demonstrated the expression of PLC γ 1 and PLC γ 2 in cultured human myometrial cells. Although these previous reports have confirmed the myometrial expression of both PLC γ isoforms, based on the studies described in the current report, the PLC γ 1 isoform appears to be the one most functionally related to spontaneous and bpV(phen) enhanced contractile activity.

PLC γ activation occurs in response to phosphorylation on specific tyrosine residues (especially tyrosine #783). These events are mediated by several different protein tyrosine kinases (PTKs) ¹⁰; some of which are membrane receptor tyrosine kinases and others are nonreceptor protein tyrosine kinases ¹¹. Growth factor receptors with intrinsic tyrosine kinase activity that have been reported to stimulate the PI-signaling pathway include the receptors for platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), nerve growth factor (NGF), and epidermal growth factor (EGF) ¹². In 1987, Gardner et al. ¹³ first reported that epidermal growth factor (EGF) was able to stimulate phasic contractions of rat myometrial tissue. Subsequently, Anwer et al. ¹⁴ described EGF stimulation of IP3 production and calcium mobilization in cultured human myometrial cells; and Carrasco et al. ¹⁵ demonstrated EGF stimulated increased in PLC γ tyrosine phosphorylation along with activation of the PI signaling pathway. Using rat myometrial cells, Boulven et al. ¹⁶ have demonstrated that platelet-derived growth factor (PDGF) stimulates the tyrosine phosphorylation of PLC γ 1.

Modulation of PLC γ tyrosine phosphorylation has also been demonstrated in response to non-receptor protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Using vascular smooth muscle cells, Schmitz et al. ¹⁷ have reported that angiotensin II stimulates tyrosine phosphorylation of PLC γ through the activation of c-Src and an unidentified 97 kD phosphoprotein. Src tyrosine kinase has also been demonstrated to generate phosphotyrosine PLC γ 1 in rat myometrial cells; an effect that is prevented by pretreatment with the protein tyrosine kinase inhibitors genistein and PP1 ¹⁸. Palmier et al. ¹⁹ in 1996 described the ability of pervanadate, a PTP inhibitor, to significantly increase PLC γ tyrosine phosphorylation, activation of the PI-signaling pathway, and the generation of phasic contractions in rat myometrial tissue. Phaneuf et al. ⁶ also demonstrated pervanadate stimulated PLC γ tyrosine

phosphorylation and PI-signaling in isolated human myometrial cells. A report from our laboratory in 1999 confirmed the ability of pervanadate to generation of phasic uterine contractions which are driven by cytosolic calcium oscillations ⁷.

The studies described in the current report have provided evidence supporting the hypothesis that spontaneous phasic myometrial contractions are associated with constitutive tyrosine phosphorylation of PLC γ 1. Inhibition of protein tyrosine phosphatase activity using bpV(phen) resulted in a significant increase in measurable phosphotyrosine-PLC γ 1 along with increased contractile activity. In contrast, inhibition of PLC γ 1 signaling using PAO was associated with significant suppression of measurable phosphotyrosine-PLC γ 1 and contractile activity. Other uterotonic agonists (e.g. oxytocin and thrombin) which are not associated with PLC γ 1 activation produced no consistent effect on phosphotyrosine-PLC γ 1 levels. Inhibitors of the phosphatidylinositol signaling pathway not associated PLC γ 1 mediated effects produced marked inhibition of contractile activity; however, these agents failed to suppress phosphotyrosine-PLC γ 1 levels. In summary, these studies have provided evidence supporting an important role for PLC γ 1 during spontaneous phasic contractions of the rat uterus.

Condensation

The studies described in this report support the hypothesis that the tyrosine phosphorylation of PLC γ 1 plays a role during spontaneous contractions of the rat uterus.

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Figure 1.

(A) *In vitro* contraction study demonstrating the effect of bpV(phen) on spontaneous contractions of the proestrus/estrus rat uterine strips. Cumulative additions of bpV(phen) into the muscle bath for the time periods indicated by the horizontal bars. Contractile activity is reported in grams of tension generated. (B) Bar graph demonstrating the effects of bpV(phen) (open bars) vs. vehicle (deionized H₂O) treated strips (hatched bars). Each bar = mean $\pm$ S.D. N = 8 experiments. (*) p<0.05 for comparison to vehicle treated strips. SP = spontaneous contractile activity preceding the 20 minute time point.

Figure 2.

(A) *In vitro* contraction study demonstrating the effects of phenylarsine oxide (PAO) or vehicle (DMSO) on spontaneous and bpV(phen)-enhanced contractions of the proestrus/estrus rat uterine strips. Treatment periods indicated by the horizontal bars. Contractile activity is reported in grams of tension generated. (B) Contraction study demonstrating the

effects of Neomycin or vehicle (deionized H₂O) on spontaneous and bpV(phen)-enhanced contractions. Treatment periods indicated by the horizontal bars.

Figure 3.

(A) *In vitro* contraction study demonstrating the effects of NCDC or vehicle (DMSO) on spontaneous and bpV(phen)-enhanced contractions. Treatment periods indicated by the horizontal bars. Contractile activity is reported in grams of tension generated. (B) Contraction study demonstrating the effects of 2-aminoethoxydiphenyl borate (2-APB) or vehicle (DMSO) on spontaneous and bpV(phen)-enhanced contractions. Treatment periods as noted. (C) Contraction study demonstrating the effects of ruthenium red (RRED) or vehicle (deionized H₂O) on spontaneous and bpV(phen)-enhanced contractions. Treatment periods as noted.

Figure 4.

(A) Western blots demonstrating phosphotyrosine PLCγ₁ (upper panels) compared to total PLCγ₁ (lower panels) in response to increasing concentrations and exposure time to bpV(phen). (B) Western blots demonstrating phosphotyrosine PLCγ₂ (upper panels) compared to total PLCγ₂ (lower panels) in response to increasing concentrations and exposure time to bpV(phen).

Figure 5. Western blots demonstrating phosphotyrosine PLC γ 1 (upper panels) compared to total PLC γ 1 (lower panels) in response to increasing concentrations of thrombin (A) and oxytocin (B).

Figure 6.

Western blots demonstrating phosphotyrosine PLCγ1 (upper panels) compared to total PLCγ1 (lower panels) in response to various inhibitors followed by the addition of bpV(phen). DMSO = dimethylsulfoxide, NCDC = 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate, dH₂O = deionized water, RRED = ruthenium red, 2-APB = 2-aminoethoxydiphenyl borate, PAO = phenylarsine oxide, and +bpv = addition of bpV(phen).

Table 1

Contractile Response to bpV(phen)

Treatment	Percent Control Activity* Median	25%ile	75%ile	p	(N)
DMSO + bpV	232.9	195.1	310.2	0.006	(10)
PAO + bpV	105.9	86.4	113.0		
dH ₂ O + bpV	343.3	277.8	393.9	0.005	(11-12)
Neo + bpV	184.4	158.6	221.4		
DMSO + bpV	204.9	191.2	297.0	0.06	(5)
NCDC + bpV	163.1	118.6	172.2		
DMSO + bpV	319.2	268.5	345.6	0.02	(6-7)
2-ABP + bpV	162.6	143.2	269.0		
dH ₂ O + bpV	275.8	188.7	419.5	0.49	(9)
RRED + bpV	231.2	171.9	288.4		

Notes: DMSO = dimethylsulfoxide (vehicle), bpV = bpV(phen), dH₂O = deionized water (vehicle), Neo = neomycin, RRED = ruthenium red.

* Control activity (= 100%) based on the area under the contraction curve during a 5-minute period preceding the addition of bpV(phen).

Table 2Phosphotyrosine PLC γ 1 Band Density

Treatment	Band Density* Median	25%ile	75%ile	p	(N)
DMSO + bpV	1.52	1.36	1.61	< 0.001	(10)
PAO + bpV	1.05	0.92	1.11		
dH ₂ O + bpV	1.49	1.16	1.75	0.004	(10)
Neo + bpV	0.84	0.60	0.95		
DMSO + bpV	1.25	1.03	1.48	0.96	(8)
NCDC + bpV	1.26	0.99	1.52		
DMSO + bpV	1.44	1.07	1.59	0.31	(10-11)
2-ABP + bpV	1.50	1.22	1.63		
dH ₂ O + bpV	1.28	1.21	1.39	0.67	(8-9)
RRED + bpV	1.03	0.92	1.46		

Notes: DMSO = dimethylsulfoxide (vehicle), bpV = bpV(phen), dH₂O = deionized water (vehicle), Neo = neomycin, RRED = ruthenium red.

* Band density = phosphotyrosine-PLC γ 1 band density / total PLC γ 1 band density