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Messenger molecules of the phospholipase signaling system have dual effects on vascular smooth muscle contraction

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Abstract

Background and methods. In order to investigate the role of phospholipases and their immediately derived messengers in agonist-induced contraction of portal vein smooth muscle, we used the addition in the organ bath of exogenous molecules such as: phospholipases C, A_2, and D, diacylglycerol, arachidonic acid, phosphatidic acid, choline. We also used substances modulating activity of downstream molecules like protein kinase C, phosphatidic acid phosphohydrolase, or cyclooxygenase. *Results*. a) Exogenous phospholipases C or A_2, respectively, induced small agonist-like contractions, while exogenous phospholipase D did not. Moreover, phospholipase D inhibited spontaneous contractions. However, when added during noradrenaline-induced plateau, phospholipase D shortly potentiated it. b) The protein kinase C activator, phorbol dibutyrate potentiated both the exogenous phospholipase C-induced contraction and the noradrenaline-induced plateau, while the protein kinase C inhibitor 1-(-5-isoquinolinesulfonyl)-2-methyl-piperazine relaxed the plateau. c) When added before noradrenaline, indomethacin inhibited both phasic and tonic contractions, but when added during the tonic contraction shortly potentiated it. Arachidonic acid strongly potentiated both spontaneous and noradrenaline-induced contractions, irrespective of the moment of its addition. d) In contrast, phosphatidic acid inhibited spontaneous contractile activity, nevertheless it was occasionally capable of inducing small contractions, and when repetitively added during the agonist-induced tonic contraction, produced short potentiations of the plateau. Pretreatment with propranolol inhibited noradrenaline-induced contractions and further addition of phosphatidic acid augmented this inhibition. Choline augmented the duration and amplitude of noradrenaline-induced tonic contraction and final contractile oscillations. *Conclusions*. These data suggest that messengers produced by phospholipase C and phospholipase A_2 contribute to achieve the onset and maintenance of contraction, while phospholipase D-yielded messengers appear to provide a delayed "on/off switch" that ultimately brings relaxation.

Keywords: phospholipases - proteinkinase C - phosphatidic acid - choline - arachidonic acid noradrenaline - portal vein - vascular smooth muscle

Introduction

Ca^{2+} mobilizing agonists activate phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD), which hydrolyze membrane phospholipids such as phosphatidylinositides (PI) and phosphatidylcholine (PC), to provide second messengers that modulate cell function [1-8]. Because of the individualistic nature of cells, the signaling spatiotemporal array of events appear to be specific, not only for the kind of agonist and/or receptor involved, but also for every cell (sub)type. This is

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also conspicuous in the various types of vascular smooth muscle cells [9-15]. In vascular smooth muscle, noradrenaline (NA) activates PLA_2, PLC and PLD, and the response comprises both transient and sustained biochemical signals [10]. However, studies dealing with the functional effects of these biochemical events are very scarce.

In agreement with the available biochemical data, our immunopharmacological experiments suggested that activation of phosphatidylinositol-specific phospholipase C (PI-PLC) was essential for triggering contraction, while phosphatidylcholine-specific phospholipase C (PC-PLC) and phospholipase A_2 (PLA_2) were subsequently activated and sustained tonic contraction. In contrast, phospholipase D (PLD) appeared to play a role in triggering relaxation [16]. A scheme of the molecular events following NA stimulation in portal vein smooth muscle is depicted in Fig. 1. Time-dependent interaction between the different signaling pathways may determine the modulation of the different phases of NA-induced response.

While the role of inositol 1,4,5-trisphosphate (IP_3) is documented as an initial trigger of Ca^{2+} release from internal stores [9, 17], there are conflicting data about the roles and timing of other messenger actions [reviews: 4, 7, 8]. It is also worth taking into account the possibility that phospholipases, and some of the molecules resulting by their action, may enrich the extracellular microenvironment and act by different mechanisms at the cell surface [12, 18].

The aim of this study was to investigate the trend and time course of signaling events conveyed by phospholipases C, A_2 and D, and some immediate messengers of phospholipase action, such as diacylglycerol (DAG)/proteinkinase C (PKC), phosphatidic acid (PA), choline (Ch), and arachidonic acid (AA), in portal vein smooth muscle cells, as hinted by the contractile behaviour of smooth muscle strips *in vitro,* in response to exogenously added molecules.

Materials and methods

Organ bath measurements of contraction

Male Wistar rats (*Rattus norvegicus*) of 200-250 g weight were killed by cervical dislocation. Portal vein was excised, placed into physiological salt solution (PSS) and dissected. Smooth muscle was cut into 5 mm

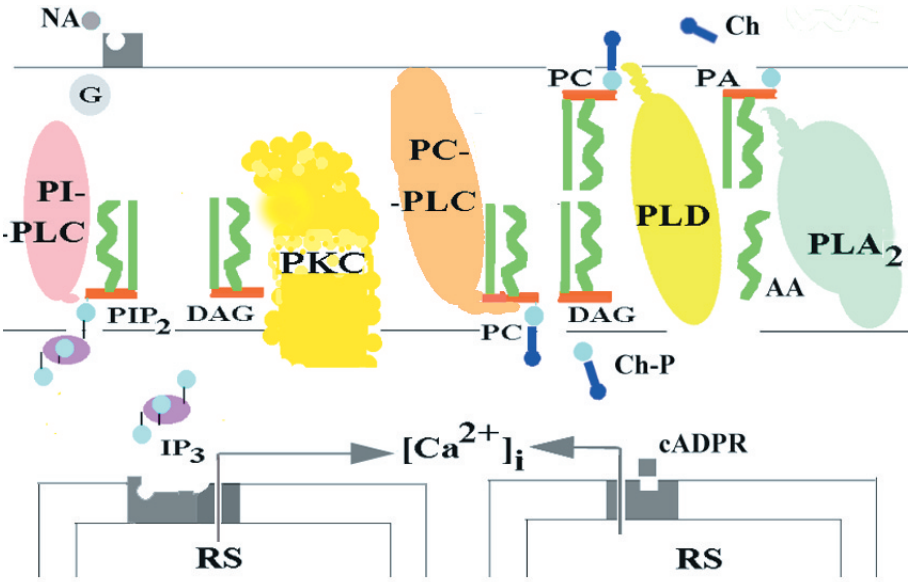

Fig. 1 Schematic representation of the main molecular events following noradrenaline stimulation of vascular smooth muscle cells. PI-PLC -phosphatidylinositide-specific phospholipase C; PC-PLC - phosphatidyl choline - specific phospholipase C; PLD-phospholipase D; PLA_2 - phospholipase A_2; G - trimeric GTP-dependent protein; PIP_2 - phosphatidyl inositol 4,5 bisphosphate; IP_3 - inositol 1,4,5-bisphosphate; DAG -diacyl glycerol; PKC - proteinkinase C; PC- phosphatidilcholine; Ch-P phosphocholine; Ch - choline; PA phosphatidic acid; AA - arachidonic acid; $[Ca^{2+}]_i$ - intracellular calcium concentration; cADPR-cyclic ADP ribose; SR - sarcoplasmic reticulum.

long and 1 mm large longitudinal strips. The strips were horizontally suspended in a 0.5 ml chamber in PSS and allowed 1 h for equilibration. The composition of PSS was as follows (mM): NaCl 130; KCl 5.6; $CaCl_2$ 2.0; $MgCl_2$ 0.24; glucose 11.0, buffered with Tris and HCl 8.3 mM at pH 7.42.

After equilibration for 1 h in PSS, strips of rat portal vein exhibited spontaneous oscillatory contractions with stable frequency and amplitude. Afterwards the muscle was stimulated with NA or other substances. The contraction was isometrically measured as previously described [19].

Definition of an integrative parameter for portal vein spontaneous contractions

Spontaneous contractile activity of portal vein smooth muscle is composed of phasic contractile oscillations of stable amplitude and frequency. Since amplitude and frequency of spontaneous contractions are sometimes pharmacologically altered in divergent directions, we defined an integrative parameter: the amplitude (A) multiplied by the frequency (F), representing the total contractile force (CF) developed on time unit (t): $CF/t = A \cdot F$

The control pattern of noradrenaline-induced contraction of rat portal vein smooth muscle

The typical NA - induced contractions are illustrated in Fig. 2 [see also ref. 16]. Low concentrations of NA (0.1 - 1 μM) augmented the frequency and amplitude of oscillatory contractions with a small increase in the basal tone. Higher concentrations of NA (2 - 3 μM) induced also an oscillatory response, in which oscillations of augmented frequency were superimposed on a large tone variation. Four phases can be separated: 1. an early phasic contraction, 2. a tonic contraction, 3. a relaxation, and 4. final oscillations, when exposure to NA exceeded 3 min.

Chemicals

PLA_2 (E.C. 3.1.1.4) from *Naja naja* venom, PC-PLC (E.C. 3.1.4.3) from *Clostridium perfringens*, PLD (E.C. 3.1.4.4) from *Streptomyces species*, arachidonic acid (AA), L-a phosphatidic acid (PA) from egg yolk, choline (Ch), DL-propranolol, indomethacin, 1-(5-isoquinolinyl sulfonyl)-2-methyl piperazine (H7), phorbol 12,13 dibutyrate (PDBu), 1-oleoyl 2-acetyl- glycerol (OAG) and all other chemicals were obtained from Sigma Chemical Co. The solvent solutions were: 100% dimethyl sulphoxide (DMSO) for indomethacin, PA and AA, and PSS for the other reagents. The final concentration of DMSO in the muscle bath was no more than 0.1%, at which concentration it caused no significant effect on the tension. Comercially available PI-PLC could not be tested, because it was delivered in a glycerol and EDTA-containing solution that drastically altered smooth muscle contractility.

Statistics

Each type of experiment was performed at least in triplicate. Values in the diagrams are expressed as mean ± SEM. Student's test was applied and a probability $p < 0.05$ was considered significant.

Results

Effects of exogenous phospholipase C and phorbol ester

PC-PLC (0.5-1 U/ml) and PDBu (1μM), respectively, induced contractions similar to that produced by low concentrations of NA (0.1-1 μm) (Fig. 3a,b). PC-PLC, but not PDBu, induced a rapid muscle "desensitization" since the effect of more 1U/ml PLC or 3μM NA applied 20-40 min later was strongly inhibited (Fig. 3a). PC-PLC (1U/ml) and PDBu (1 μM), subsequently added, at

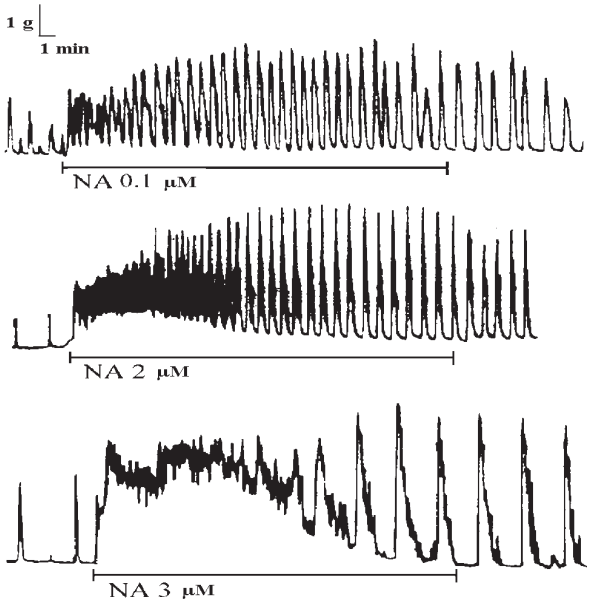

Fig. 2 Portal vein smooth muscle contractions induced by different physiological concentrations of noradrenaline (NA).

a 2 min interval, mimicked the contraction induced by 3 μM NA: PC-PLC induced a small phasic contraction, followed by a low oscillatory plateau, and PDBu elicited a superimposed tonic contraction (Fig. 3c). PDBu (0.1-2 μM) also strongly potentiated, in a dose-dependent manner, the tonic contraction induced by NA (3 μM) (Fig. 4a). However, prolonged (90 min) applications of PDBu (1 μM), used as a negative control, progresssively inhibited all phases of the contraction induced by 3 μM NA (Fig. 4b). OAG (20μM) strongly stimulated spontaneous rhythmic contractions (not shown), but had no significant effect on NA-induced contraction.

H7 (10-30 μM), the widely used PKC inhibitor, relaxed in a stepwise manner the tonic contraction; repetitive additions were needed for complete relaxation (Fig. 3d).

Effect of exogenous phospholipase D, phosphatidic acid, and choline

PLD (1-6 U/ml) alone did not induce any contractile response but progressively inhibited the spontaneously oscillatory contractions (Fig. 5a,c). However, when repetitively addded on the plateau of the NA-induced contraction, PLD (3U/ml) only produced small and transitory potentiations followed by relaxations to the initial plateau level (Fig. 5b).

In some experiments, addition of PA (20 μM) progressively inhibited the spontaneous contractile oscillations in a manner similar to that of PLD (Fig. 6a), but showing a weak inhibitory activity in the concentration domain 60-80 mM (Fig. 7a).

In other experiments, after abruptly inhibiting spontaneous contractions, PA (40-120 μM) was able to induce a transient contraction (Fig. 6b). The contraction amplitude was dose-dependent (Fig. 7b). Notably, immmediately afterwards, smooth muscle was able to respond to 3μM NA. PA (40-120 μM) added during the tonic contraction induced by NA (3 μM) produced a short potentiation of the plateau (Fig. 6b,c). Subsequently, PDBu (100 nM) additively potentiated the plateau (Fig. 6b). However, potentiation of the plateau by PDBu after muscle treatment with PA was weaker than potentiation by PDBu alone (Fig.8).

When added during the plateau, propranolol (100-240 μM), a PA phosphohydrolase inhibitor, did not significantly alter the amplitude of NA induced contraction, but clearly supressed the final oscillations (Fig. 6d). On the other hand, previously added propranolol (100-300 μM) had a clear inhibitory effect on the NA-induced contraction and abolished any potentiation by PA (40-80μM) (Fig. 6e). Choline (2 mM), added during the NA-induced tonic contraction, augmented the frequency and

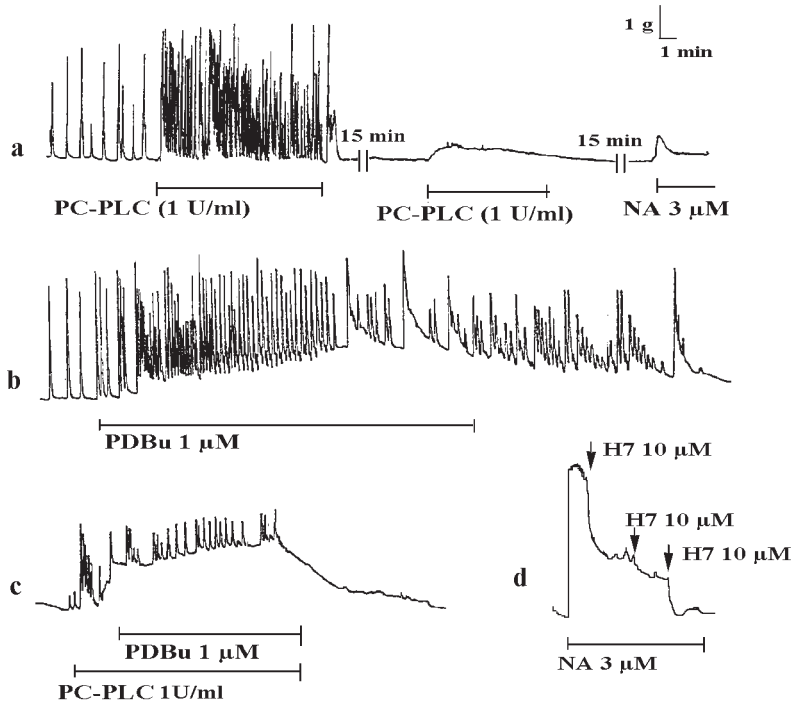

Fig. 3 Effect of exogenous phospholipases and phorbol ester on the contractile activity of portal vein. **(a)** PC-specific PLC (1U/ml) induced a contraction similar to that produced by a low concentration (0.1 μM) of noradrenaline. Further applications of PLC (1U/ml, 40 min after the first one) and noradrenaline (3 μM, 10 min after PLC) induced small contractions. **(b)** Phorbol dibutyrate (PDBu, 1μM) induced a contraction similar to that produced by PLC. **(c)** PC-specific PLC (1U/ml) and PDBu (1 μM) subsequently added at 2 min interval mimicked the contraction induced by 3 μM noradrenaline. **(d)** H7 (10 μM) inhibited the noradrenaline-induced contraction in a concentration-dependent manner.

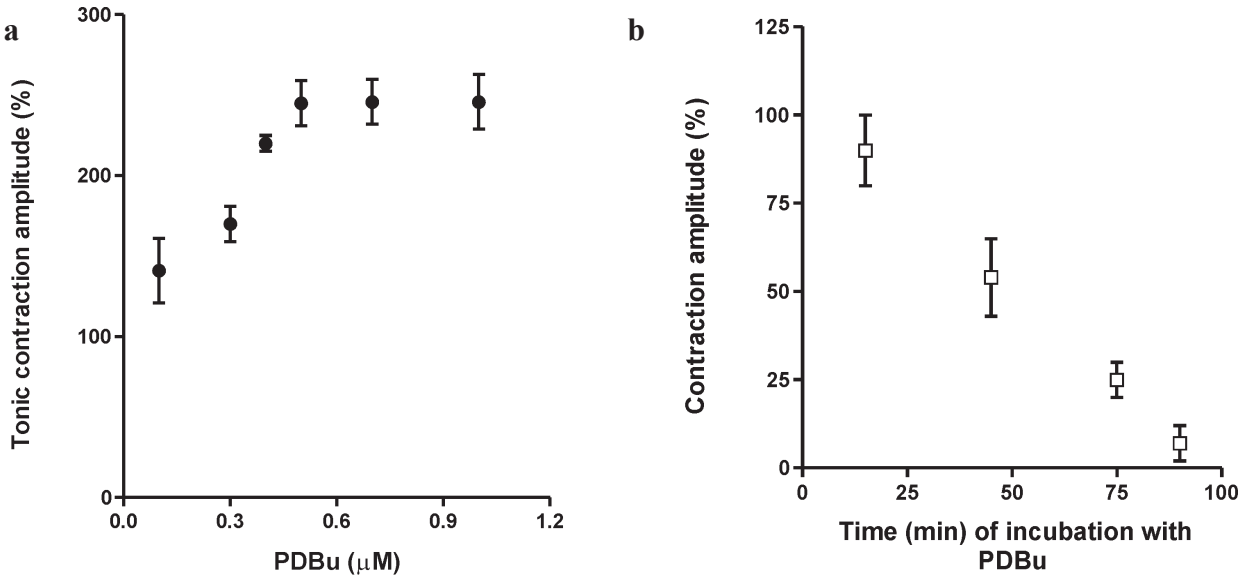

Fig. 4 Effect of PDBu on noradrenaline-induced contraction in two different (a) and (b) experimental situations: **(a)** Addition of PDBu on the plateau of the noradrenaline-induced contraction. Concentration-effect relationship of the rapid potentiation by PDBu of the noradrenaline-induced tonic contraction. Additive contraction produced by PDBu is expressed as a percentage of the tonic contraction produced by 3 μM noradrenaline, taken as 100%. Data shown are means ± s.e. mean (n=4). **(b)** Muscle incubation with PDBu before stimulation with noradrenaline. Inhibitory effect of 1 μM PDBu on the noradrenaline-induced contraction as a function of the time of previous incubation with PDBu. Noteworthy, the inhibitory effect of PDBu appeared after at least 15 min of incubation. Contraction is expressed as a percentage of the peak contraction produced by 3 μM noradrenaline, taken as 100%. Data shown are means ± s.e. mean (n=4).

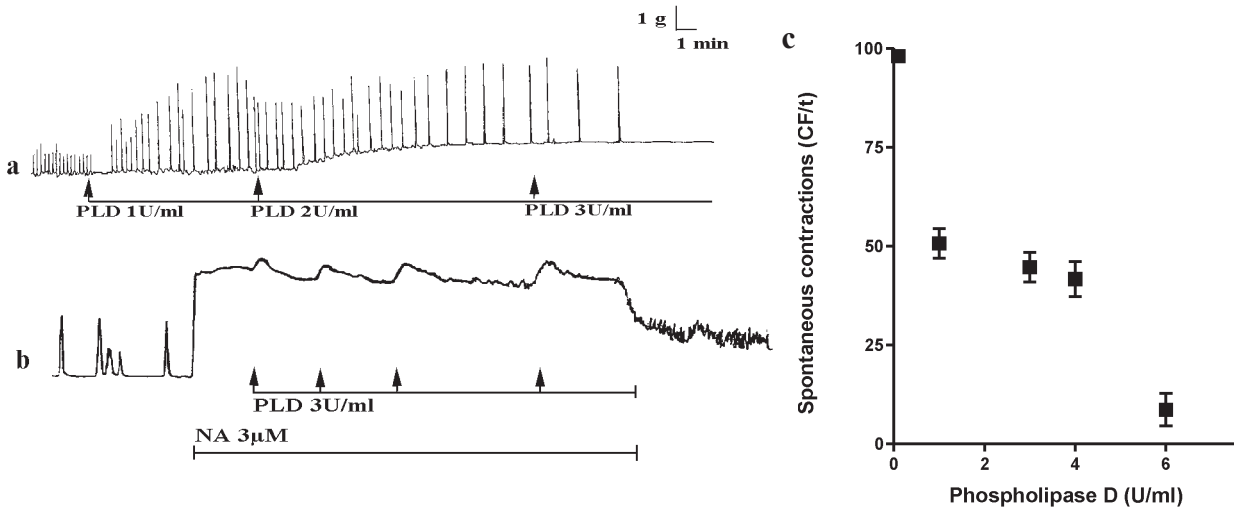

Fig. 5 Effect of phospholipase D on portal vein smooth muscle contraction. **(a)** Phospholipase D (PLD) (1 - 6 U/ml) progressively inhibited the spontaneous contractions. **(b)** When applied on the plateau of the noradrenaline-induced contraction, increasing concentrations of PLD produced transitory contractions followed by slight relaxations. **(c)** Diagram of the concentration dependent inhibitory effect of PLD on spontaneous contractions of portal vein.

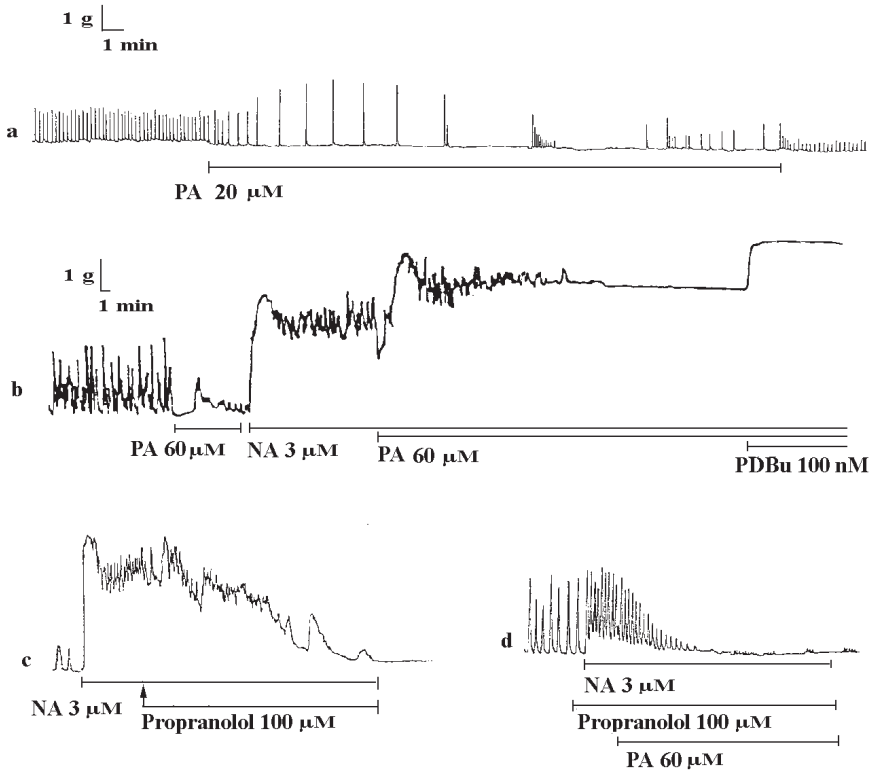

Fig. 6 Effect of second messengers derived from phospholipase D action on noradrenaline-induced contraction. **(a)** Phosphatidic acid (PA, 40 μM) alone inhibited the spontaneous contractions and induced a transient contraction. **(b)** Added during the tonic contraction produced by 3 μM noradrenaline, PA (120 μM) produced transitory potentiation. **(c-d)** Propranolol (100 μM), a PA phosphohydrolase inhibitor, suppressed the stimulatory effect of PA and inhibited the noradrenaline-induced contraction.

lowered the amplitude of superimposed oscillations, and prolonged by approximately 100% the duration of the plateau and of the final oscillations (Fig. 9a). NA (3 μM) added during these prolonged final oscillations failed to elicit a further contraction (not shown), even at 45 min from the preceding stimulation by NA, so that these final oscillations might be defined as a refractory period. The ability of muscle to respond to NA recovered only after 90 min washing-out. Choline also significantly stimulated spontaneous contractions of portal vein smooth muscle (Fig. 9b).

Effect of phospholipase A_2 and arachidonic acid

PLA_2 (2U/ml) induced contractions which exhibited phasic, tonic and oscillatory phases (Fig. 10a) but their amplitude was 45-50% ± 2-5% of the amplitude of 3 μM NA-induced contraction.

AA was able to increase the amplitude and frequency of spontaneous contractions and slightly augmented the basal tone (Figs. 10b,b'). AA had a very strong stimulatory effect of all phases of the NA-induced contraction if added 10 min before NA (not shown), and if added 2 min after NA, shortly potentiated the plateau (Fig. 10c).

Indomethacin (20 μg/ml) reduced all the phases of the NA-induced contraction when added 1h before NA (Fig. 10d), but transiently potentiated the plateau if added 2 min after NA (not shown).

Discussion

Effect of exogenous phospholipases

As previously shown, exogenous PLA_2 and PLC are able to contract intestinal and uterine smooth muscles [20, 21]. Since exogenous PC-PLC and PLA_2 respectively were able to induce a contractile response also in portal vein smooth muscle, the possibility these phospholipases could be directly

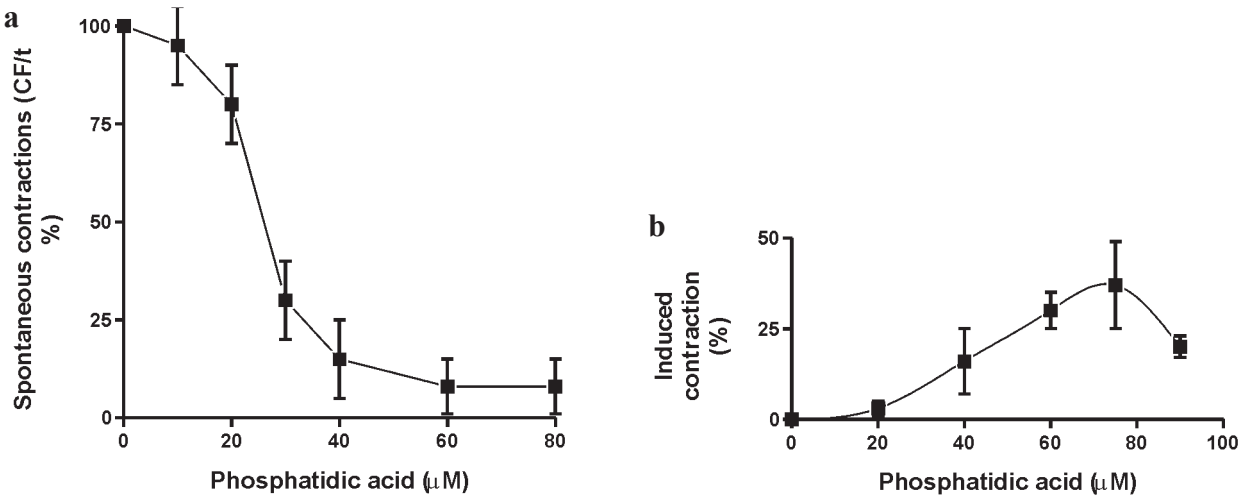

Fig. 7 Effect of phosphatidic acid on portal vein smooth muscle: **(a)** inhibition of spontaneous contractions; **(b)** contraction (% of the standard noradrenaline-induced contraction).

activated *via* receptors cannot be excluded, but in that case, the question is raised whether or not cellular response includes also activation of a PI-PLC.

Exogenous PLD alone had no contractile effect. On the contrary, PLD inhibited spontaneous contractile oscillations. However, when added after NA, exogenous PLD induced short, transient potentiations of the plateau. This indicates that PLD activity may provide a fine regulatory balance between contraction and relaxation, which appear to be dependent of the onset of other simultaneous events. To our knowledge, up-to-date published studies indicate exogenous PLD produced an increase in cardiac ventricular contractile force [22], but do not indicate any contractile effect on smooth muscle.

How do phospholipase-derived messengers contribute to the different phases of the NA-induced contraction?

Phospholipase C-derived messengers

Evidence shows that phasic contraction occurs as a consequence of Ca^{2+} release from the sarcoplasmic reticulum by IP_3, derived from PI-PLC action on PIP_2. In portal vein smooth muscle this initial phase appear to be a prerequisite for the subsequent development of the tonic contraction [16, 17].

The sustained phase of DAG production from PC by a PC-PLC may account for the tonic phase of contraction [5,10] and it is now generally accepted that activation of PKC plays a key role in tonic contraction of arterial smooth muscle [23,24]. In portal vein smooth muscle our results confirm these findings: PDBu, a PKC activator, strongly potentiated the NA - induced tonic contraction and augmented the small tonic contraction induced by PC-PLC. H7, a PKC inhibitor, relaxed the NA-induced contraction. Down-regulation of PKC produced by prolonged exposure to strong concentrations of PDBu also drastically inhibited the contraction.

Phospholipase D-derived messengers

According to the available biochemical studies concerning the arterial smooth muscle cells [10, 25], increasing choline and PA production by PLD takes place at 1 to 5 min, and their maximal production at 5 to 20 min from agonist application. Production of phosphocholine and DAG, due to PC-PLC action is smaller and reaches a plateau during 2 to 20 min from agonist application [25]. We may infer that the time course of the biochemical events may be quite similar in the portal vein smooth muscle. Thus, considering the time course of NA-induced contraction, the development of the tonic phase may correspond to maximal activation of PKC and to increasing PA and choline production. Maximal production of PA

and choline may correspond to the relaxation of the tonic contraction and development of the final oscillations. Therefore, the physiological response during 2 to 20 min from agonist stimulation should result from the combined effect of the produced second messengers (DAG and phosphocholine; PA and choline) and from the ratio of their production.

Interestingly, PLD activity appears to be preferentially performed in the external leaflet of the lipid bilayer, since choline is mostly delivered into the exacellular space, while PA, simultaneously generated, may also be localized in the outer leaflet of the sarcolemma [25]. Therefore, testing the effects of exogenous PA and choline must be of physiological interest, since these messengers are still in search of a definite function [26 - 29].

In our experiments PA had dual effects: arrested spontaneous oscillatory contractions, but also occasionaly produced small contractions of portal vein smooth muscle, or induced transient potentiations of the NA-induced plateau. However, PA failed to potentiate the plateau of the NA-induced contraction in the presence of propranolol. This dual effect and the spontaneous reversibility of some effects may possibly be explained by the fact that PA is subject to rapid metabolization by PLD itself by transphosphatidylation. The nature and the effects of the molecules implicated in transphosphatidylation in portal vein smooth muscle remain to be established. PA is also processed by PA phosphohydrolase that converts PA to DAG with subsequent activation of PKC [30]. Noteworthy, propranolol, an inhibitor of PA phosphohydrolase had the same inhibitory effect as exogenous PA on spontaneous oscillatory contractions (unpublished results) and

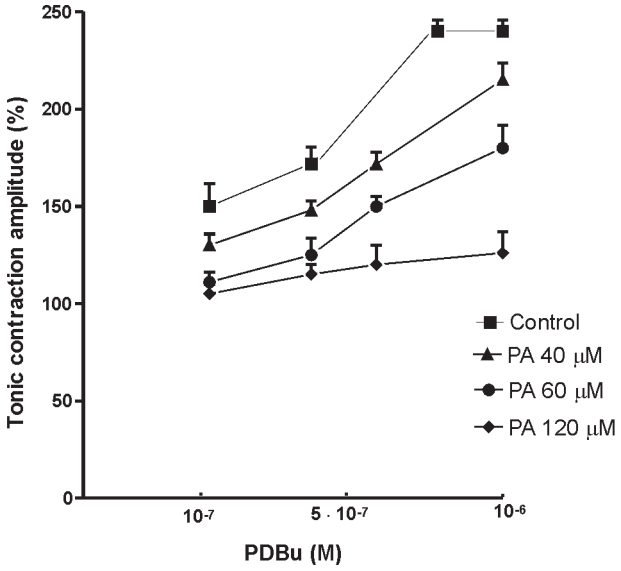

Fig. 8 Potentiation of noradrenaline-induced tonic contraction by phorbol dibutirate (PDBu) in the absence (control) and in the presence of phosphatidic acid (PA).

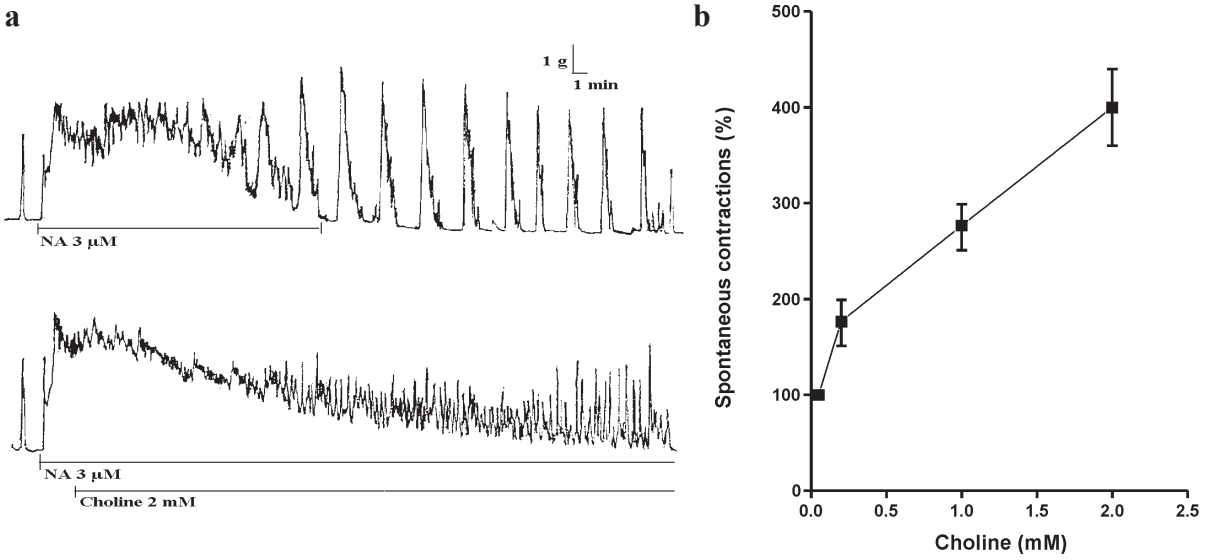

Fig. 9 (a) Choline (2 mM) added during the noradrenaline-induced tonic contraction prolonged the duration of the plateau and of the final oscillations. **(b)** Choline strongly stimulated spontaneous contraction of portal vein smooth muscle.

had a clear relaxant effect on the NA-induced contraction. Propranolol abolished the potentiation of the tonic contraction by exogenous PA. This may indicate that the potentiating effect of PA may be attributed to PA processing to DAG by PA phosphohydrolase. Moreover, the relaxant effect of propranolol was greatly augmented by PA, thus a relaxant effect of PA being probably unmasked. Moreover, PA relaxed the plateau of the NA-induced contraction in the presence of the anti-relaxant anti-PLD antibodies, as previously shown [16].

It was claimed that choline, which is abundant in the extracellular space (about 15 mM in plasma, [31]), would be of little physiological significance. However, exogenous choline (60 μM-2 mM) had a clear stimulatory effect on spontaneous oscillations frequency. Choline also prolonged the sustained phase of the NA-induced contraction, potentiated the plateau, augmented the frequency of final oscillations. Noteworthy, final oscilllations appeared to be a "refractory" state during which agonist stimulation failed to elicit a new response. Therefore, choline favors a "desensitized" state that prevents supramaximal stimulation.

Phospholipase A_2-derived messengers

AA is the product of PLA_2-mediated hydrolysis of phospholipids which are rich in AA [ref. 32, for review]. In our experiments, if added 2 min after NA, AA instantaneously potentiated the tonic contraction and therefore appeared to be directly implicated in the sustaining of the tonic phase. If added before NA, AA strongly potentiated all phases of the contraction, as also shown in some arterial beds [33]. It is already described its stimulatory action on

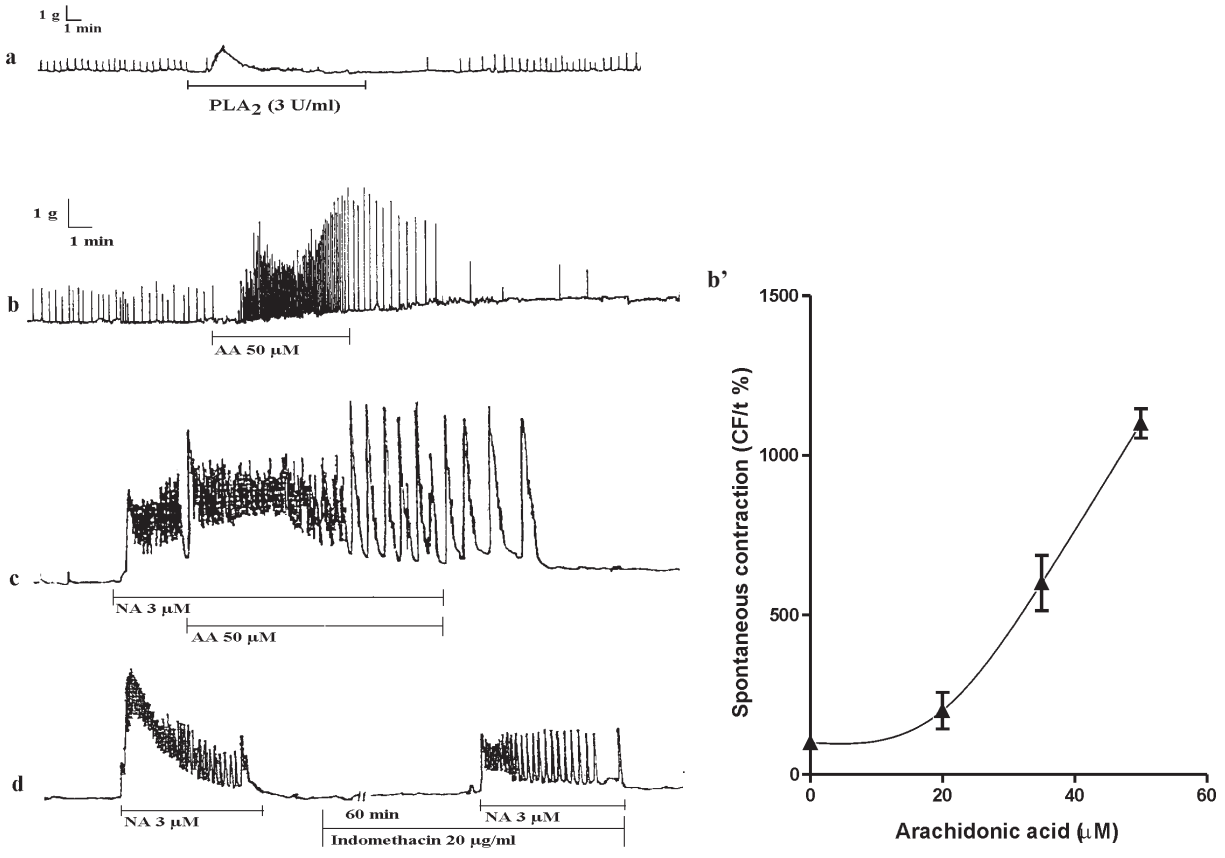

Fig. 10 Effect of phospholipase A_2 and arachidonic acid on portal vein contraction. **(a)** Phospholipase A_2 (PLA_2) induced a low amplitude contraction of portal vein smooth muscle. **(b)(b')** Arachidonic acid (AA) increased the amplitude and frequency of spontaneous contractions. **(c)** AA potentiated the plateau of the noradrenaline-induced contraction. **(d)** Indomethacin (20 μg/ml) reduced all phases of the noradrenaline-induced contraction when applied 1 h before noradrenaline (3 μM) addition.

Ca^{2+} channels, but considering the intensity of the stimulatory effect, further investigations are needed to detect the other sites and mechanisms of its cellular action. The products of the AA cascade also may contribute to the contraction development, since indomethacin, the well-known cyclooxygenase blocker, had a clear inhibitory effect if added 1 hr before NA. Indomethacin, added 2 min after NA potentiated the plateau, presumably because of AA acumulation by lack of metabolization by cyclooxygenase.

Conclusion

Our results show that messengers produced by phospholipase C and phospholipase A_2 contribute to achieve the onset and maintenance of contraction, while phospholipase D-yielded messengers appear to provide a delayed "on/off switch" that ultimately brings relaxation.

These functional data may suggest a more time-related biochemical approach of molecular events in portal vein, to provide an insight in the temporal order of the fine balanced, dual array of signaling activities for smooth muscle contraction and relaxation.

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