Stimulation of G-protein coupled receptors in vascular smooth muscle cells induces tyrosine kinase dependent increases in calcium without tyrosine phosphorylation of phospholipase C γ-1

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Abstract It is often believed that increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) resulting from stimulation of G-protein coupled receptors in vascular smooth muscle cells (VSMC) require activation of the β1 isoform of phospholipase C (PLC). However, recent studies showed that rat aortic VSMC do not express PLC β-1 and that stimulation with angiotensin-II induces tyrosine kinase dependent increases in $[Ca^{2+}]_i$ and tyrosine phosphorylation of PLC γ-1. Whether this pathway is activated by other vasoactive agents that stimulate G-protein coupled receptors is unknown. Here, we show that A10 VSMC express PLC β-2, PLC β-3, PLC δ-1, and PLC γ-1. The cells also expressed $G\alpha_{q/11}$. However, neither PLC β-1 nor PLC β-4 was detected. Stimulation with angiotensin-II, vasopressin, serotonin, or endothelin induced tyrosine kinase dependent increases in $[Ca^{2+}]_i$. However, tyrosine phosphorylation of PLC γ-1 did not occur. In contrast, stimulation with platelet derived growth factor increased $[Ca^{2+}]_i$ and tyrosine phosphorylation of PLC γ-1. The results show that tyrosine phosphorylation of PLC γ-1 is not required for tyrosine kinase dependent increases in $[Ca^{2+}]_i$ resulting from stimulation of diverse G-protein coupled receptors in VSMC.

Key words: Angiotensin-II; Cellular Ca^{2+}; Endothelin; G-protein coupled receptor; Fura-2; Genistein; Phospholipase C; Platelet derived growth factor; Protein tyrosine phosphorylation; Serotonin; Vascular smooth muscle cell; Vasopressin

1. Introduction

Phospholipase C (PLC) has been assigned a key role in mediating increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) that occur in vascular smooth muscle cells (VSMC) when G-protein coupled receptors are stimulated with vasoactive agents [1–4]. Activated PLC generates two second messengers: one, inositol 1,4,5-trisphosphate, induces release of Ca^{2+} from the sarcoplasmic reticulum, while the other, diacylglycerol, activates protein kinase C.

However, because multiple isoforms of PLC exist, uncertainties arise regarding the identity of the relevant isoform in VSMC and how it is activated. At least 10 isoforms of PLC belonging to three families designated β, γ, and δ have been identified in mammalian cells [4–6]. Activation of PLC β isoforms is linked to stimulation of G-protein coupled receptors, whereas activation of PLC γ isoforms is largely mediated by its direct tyrosine phosphorylation. Mechanisms for activation of δ isoforms are unknown. The currently prevalent view for VSMC is that stimulation induces activation of a trimeric G-protein containing an α subunit of the *q* type which, in turn, activates the β-1 isoform of PLC. Recently, this view has been questioned because PLC β-1 was not detected in several VSMC preparations [7–9]. Moreover, growing evidence suggests that protein tyrosine kinase activity may modulate increases in $[Ca^{2+}]_i$ evoked by stimulation of diverse G-protein coupled receptors [7,10–13]. In this context, stimulation of rat VSMC with angiotensin II induced tyrosine phosphorylation of PLC γ-1, generation of inositol 1,4,5-trisphosphate, and increases in $[Ca^{2+}]_i$ [7]. However, whether or not angiotensin-II induces tyrosine phosphorylation of PLC γ-1 in different VSMC that lack PLC β-1 is unknown. Similarly, whether or not tyrosine phosphorylation of PLC γ-1 is a common mechanism for linking stimulation of different G-protein coupled receptors to $[Ca^{2+}]_i$ also is unknown.

Our earlier studies showed that tyrosine kinase dependent increases in $[Ca^{2+}]_i$ were evoked in A10 and other VSMC stimulated with diverse vasoactive agents [10–15]. However, PLC expression was not determined. In this study, we show that although A10 cells express several isoforms of PLC, PLC β-1 was not detected. Expectedly [7–10], angiotensin-II evoked increases in $[Ca^{2+}]_i$ were tyrosine kinase dependent. Surprisingly, no tyrosine phosphorylation of PLC γ-1 occurred. Moreover, no tyrosine phosphorylation of PLC γ-1 occurred when increases in $[Ca^{2+}]_i$ were induced with three other vasoactive agents that stimulate different G-protein coupled receptors. These new data show that tyrosine phosphorylation of PLC γ-1 is not obligatory for tyrosine kinase dependent increases in $[Ca^{2+}]_i$ that occur in response to stimulation of G-protein coupled receptors in A10 VSMC.

2. Materials and methods

2.1. Cell culture and fura-2 fluorescence analysis

Methods for culturing A10 aortic smooth muscle cells (ATCC) and for radiometric fluorescence analysis of changes in $[Ca^{2+}]_i$ in fura-2 loaded cells were described previously [12,14,15]. The cells were excited at 340 and 380 nm and emission was monitored at 510 nm. Changes in the 340/380 fluorescence ratio were used as a measure of changes in $[Ca^{2+}]_i$.

The concentrations of agonists tested were: 50 nM angiotensin-II (Peninsula Labs), 20 nM [arg^8]-vasopressin (Sigma), 1 μM serotonin (Calbiochem), and 400 nM endothelin (Peninsula Labs.). Responses evoked by 1 nM platelet derived growth factor (PDGF; AB isomer, UBI) were also examined as a representative agent that acts through a tyrosine kinase receptor rather than a G-protein coupled receptor [16]. To assess the requirement for tyrosine kinase activity, experiments were performed before and after preincubation with 105 μM genistein (Calbiochem). Earlier studies showed that this concentration of genistein virtually abolished increases in tyrosine phosphorylation and $[Ca^{2+}]_i$ that were evoked by diverse vasoactive agents in different VSMC without altering the activity of several serine/threonine kin-

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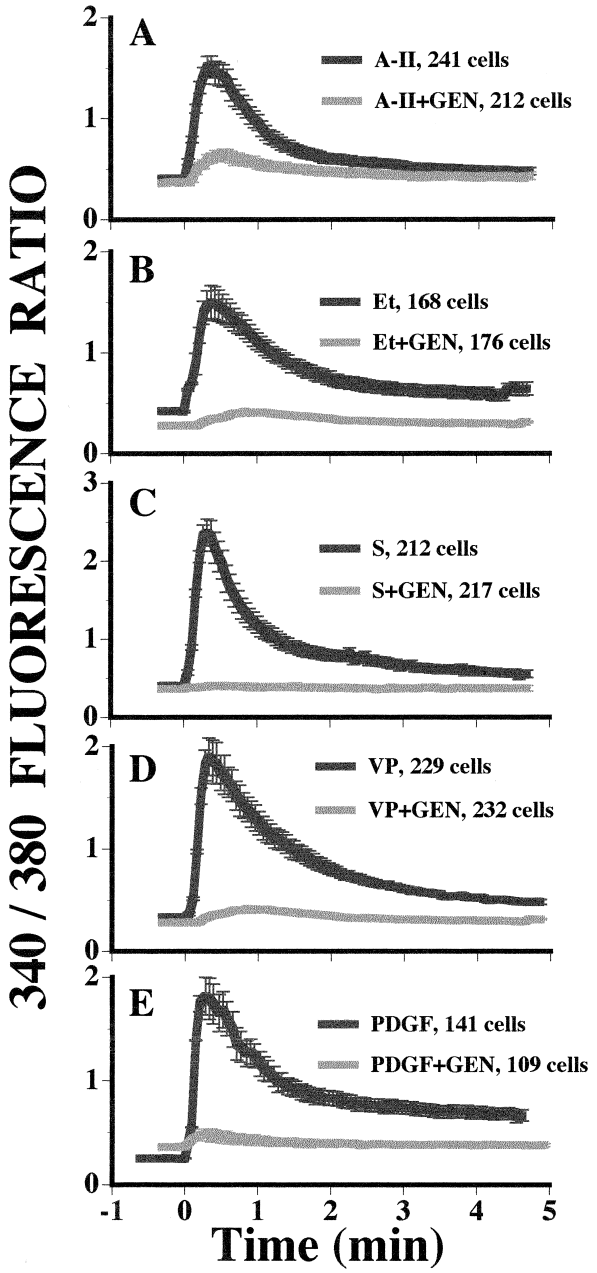

Fig. 1. Genistein (105 μM/45 min) markedly inhibits transient and sustained increases in $[Ca^{2+}]_i$ (340/380 fluorescence ratio) evoked by stimulation of A10 VSMC with either 50 nM angiotensin-II (A-II, panel A), 400 nM endothelin (Et, panel B), 1 μM serotonin (S, panel C), 20 nM vasopressin (VP, panel D) or 1 nM PDGF (panel E). Each curve shows the mean response for the number of cells listed under each condition. Small vertical bars are ±1 S.E.M.

ases, including myosin light chain kinase and cAMP dependent protein kinase [10–15].

2.2. Immunoblotting: expression of PLC isoforms

Extracts were prepared from cells grown to 80–90% confluency in 75 cm^2 T-flasks, serum deprived for 48 h, and lysed in buffer containing 1% Triton X, 25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM Na vanadate, 10 mM Na pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. The lysate was centrifuged, and the supernatant fraction was assayed for protein (Pierce's BCA protein reagent). Immunoblotting was performed by subjecting 10 μg of solubilized protein to SDS-PAGE and electrophoretic transfer of proteins to nitrocellulose membranes [17]. Antibodies for PLC β-1, PLC β-2, PLC β-3, PLC β-4, PLC δ-1, PLC γ-1, and $G\alpha_{q/11}$ were from Santa Cruz, Transduction Labs, and UBI. Blotted proteins were visualized using enhanced chemiluminescence (Amersham).

2.3. Immunoprecipitation of PLC γ-1: determination of protein tyrosine phosphorylation

Each flask in a series of cultures was challenged with the desired vasoactive agent and processed for extraction of protein as described earlier. Then, 100 μg protein from each sample was incubated for 30 min with protein A coupled Sepharose beads to adsorb proteins that non-specifically bind to the beads. Following centrifugation, antibody for PLC γ-1 was added to the supernatant. The mixture was incubated overnight at 4°C. After addition of fresh protein A coupled Sepharose beads, immunoprecipitates were sedimented, subjected to SDS electrophoresis and transferred to nitrocellulose membranes. The membranes were first blotted for tyrosine phosphorylated proteins (UBI), stripped of phosphotyrosine antibody, and then blotted for PLC γ-1.

3. Results

3.1. Increases in $[Ca^{2+}]_i$ evoked before and after incubation with genistein

Marked increases in $[Ca^{2+}]_i$ occurred when the cells were stimulated with either angiotensin-II, endothelin, serotonin, or vasopressin (Fig. 1A–D). The increases in $[Ca^{2+}]_i$ were characterized by an early rapid transient increase in the 340/380

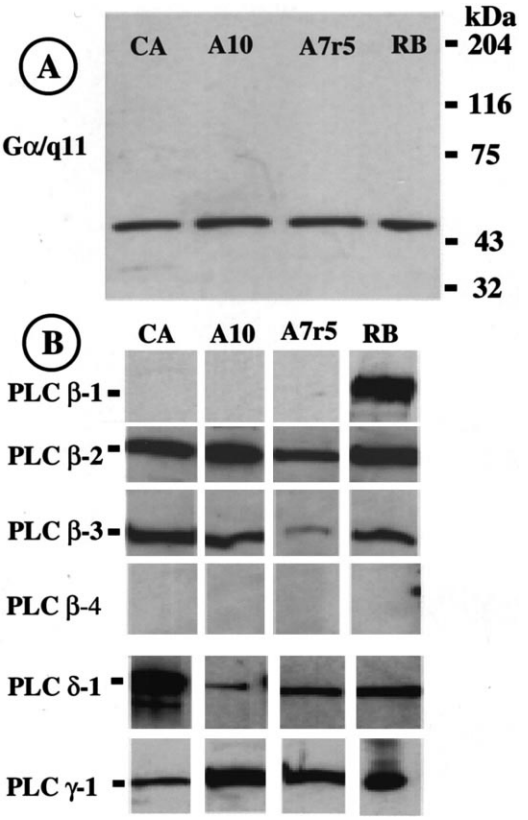

Fig. 2. Expression of $G\alpha_{q/11}$ (panel A) and different isoforms of PLC (panel B). Extracts from canine carotid artery (CA), A10 and A7r5 VSMC, and rat brain (RB) were processed and immunoblotted. The entire immunoblotted gel is shown in panel A, whereas each row in panel B shows lanes from a different gel that was immunoblotted for the specific isoform of PLC indicated. The apparent M_r for each protein was: $G\alpha_{q/11}$, 47000; PLC β-1, 150000; PLC β-2, 90000–100000; PLC β-3, 140000; PLC δ-1, 88000; PLC γ-1, 142000.

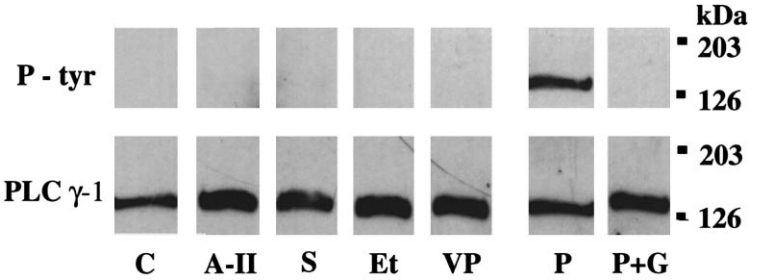

Fig. 3. Tyrosine phosphorylation of PLC γ-1 is not apparent in control resting A10 VSMC (C) or in cells stimulated for 30 s with angiotensin-II (A-II), endothelin (Et), serotonin (S), or vasopressin (VP). In contrast, tyrosine phosphorylation is evident in response to PDGF (P). PDGF induced phosphorylation was blocked by genistein (P+G). PLC γ-1 was immunoprecipitated from cell extracts and assessed for tyrosine phosphorylation. The top panel (P-tyr) shows the immunoblot for tyrosine phosphorylated protein. The blot was stripped and reblotted for PLC γ-1 as shown in the lower panel. The same results were obtained in three different experiments. No evidence for tyrosine phosphorylation of PLC γ-1 was obtained in cells stimulated with vasopressin for 15–480 s (not shown).

fluorescence ratio that was followed by a slow decline to a lower sustained value. Preincubation with 105 μM genistein, to inhibit tyrosine kinase activity [7,10–15,18], markedly suppressed the early transient increases in $[Ca^{2+}]_i$ and virtually abolished the later and smaller sustained increases in $[Ca^{2+}]_i$. Cells stimulated with PDGF, to activate a receptor that exhibits ligand dependent tyrosine kinase activity [7–16], also induced an increase in $[Ca^{2+}]_i$ that was blocked with genistein (Fig. 1E).

3.2. PLC isoforms in VSMC and intact vascular smooth muscle

Proteins corresponding to PLC β-2, PLC β-3, PLC δ-1, PLC γ-1, and $G\alpha_{q/11}$ were demonstrable in extracts from A10 VSMC, A7r5 VSMC and from canine carotid artery (Fig. 2). In contrast, neither PLC β-1 nor PLC β-4 could be detected in any of these preparations.

3.3. Tyrosine phosphorylation of PLC γ-1

No tyrosine phosphorylation of PLC γ-1 was apparent in control cells or in response to either angiotensin-II, endothelin, serotonin, or vasopressin (Fig. 3). In contrast, genistein sensitive tyrosine phosphorylation of PLC γ-1 occurred when the cells were treated with PDGF.

4. Discussion

The apparent lack of PLC β-1 in intact carotid arterial smooth muscle and either A10 or A7r5 clonal VSMC (Fig. 2) is consistent with reports from several laboratories showing that the enzyme is not expressed in VSMC from rat [7] or rabbit aorta [8] or in extracts from rat mesenteric artery and aorta [9]. This suggests that we need to re-examine our views of mechanisms that link activation of G-protein coupled receptors and increases in $[Ca^{2+}]_i$. In this context, Marrero et al. [7] showed that tyrosine phosphorylation of PLC γ-1 was functionally linked to increases in $[Ca^{2+}]_i$ induced by angiotensin-II in primary VSMC from adult rats.

In contrast, this study shows that tyrosine phosphorylation of PLC γ-1 (Fig. 3) does not occur during tyrosine kinase dependent increases in $[Ca^{2+}]_i$ evoked by stimulation of A10 VSMC with angiotensin-II. The absence of such phosphorylation is not reflective of procedural limitations because tyrosine phosphorylation of PLC γ-1 was easily demonstrated during stimulation of the cells with PDGF (Fig. 3). These results suggest that angiotensin-II may increase $[Ca^{2+}]_i$ by activating different tyrosine kinase dependent signaling pathways in different types of VSMC.

Stimulation of G-protein coupled receptors for endothelin, serotonin, or vasopressin also evoked tyrosine kinase dependent increases in $[Ca^{2+}]_i$ without inducing tyrosine phosphorylation of PLC γ-1 (Figs. 1 and 3). Nevertheless, the possibility that tyrosine kinase dependent activation of PLC γ-1 can occur in the absence of its direct phosphorylation cannot be discounted. Recent studies in Exton's laboratory show that unphosphorylated PLC γ-1 can be activated by forming a complex with tyrosine phosphorylated PDGF receptor [19]. Conceivably, tyrosine phosphorylation of an unidentified upstream regulatory protein may promote activation of unphosphorylated PLC γ-1 in some types of VSMC. Further studies are required to accept or reject this hypothesis.

Alternatively, it is also possible that a different isoform of PLC participates in tyrosine kinase dependent increases in $[Ca^{2+}]_i$. Potential participants include the isoforms we identified (i.e. PLC β-2, β-3, and δ-1; Fig. 2) and perhaps other isoforms that may be present in VSMC. Both, PLC β-2 and β-3 are attractive candidates because each of the preparations examined also express $G\alpha_{q/11}$ (Fig. 2), a subunit(s) of trimeric G-proteins known to activate PLC β(s) [4–6]. These possibilities assume heightened interest in view of recent observations indicating that activation of $G\alpha_{q/11}$ may involve tyrosine phosphorylation [20].

Taken together, these new data underscore the complexity of mechanisms that may link stimulation of diverse G-protein coupled receptors in VSMC to tyrosine kinase dependent activation of PLC and increased $[Ca^{2+}]_i$.

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