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Biochemical Pharmacology 65 (2003) 2091–2098
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Modification of intracellular free calcium in cultured A10 vascular smooth muscle cells by exogenous phosphatidic acid

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Received 12 February 2002; accepted 9 December 2002

Abstract

Exogenous phosphatidic acid (PA) was observed to produce a concentration-dependent increase in $[Ca^{2+}]_i$ in cultured A10 vascular smooth muscle cells. Preincubation of cells with sarcoplasmic reticulum Ca^{2+}-ATPase inhibitors (cyclopiazonic acid and thapsigargin), a phospholipase C inhibitor (2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate), inositol 1,4,5-trisphosphate receptor antagonists (2-aminoethoxydiphenyl borate and xestospongin), and an activator of protein kinase C (PKC) (phorbol 12-myristate 13-acetate) depressed the PA-evoked increase in $[Ca^{2+}]_i$. Although EGTA, an extracellular Ca^{2+} chelator, decreased the PA-induced increase in $[Ca^{2+}]_i$, sarcolemmal Ca^{2+}-channel blockers (verapamil or diltiazem) did not alter the action of PA. On the other hand, inhibitors of PKC (bisindolylmaleimide I) and G_i-protein (pertussis toxin) potentiated the increase in $[Ca^{2+}]_i$ evoked by PA significantly. These results suggest that the PA-induced increase in $[Ca^{2+}]_i$ in vascular smooth muscle cells may occur upon the activation of phospholipase C and the subsequent release of Ca^{2+} from the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool in the sarcoplasmic reticulum. This action of PA may be mediated through the involvement of PKC.

Keywords: Phosphatidic acid; Vascular smooth muscle cell; Ca^{2+}-handling; SR Ca^{2+}-ATPase; Sarcoplasmic reticulum

1. Introduction

PA, an active phospholipid, is formed in membranes by the activation of phospholipase D (PLD), as well as by the phosphorylation of 1,2-diacylglycerol, and is released from platelets during clotting [1]. It has been considered to serve as a second messenger for several bioactive agents [2]. Exogenous PA has been shown to increase the activity of Ca^{2+} transport in a variety of cellular systems including synaptosomes [3], neuroblastoma cells [4], freshly isolated adult cardiomyocytes [2], and cultured neonatal rat cardiomyocytes [5]. Putney *et al.* [6] have demonstrated that PA acts as a Ca^{2+} ionophore, whereas Ohsako and Deguchi [4] have suggested that PA opens voltage-activated Ca^{2+} channels in various membranes. Some studies with artificial membranes have ruled out the role of PA as a Ca^{2+} transporter or ionophore and, in fact, have hypothesized that PA may be altering Ca^{2+}-channel activity by binding to the cellular membrane [7]. Although exogenous PA has been shown to regulate Ca^{2+} transport in platelets [8] and rat cardiomyocytes [2,5], the role of exogenous PA in the regulation of Ca^{2+}-transport activity in vascular smooth muscle cells (VSMCs) has not been investigated previously. Therefore, the present study was undertaken to examine the effect of exogenous PA on $[Ca^{2+}]_i$ in cultured A10 VSMCs, as well as its mechanism of action. Furthermore, some

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; Bin-I, bisindolylmaleimide I; CPA, cyclopiazonic acid; Fura 2-AM, Fura-2 acetoxymethyl/ester; NCDC, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate; PA, phosphatidic acid; L-3-PA, L-3-phosphatidic acid dicapryl; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PTx, pertussis toxin; SR, sarcoplasmic reticulum; TG, thapsigargin; VSMCs, vascular smooth muscle cells; Xe C, xestospongin.

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doi:10.1016/S0006-2952(03)00201-6

experiments were carried out to test the hypothesis that the PA-induced increase in $[Ca^{2+}]_i$ in VSMCs may be due to the release of Ca^{2+} from the sarcoplasmic reticulum (SR) through the phospholipase C (PLC)–PKC pathway.

2. Materials and methods

2.1. Materials

PA (L-α-phosphatidic acid, from egg yolk lecithin, sodium salt), cyclopiazonic acid (CPA), thapsigargin (TG), diltiazem, verapamil, EGTA, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC), phorbol 12-myristate 13-acetate (PMA), pertussis toxin (PTx), HEPES, 2-aminoethoxydiphenyl borate (2-APB), and BSA were purchased from the Sigma Chemical Co; bisindolylmaleimide I (Bin-I) and xestospongin (Xe C) were obtained from Calbiochem and Fura-2 acetoxymethyl/ester (Fura 2-AM) was purchased from Molecular Probes. L-3-Phosphatidic acid dicapryl (L-3-PA) was purchased from the Doosan Serdary Research Laboratory. Culture medium, fetal bovine serum, trypsin, and other related products were from GIBCO (Life Technologies). All other reagents were of analytical grade and purchased from either Sigma or Fisher Scientific.

2.2. Cell culture

A continuous line of A10 VSMCs was obtained from the American Type Culture Collection. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Life Technologies), 3.7 mg/mL of $NaHCO_3$, and 100 μg/mL of gentamicin at 37° in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed initially after 48 hr and then every 2–3 days. When the cells had formed a confluent monolayer after 7–9 days, they were made quiescent by incubation in serum-free medium for 24 hr. Then they were harvested by the addition of 0.05% trypsin and 0.1% fetal bovine serum. The cell culture was continued up to eight passages, and the cells were observed to maintain uniform growth and response characteristics during this period.

2.3. Measurement of $[Ca^{2+}]_i$

The cultured A10 VSMCs harvested with trypsin and fetal bovine serum were centrifuged at 240 *g* for 5 min at room temperature. The supernatant was removed, and the cells were washed twice in a HEPES buffer physiological salt solution (mM/L: NaCl 145, KCl 4.5, $CaCl_2$ 1.0, $MgSO_4{\cdot}7H_2O$ 1.0, HEPES 10, *d*-glucose 5, BSA 0.1%, KH_2PO_4 1.0, pH 7.4). The cells were then incubated for 40 min at 37° with 10 μM Fura 2-AM in HEPES buffer and washed twice to remove any extracellular dye. The cell number was adjusted to 0.5×10^6/mL; the final concentration in the cuvette was approximately 0.3×10^6 cells/mL.

The fluorescence intensity at 510 nm was monitored by an SLM DMX-1100 dual-wavelength spectrofluorometer; the ratio (R) of the fluorescence signals at 340/380 (nm) was calculated automatically. The R_{max} and R_{min} values were determined by the addition of 40 μL Triton X-100 (10%) and 40 μL EGTA (400 nM), respectively. The $[Ca^{2+}]_i$ levels at rest as well as maximal increases evoked by PA were calculated according to the following formula:

$$[Ca^{2+}]_i = 224\left(\frac{R - R_{min}}{R_{max} - R}\right)\frac{Sf_2}{Sb_2}$$

where Sf_2 and Sb_2 are the fluorescence proportionality coefficients obtained at 380 nm under R_{min} and R_{max} conditions, respectively. Each cuvette containing approximately 0.3×10^6 cells/mL was exposed to a single concentration of PA, and the ratio of the response of Fura 2-AM fluorescence at 510 nm, when excited at 340 nm vs. 380 nm, was monitored for a total period of 5 min after the addition of PA. Studies involving various pharmacological agents for the modification of $[Ca^{2+}]_i$ in VSMCs by exogenous PA were performed by placing Fura 2-AM loaded cells in the buffer containing the indicated concentration of a drug for a particular time (as mentioned in Section 3) prior to the measurement of fluorescence. The increase in $[Ca^{2+}]_i$ at peak $[Ca^{2+}]_i$ was calculated as the net increase above the basal value in each experiment. This method of $[Ca^{2+}]_i$ measurement is similar to that described elsewhere [2].

2.4. Statistical analysis

All results are expressed as means ± SEM. Statistical analysis was performed using Microcal Origin Version 6 (Microcal Software Inc.). The comparison of mean values was performed by the use of one-way ANOVA. P values less than 0.05 were considered to reflect a significant difference.

3. Results

3.1. Effect of PA on $[Ca^{2+}]_i$ in VSMCs

The effect of different concentrations of PA (1–100 μM) was studied on $[Ca^{2+}]_i$ in cultured A10 VSMCs, and the data are shown in Fig. 1. PA increased the $[Ca^{2+}]_i$ in a concentration-dependent manner; the maximal increase in $[Ca^{2+}]_i$ was 138 ± 3.5 nM. The concentration of PA required for a 50% (EC_{50}) increase in $[Ca^{2+}]_i$ was 9.2 ± 1.5 μM. As shown in Fig. 2, L-3-PA (a PA analogue) also caused a concentration-dependent increase in $[Ca^{2+}]_i$; the maximal increase was 37 ± 5 nM. The EC_{50} value was

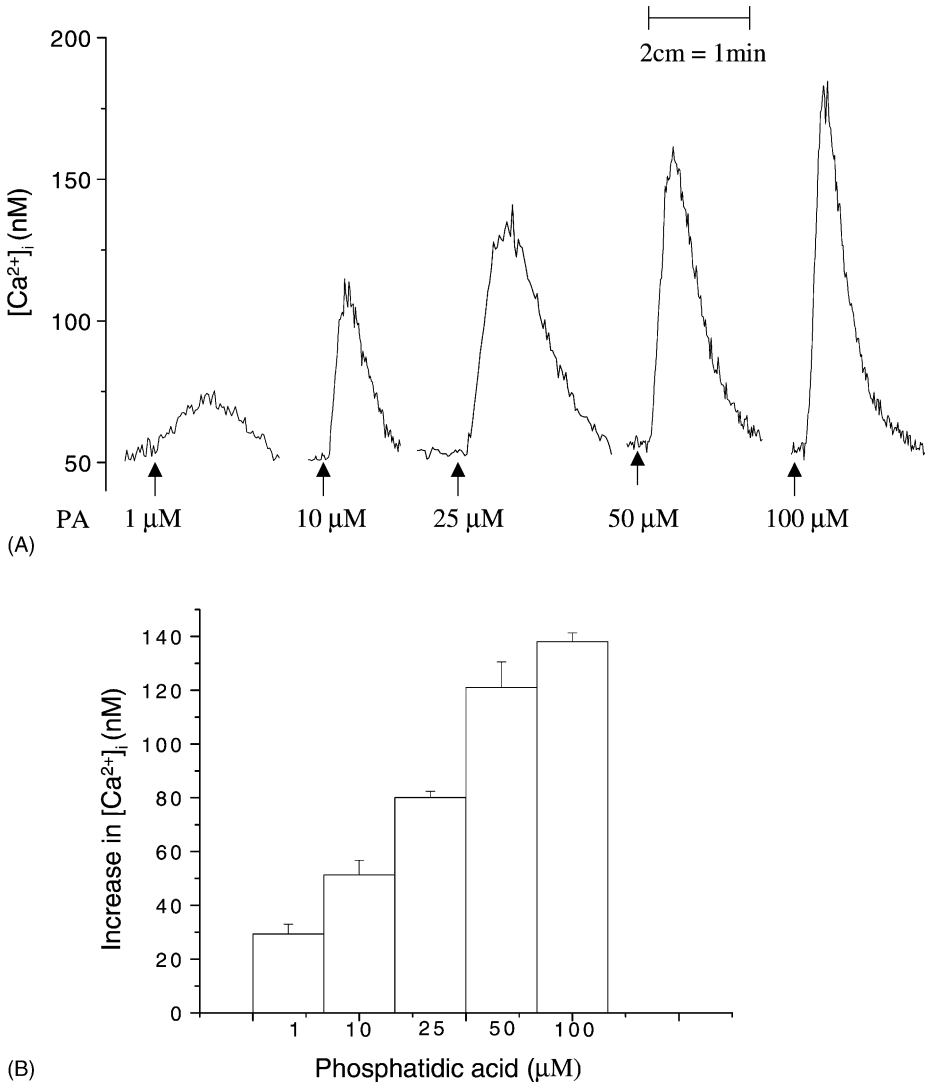

Fig. 1. Effect of different concentrations of PA on intracellular calcium ($[Ca^{2+}]_i$) in VSMCs. (A) $[Ca^{2+}]_i$ tracings exposed to various concentrations of PA. (B) Bar diagram representing the concentration response for PA-evoked increases in $[Ca^{2+}]_i$. Values are the means ± SEM of six experiments.

145 ± 20 μM. In addition, PA caused a transient increase in $[Ca^{2+}]_i$, while the PA analogue caused a sustained increase in $[Ca^{2+}]_i$.

3.2. Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with EGTA, verapamil, and diltiazem

To study whether the increase in $[Ca^{2+}]_i$ induced by PA was due to an influx of Ca^{2+} from an extracellular source, some inhibitors of Ca^{2+} influx [2,9–11] were used. On the one hand, preincubation of cultured A10 VSMCs for 30 s or 3 min with EGTA (1 mM) led to a significant diminution of both the basal $[Ca^{2+}]_i$ and the PA (10 μM) evoked increase in $[Ca^{2+}]_i$ (Table 1). On the other hand, preincubation of the cells for 10 min with the Ca^{2+}-channel blockers verapamil (5 and 10 μM) or diltiazem (5 and 10 μM) did not produce any significant change in the basal $[Ca^{2+}]_i$ or the PA-evoked increase in $[Ca^{2+}]_i$ (Table 1).

3.3. Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with CPA or TG

To examine if the PA-induced increase in $[Ca^{2+}]_i$ was due to the release of Ca^{2+} from the SR, different inhibitors of SR Ca^{2+}-ATPase [12,13] were used. Preincubation of cultured A10 VSMCs for 10 min with CPA (25 and 50 μM) or TG (10 and 20 μM) produced an insignificant change in the basal $[Ca^{2+}]_i$ [control: 49 ± 2 nM; CPA (25 μM): 45 ± 2 nM; CPA (50 μM): 45 ± 3 nM; TG (10 μM): 48 ± 5 nM; TG (20 μM): 48 ± 5 nM]. However, the SR Ca^{2+}-pump inhibitors (CPA or TG) almost abolished the increase in $[Ca^{2+}]_i$ evoked by 10 μM PA (Fig. 3A).

3.4. Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with NCDC

It was demonstrated previously that stimulation of PLC by PA leads to the production of inositol 1,4,5-trisphosphate

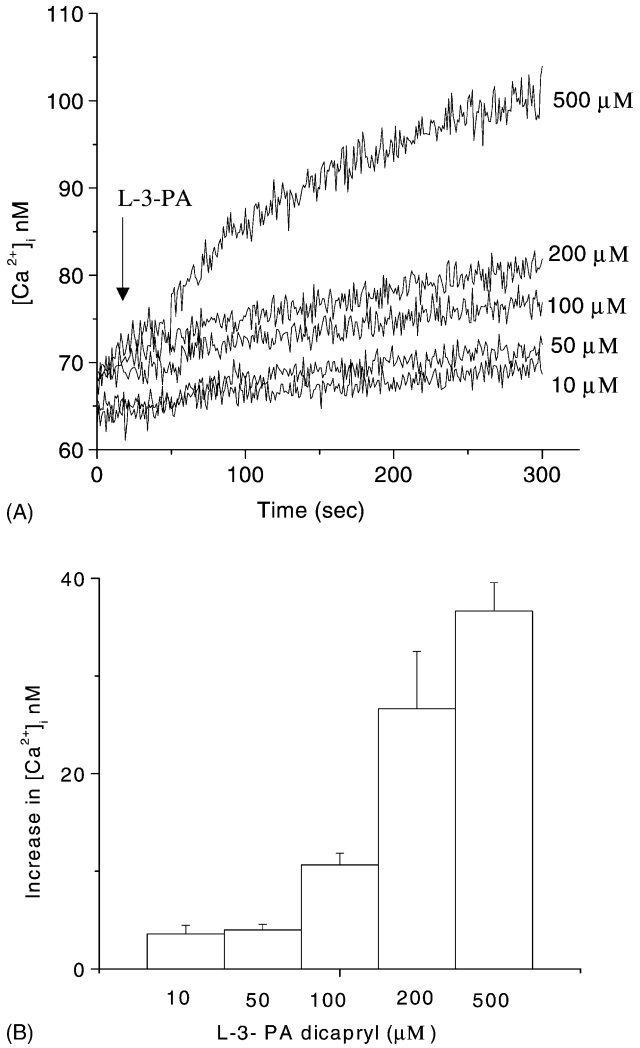

Fig. 2. Effect of different concentrations of L-3-PA on $[Ca^{2+}]_i$ in VSMCs. (A) $[Ca^{2+}]_i$ tracing exposed to various concentrations of L-3-PA. (B) Bar diagram representing the concentration response for L-3-PA-evoked increases in $[Ca^{2+}]_i$. Values are the means ± SEM of six experiments.

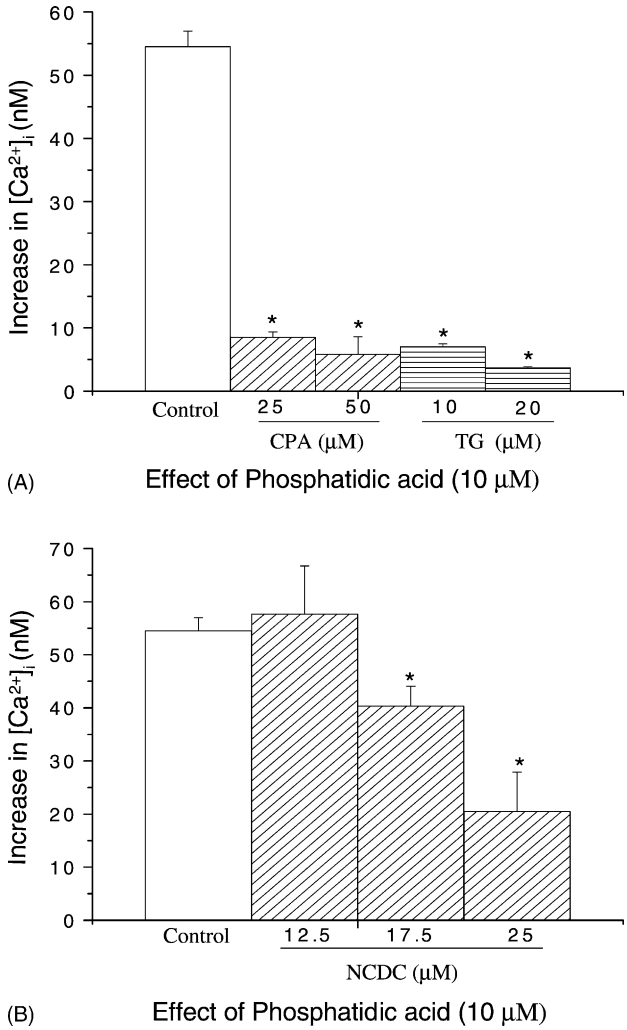

Fig. 3. (A) Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with CPA (25 and 50 µM) and TG (10 and 20 µM). (B) Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with NCDC (12.5–25 µM). Bars in (A) and (B) represent means ± SEM of the PA-evoked increase in peak $[Ca^{2+}]_i$ above basal for six experiments. Key: (*) $P < 0.05$ vs. control.

Table 1
Basal and PA-evoked increase in free intracellular calcium ($[Ca^{2+}]_i$) in control smooth muscle cells and cells preincubated with EGTA or calcium-channel blockers (verapamil and diltiazem)

	Basal $[Ca^{2+}]_i$ (nM)	PA-evoked increase in $[Ca^{2+}]_i$ (nM)
Control	50 ± 0.5	54 ± 4
EGTA (1 mM; 30 s)	26 ± 2*	27 ± 1*
EGTA (1 mM; 3 min)	27 ± 2*	25 ± 3
Verapamil (5 µm)	53 ± 3	51 ± 3
Verapamil (10 µm)	53 ± 3	63 ± 1
Diltiazem (5 µm)	50 ± 3	51 ± 4
Diltiazem (10 µm)	50 ± 4	59 ± 5

Values are the means ± SEM of 12 experiments for the control and six experiments each for all other groups. The PA (10 µM)-evoked increase in peak $[Ca^{2+}]_i$ was above the base value. Cells were treated with verapamil and diltiazem for 10 min.

* $P < 0.5$ vs. the respective control.

($InsP_3$) in cardiomyocytes [14] and that $InsP_3$ may result in the release of Ca^{2+} from the SR stores [15]. Thus, it was considered worthwhile to study whether a similar mechanism is involved in the PA-evoked Ca^{2+} mobilization in cultured A10 VSMCs. Preincubation of A10 VSMCs for 10 min with 12.5–25 µM NCDC, an inhibitor of PLC [16], produced an insignificant change in the basal $[Ca^{2+}]_i$ [control: 50 ± 2 nM; NCDC (12.5 µM): 49 ± 1 nM; NCDC (17.5 µM): 51 ± 1 nM; NCDC (25 µM): 52 ± 4 nM]. These results are consistent with earlier studies showing no change in the resting levels of $[Ca^{2+}]_i$ upon the addition of 1–100 µM NCDC in cardiomyocyte suspension [2]. The A10 cells preincubated with 12.5 µM NCDC did not reveal any significant change in the increase in $[Ca^{2+}]_i$ evoked by 10 µM PA, whereas the cells preincubated with 17.5 or 25 µM NCDC showed

a significant decrease in the PA-induced increase in $[Ca^{2+}]_i$ (Fig. 3B).

3.5. Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with Xe C and 2-APB

A10 VSMCs were preincubated for 30 min with 7.5 or 10 μM Xe C, a potent membrane permeable blocker of $InsP_3$-mediated Ca^{2+} release in intact cells [17]. Xe C produced an insignificant change in basal $[Ca^{2+}]_i$ [control: 49 ± 2 nM; Xe C (7.5 μM): 51 ± 1 nM; Xe C (10 μM): 53 ± 1 nM]. The cells preincubated with Xe C at concentrations of 7.5 and 10 μM when challenged with exogenous 10 μM PA showed a significant decrease in the PA-evoked increase in $[Ca^{2+}]_i$ (Fig. 4A). As shown in Fig. 4B, 2-APB [18], an $InsP_3$ receptor antagonist, also caused a concentration-dependent inhibition of PA-induced Ca^{2+} mobilization, indicating that PA caused the release of Ca^{2+} from the SR through $InsP_3$ receptors.

3.6. Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with Bin-I and PMA

To determine whether PKC, which is known to affect SR Ca^{2+} uptake [11], was involved in the PA-evoked increase in $[Ca^{2+}]_i$ in A10 VSMCs, cells were preincubated for 20 min with Bin-I (also known as Gf-109203x or GF-I), a potent inhibitor of phorbol ester-sensitive isoforms of PKC [19], at various concentrations ranging from 50 to 200 nM. After preincubation with low concentrations of Bin-I, the Fura 2 fluorescence ratio was determined but no significant change in basal $[Ca^{2+}]_i$ was observed as compared to the control [control: 50 ± 2 nM; Bin-I (50 nM): 53 ± 2 nM;

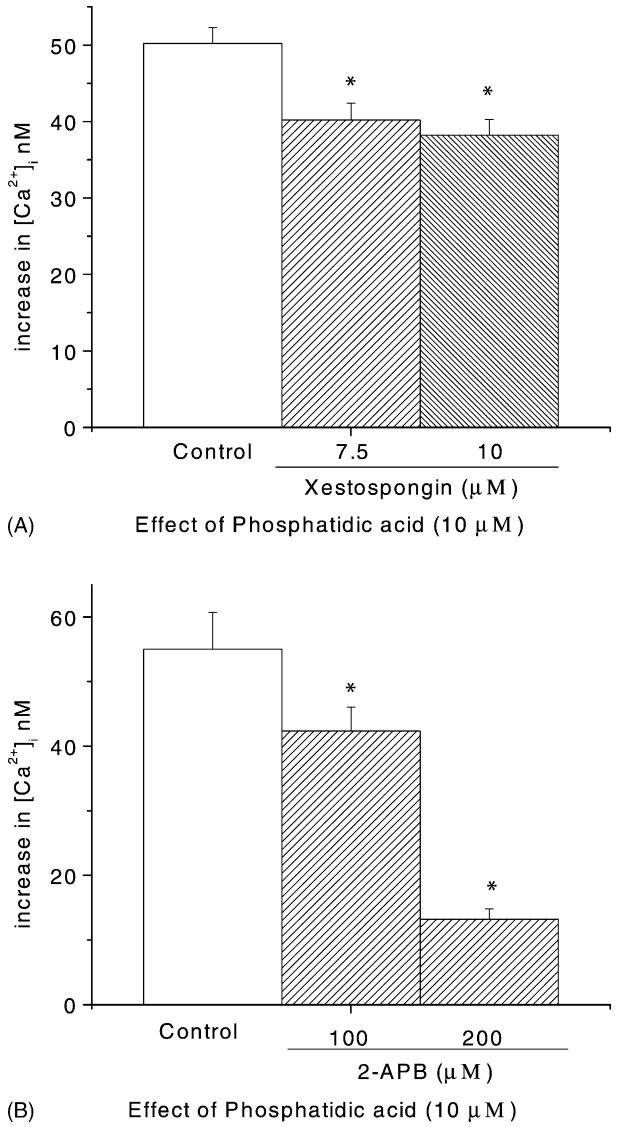

Fig. 4. (A) Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with Xe C (7.5 and 10 μM). (B) Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with 2-APB (100 and 200 μM). Bars in (A) and (B) represent means ± SEM of the PA-evoked increase in peak $[Ca^{2+}]_i$ above basal for six experiments. Key: (*) $P < 0.05$ vs. control.

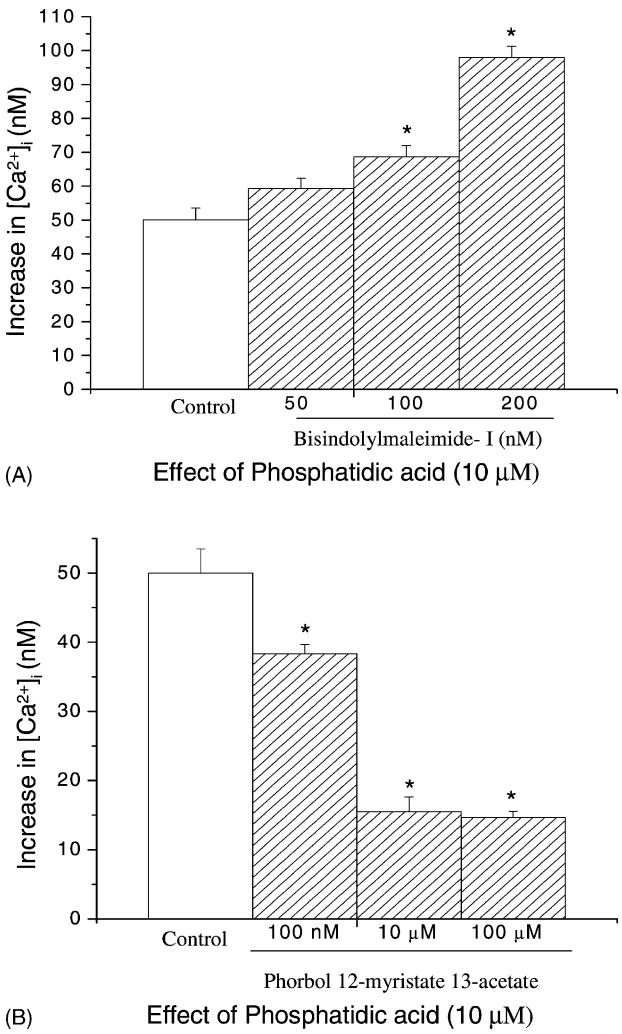

Fig. 5. (A) Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with Bin-I (50–200 nM). (B) Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with PMA (100 nM to 100 μM). Bars in (A) and (B) represent means ± SEM of the PA-evoked increase in peak $[Ca^{2+}]_i$ above basal for six experiments. Key: (*) $P < 0.05$ vs. control.

Bin-I (100 nM): 53 ± 2 nM]. However, when the cells were preincubated with a higher concentration of Bin-I (200 nM), there was a significant increase in basal $[Ca^{2+}]_i$ (82 ± 4 nM). When the cells were preincubated with different concentrations of Bin-I, there was an augmentation of the increase in $[Ca^{2+}]_i$ due to 10 μM PA in a concentration-dependent manner (Fig. 5A). To further understand the role of PKC in the PA-evoked increase in $[Ca^{2+}]_i$ in the A10 VSMCs, we used cells preincubated for 10 min with 100 nM to 100 μM PMA, an activator of PKC [11]. No change in the basal $[Ca^{2+}]_i$ was observed with the different concentrations of PMA [control: 52 ± 1 nM; PMA (100 nM): 52 ± 2 nM; PMA (10 μM): 52 ± 4 nM; PMA (100 μM): 54 ± 2 μM]. However, a significant inhibition of the increase in $[Ca^{2+}]_i$ evoked by 10 μM PA was evident when the cells were treated with different concentrations of PMA (Fig. 5B).

3.7. Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with PTx

Since the responses of different cells to lysophosphatidic acid (LPA) have been shown to involve G_i-proteins [20], it is likely that the PA-induced increase in $[Ca^{2+}]_i$ may be mediated through G_i-proteins. To determine whether a G_i-protein-dependent pathway is involved in the PA-induced increase in $[Ca^{2+}]_i$, the A10 VSMCs were preincubated with different concentrations of PTx (12.5–100 ng/mL) for 10 min before the determination of the Fura 2 fluorescence ratio. There was no significant change in basal $[Ca^{2+}]_i$ in the PTx-preincubated cells [control: 48 ± 2 nM; PTx (12.5 ng/mL): 50 ± 2 nM; PTx (25 ng/mL): 51 ± 3 nM; PTx (50 ng/mL): 49 ± 2 nM; PTx (100 ng/mL): 50 ± 3 nM]. However, there was a significant augmentation of the increase in $[Ca^{2+}]_i$ evoked by 10 μM PA at all concentrations of PTx (Fig. 6).

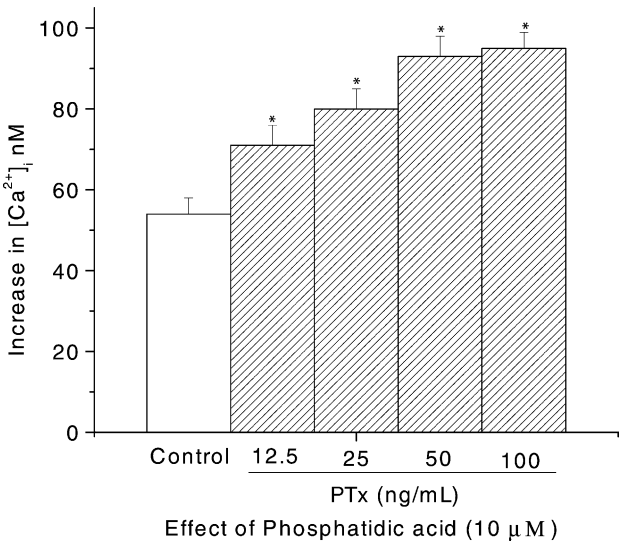

Fig. 6. Effect of PA on $[Ca^{2+}]_i$ after preincubation of VSMCs with PTx (12.5–100 ng/mL). Bars represent means ± SEM of the PA-evoked increase in peak $[Ca^{2+}]_i$ above basal for six experiments. Key: (*) $P < 0.05$ vs. control.

4. Discussion

In the present study, exogenous PA (1–100 μM) was found to produce a concentration-dependent increase in $[Ca^{2+}]_i$ in the A10 VSMCs. We employed a 10 μM concentration of PA for studying its mechanism of action because similar concentrations of PA were used by other investigators in their studies [3,14,21,22]. It is difficult to compare the concentrations of PA employed exogenously with those present endogenously under different physiological and pathological conditions as PA remains in the bound form with various membrane systems in the cell. However, the EC_{50} value of PA is about 9 μM, while the EC_{50} value of the PA analogue, L-3-PA, is about 145 μM, indicating that the Ca^{2+} response to PA in VSMCs may be mediated through a PA receptor.

It was interesting to observe that preincubation of cells with EGTA lowered the PA-evoked increase in $[Ca^{2+}]_i$; this finding appears to indicate that the PA-induced increase in $[Ca^{2+}]_i$ may be partially dependent on $[Ca^{2+}]_e$. Although PA has been suggested previously to mobilize $[Ca^{2+}]_i$ through the stimulation of L-type Ca^{2+} channels in neuroblastoma cells [4] and cardiomyocytes [2], incubation with the Ca^{2+}-channel blockers verapamil and diltiazem did not reduce the PA-evoked increase in $[Ca^{2+}]_i$ in quiescent A10 VSMCs. The discrepancy in these results could be due to the difference in cell type employed in these studies. Thus, it is reasonable to assume that the PA-evoked increase in $[Ca^{2+}]_i$ in the quiescent A10 VSMCs may not be linked to the opening of L-type Ca^{2+} channels in the sarcolemmal membrane. Because EGTA also produced a decrease in the basal levels of $[Ca^{2+}]_i$, in view of the fact that the influx of Ca^{2+} is obligatory to replenish the SR Ca^{2+} stores, it may be that the intracellular Ca^{2+} stores become depleted by the use of EGTA and this may result in depressing the PA-induced Ca^{2+} release from SR in the A10 VSMCs treated with EGTA. However, the role of store-operated Ca^{2+} channel [18] in PA-induced mobilization in A10 VSMCs remains to be investigated.

To understand the exact mode of action of exogenous PA on A10 VSMCs, we incubated the cells with CPA and TG, which deplete the SR Ca^{2+} stores by inhibiting SR Ca^{2+}-ATPase [2,23]. It was observed that the PA-evoked increase in $[Ca^{2+}]_i$ was depressed by CPA and TG. Such observations indicated that Ca^{2+} release from the SR may be involved in the PA-induced Ca^{2+} mobilization. It is pointed out that PA has been suggested to activate the $InsP_3$ pathway in fibroblasts [24], neutrophils [23], adult cardiomyocytes [2,14], and neonatal cardiomyocytes [5], and this may lead to the release of Ca^{2+} from the SR. Thus, it is concluded that the PA-evoked increase in $[Ca^{2+}]_i$ may be associated with the release of Ca^{2+} from the SR Ca^{2+} store,

which has been proposed to be the site of PA action in neonatal rat cardiomyocytes [5].

Patel *et al*. [25] suggested that stimulation by many agonists, which bind to G-protein coupled receptors in A10 VSMCs, results in the activation of PLC and the metabolism of phosphatidyl-inositol phosphate to $InsP_3$. The $InsP_3$ thus formed then activates Ca^{2+} release by its action on SR Ca^{2+}-release channels [26]. It has been demonstrated by a number of investigators that NCDC decreases the $InsP_3$ level and $[Ca^{2+}]_i$ by inhibiting PLC [2,16,27–29]. Our results in VSMCs preincubated with NCDC indicated that the PA-evoked increase in $[Ca^{2+}]_i$ was depressed by NCDC in a concentration-dependent manner. Accordingly, it is concluded that the PA-induced increase in $[Ca^{2+}]_i$ may be elicited through the activation of PLC and the formation of $InsP_3$.

To further confirm the role of exogenous PA in the release of Ca^{2+} from the SR through the involvement of $InsP_3$-sensitive SR Ca^{2+} pools, the A10 VSMCs were preincubated with two $InsP_3$ receptor blockers, Xe C and 2-APB [17,18,26]. The PA-evoked increase in $[Ca^{2+}]_i$ in cells preincubated with Xe C or 2-APB was decreased significantly as compared to the control, suggesting that $InsP_3$-sensitive Ca^{2+} channels may be involved in the PA-evoked increase in $[Ca^{2+}]_i$. Liu *et al*. [30] have reported that vasopressin-induced Ca^{2+} mobilization in cardiomyocytes is depressed by PLC inhibitors, but not by ryanodine or caffeine, suggesting that there are two Ca^{2+} pools in the SR, namely ryanodine-sensitive and $InsP_3$-sensitive pools. Our studies using different agents to modify the action of PA suggest that PA may release Ca^{2+} from the $InsP_3$-sensitive Ca^{2+} pool in the SR; further research is required to investigate the role of the ryanodine-sensitive Ca^{2+} pool in the SR in PA-induced Ca^{2+} mobilization in A10 VSMCs.

Since PKC activators have been shown to reduce Ca^{2+} accumulation in the SR in cardiomyocytes [11], activation of PKC can be seen to decrease the Ca^{2+} store in the SR and thus less Ca^{2+} will be released in the cell upon stimulation. This view is consistent with our results demonstrating a decrease in the PA-evoked increase in $[Ca^{2+}]_i$ in A10 VSMCs preincubated with PMA, an activator of PKC. It has been reported by Stasek *et al*. [31,32] that PA directly activates PKC in the pulmonary artery endothelial cell line CCL-209. To further confirm the role of PKC in the involvement of Ca^{2+} mobilization induced by PA in A10 VSMCs, it was observed that the cells preincubated with Bin-I, a specific PKC inhibitor [20], and later challenged with PA, showed an augmentation of the PA-induced increase in $[Ca^{2+}]_i$. However, the present study does not rule out other mechanisms such as the effect of PKC on SR Ca^{2+} release or PA receptor down-regulation in A10 VSMCs. It should be pointed out that our data for the effects of PKC activation on basal $[Ca^{2+}]_i$ levels, as well as the PA-induced increase in $[Ca^{2+}]_i$, are different from those reported by others [33–35]. In this regard, PMA has been shown to increase the level of $[Ca^{2+}]_i$ and increase Ca^{2+} waves in VSMCs [33,34,36,37]. In addition, PMA was observed to increase the angiotensin II (AII)- or homocysteine-evoked increase in $[Ca^{2+}]_i$ in VSMCs [35,38]. Since the inhibition of PKC has been reported to enhance the endothelin-1 response and to attenuate the vasopression- or AII-induced elevation of $[Ca^{2+}]_i$ in cardiomyocytes [39], it appears that the role of PKC activation or inhibition in modifying the level of $[Ca^{2+}]_i$ may be agonist-specific.

The role of G-proteins in PA-induced Ca^{2+} mobilization has not been reported; however, in the present study, PTx treatment augmented the $[Ca^{2+}]_i$ response to PA in VSMCs. Although the mechanism of this action of PA is not clear, Paolucci *et al*. [20] have demonstrated that prior treatment of either Swiss 3T3 cells or rat-1 cells with low concentrations of PTx leads to the activation of PLD in response to LPA by inhibiting the G_i-protein and thus causing an increase in cyclic AMP levels. Our results with PTx are likely to be mediated through a cyclic AMP-dependent mechanism as suggested by Paolucci *et al*. [20]. Furthermore, Freeman *et al*. [40,41] reported that AII-induced growth of VSMCs was dependent upon the activation of PLD and the formation of PA. Thus, PA could serve as an important second messenger in the regulation of proliferation as well as Ca^{2+} homeostasis in VSMCs. The results from the present study substantiate this viewpoint. However, it is noted that some caution should be exercised while interpreting the data from the A10 VSMC line *in vitro* to vascular smooth muscle signal transduction mechanisms *in vivo*.

Acknowledgments

This work was supported by the Canadian Institute of Health Research (CIHR) Group in Experimental Cardiology. N.S.D. holds the CIHR/Research & Development Chair in Cardiovascular Research supported by Merck Frosst Canada. Dr. P. Bhugra was a visiting scientist from the Department of Pharmacology, Pramukhswami Medical College, Karamsad, Gujarat, India.

References

[1] English D, Garcia JGN, Brindley DN. Platelet-released phospholipids link haemostasis and angiogenesis. Cardiovasc Res 2001;49:588–99.

[2] Xu YJ, Panagia V, Shao Q, Wang X, Dhalla NS. Phosphatidic acid increases intracellular free Ca^{2+} and cardiac contractile force. Am J Physiol Heart Circ Physiol 1996;271:H651–9.

[3] Harris RA, Schmidt J, Hitzemann BA, Hitzemann RJ. Phosphatidate as a molecular link between depolarization and neurotransmitter release in the brain. Science 1981;212:1290–1.

[4] Ohsako S, Deguchi T. Stimulation by phosphatidic acid of calcium influx and cyclic GMP synthesis in neuroblastoma cells. J Biol Chem 1981;256:10945–8.

[5] Liu P, Xu YJ, Hopfner RL, Gopalakrishnan V. Phosphatidic acid increases inositol-1,4,5-trisphosphate and $[Ca^{2+}]_i$ levels in neonatal rat cardiomyocytes. Biochim Biophys Acta 1999;1440:89–99.
[6] Putney Jr JW, Weiss SJ, Van De Walle CM, Haddas RA. Is phosphatidic acid a calcium ionophore under neurohumoral control? Nature 1980;284:345–7.
[7] Holmes RP, Yoss NL. Failure of phosphatidic acid to translocate Ca^{2+} across phosphatidylcholine membranes. Nature 1983;305:637–8.
[8] Hashizume T, Sato T, Fujii T. Phosphatidic acid with medium-length fatty acyl chains synergistically stimulates phospholipase C with Ca^{2+} in rabbit platelets. Biochem Int 1992;26:491–7.
[9] Karaki H, Ozaki H, Hori M, Mitsui-Saito M, Amano K, Harada K, Miyamoto S, Nakazawa H, Won KJ, Sato K. Calcium movements, distribution, and functions in smooth muscle. Pharmacol Rev 1997;49: 157–230.
[10] Kuriyama H, Kitamura K, Nabata H. Pharmacological and physiological significance of ion channels and factors that modulate them in vascular tissues. Pharmacol Rev 1995;47:387–473.
[11] Rogers TB, Gaa ST, Massey C, Dosemeci A. Protein kinase C inhibits Ca^{2+} accumulation in cardiac sarcoplasmic reticulum. J Biol Chem 1990;265:4302–8.
[12] Seidler NW, Jona I, Vegh M, Martonosi A. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. J Biol Chem 1989;264:17816–23.
[13] Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci USA 1990;87:2466–70.
[14] Henry RA, Boyce SY, Kurz T, Wolf RA. Stimulation and binding of myocardial phospholipase C by phosphatidic acid. Am J Physiol Cell Physiol 1995;269:C349–58.
[15] Berridge MK. Inositol trisphosphate and calcium signalling. Nature 1993;361:315–25.
[16] Takei M, Ueno M, Endo K, Nakagawa H. Effect of NCDC, a protease inhibitor, on histamine release from rat peritoneal mast cells induced by anti-IgE. Biochem Biophys Res Commun 1991;181:1313–22.
[17] Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN. Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. Neuron 1997;19: 723–33.
[18] Ma HT, Venkatachalam K, Parys JB, Gill DL. Modification of store-operated channel coupling and inositol trisphosphate receptor function by 2-aminoethoxydiphenyl borate in DT40 lymphocytes. J Biol Chem 2002;277:6915–22.
[19] Toullec D, Pianetti P, Coste H, Bellevergue P, Grandperret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J. The bisindolylmaleimide Gf-109203x is a selective inhibitor of protein kinase C. J Biol Chem 1991;266:15771–81.
[20] Paolucci L, Sinnett-Smith J, Rozengurt E. Lysophosphatidic acid rapidly induces protein kinase D activation through a pertussis toxin-sensitive pathway. Am J Physiol Cell Physiol 2000;278:C33–9.
[21] Kurz T, Wolf RA, Corr PB. Phosphatidic acid stimulates inositol 1,4, 5-trisphosphate production in adult cardiac myocytes. Circ Res 1993; 72:701–16.
[22] Qualliotine-Mann D, Agwu DE, Ellenburg MD, McCall CE, McPhail LC. Phosphatidic acid and diacylglycerol synergize in a cell-free system for activation of NADPH oxidase from human neutrophils. J Biol Chem 1993;268:23843–9.
[23] Siddiqui RA, English D. Phosphatidic acid elicits calcium mobilization and actin polymerization through a tyrosine kinase-dependent process in human neutrophils: a mechanism for induction of chemotaxis. Biochim Biophys Acta 1997;1349:81–95.
[24] Gonzalez-Gronow M, Gawdi G, Pizzo SV. Plasminogen activation stimulates an increase in intracellular calcium in human synovial fibroblasts. J Biol Chem 1993;268:20791–5.
[25] Patel S, Joseph SK, Thomas AP. Molecular properties of inositol 1,4, 5-trisphosphate receptors. Cell Calcium 1999;25:247–64.
[26] Sanders KM. Invited review: mechanisms of calcium handling in smooth muscles. J Appl Physiol 2001;91:1438–49.
[27] Saetrum Opgaard O, Nothacker H, Ehlert FJ, Krause DN. Human urotensin II mediates vasoconstriction via an increase in inositol phosphates. Eur J Pharmacol 2000;406:265–71.
[28] Tostes RC, Storm DS, Chi DH, Webb RC. Intracellular calcium stores and oscillatory contractions in arteries from genetically hypertensive rats. Hypertens Res 1996;19:103–11.
[29] Tanaka Y, Hata S, Ishiro H, Ishii K, Nakayama K. Quick stretch increases the production of inositol 1,4,5-trisphosphate (IP_3) in porcine coronary artery. Life Sci 1994;55:227–35.
[30] Liu P, Hopfner PL, Xu YJ, Gopalakrishnan V. Vassopressin-evoked $[Ca^{2+}]_i$ responses in neonatal rat cardiomyocytes. J Cardiovasc Pharmacol 1999;34:540–6.
[31] Stasek Jr JE, Natarajan V, Garcia JG. Phosphatidic acid directly activates endothelial cell protein kinase C. Biochem Biophys Res Commun 1993;191:134–41.
[32] Stasek Jr JE, Patterson CE, Garcia JGN. Protein kinase C phosphorylates caldesmon$_{77}$ and vimentin and enhances albumin permeability across cultured bovine pulmonary artery endothelial cell monolayers. J Cell Physiol 1992;153:62–75.
[33] Neusser M, Tepel M, Zidek W. Angiotensin II responses after protein kinase C activation in vascular smooth muscle cells of spontaneously hypertensive rats. J Cardiovasc Pharmacol 1993;21:749–53.
[34] Caramelo C, Okada K, Tsai P, Schrier RW. Phorbol esters and arginine vasopressin in vascular smooth muscle cell activation. Am J Physiol 1989;256:F875–81.
[35] Mujumdar VS, Hayden MR, Tyagi SC. Homocyst(e)ine induces calcium second messenger in vascular smooth muscle cells. J Cell Physiol 2000;183:28–36.
[36] Jagger JH, Nelson MT. Differential regulation of Ca^{2+} waves by UTP in rat cerebral artery smooth muscle cells. Am J Physiol 2000;279: C1528–39.
[37] Gokina NI, Knot HJ, Nelson MT, Osol G. Increased Ca^{2+} sensitivity as a key mechanism of PKC-induced constriction in pressurized cerebral arteries. Am J Physiol 1999;277:H1178–88.
[38] Bonev AD, Jagger JH, Rubart M, Nelson MT. Activators of protein kinase C decrease Ca^{2+} spark frequency in smooth muscle cells from cerebral arteries. Am J Physiol 1997;273:C2090–5.
[39] Xu Y, Sandirasegarane L, Gopalkrishnan V. Protein kinase C inhibitors enhance endothelin-1 and attenuate vasopressin and angiotensin II evoked $[Ca^{2+}]_i$ elevation in rat cardiomyocytes. Br J Pharmacol 1993;108:6–8.
[40] Freeman EJ. The Ang II-induced growth of vascular smooth muscle cells involves a phospholipase D-mediated signaling mechanism. Arch Biochem Biophys 2000;374:363–70.
[41] Freeman EJ, Chisolm GM, Tallant EA. Role of calcium and protein kinase C in the activation of phospholipase D by angiotensin II in vascular smooth muscle cells. Arch Biochem Biophys 1995;319:84–92.