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α_1-Adrenoceptor-Mediated Formation of Glycerophosphoinositol 4-phosphate in Rat Heart: Possible Role in the Positive Inotropic Response

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ABSTRACT. In the present study, we investigated whether phospholipase A_2 (PLA_2)/lysophospholipase activity producing glycerophosphoinositols from phosphoinositides was operating in rat heart and could be stimulated by α_1-adrenergic agonists. PLA_2/lysophospholipase activity was found in homogenates from rat right ventricles. The stimulation of PLA_2/lysophospholipase activity by noradrenaline (NA) was prevented either by the α_1-adrenergic antagonist prazosin or arachidonyl trifluoromethyl ketone, a selective inhibitor of the 85–110 kDa, *sn*-2-arachidonyl-specific cytosolic PLA_2. The selective α_1-adrenergic agonist phenylephrine induced a concentration- and time-dependent increase in glycerophosphoinositol (GroPIns) and glycerophosphoinositol 4-phosphate (GroPIns4P) in rat right ventricle slices prelabelled with D-*myo*-[^{3}H]inositol. In electrically driven strips of rat right ventricles, prelabelled with D-*myo*-[^{3}H]inositol, the positive inotropic effect induced by 20 μM NA in the presence of propranolol was accompanied by the formation of GroPIns and GroPIns4P. The concentration of the formed GroPIns4P (1.33 ± 0.12 μM, N = 6) was similar to that previously reported to inhibit the Na^+/Ca^{2+} exchanger in cardiac sarcolemmal vesicles (Luciani S, Antolini M, Bova S, Cargnelli G, Cusinato F, Debetto P, Trevisi L and Varotto R, *Biochem Biophys Res Commun* **206:** 674–680, 1995). These findings show that the stimulation of α_1-adrenoceptors in rat heart is followed by an increase in the formation of GroPIns4P, which may contribute to the positive inotropic effect of α_1-adrenergic agonists by inhibition of the Na^+/Ca^{2+} exchanger. BIOCHEM PHARMACOL **58**;9:1437–1446, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. noradrenaline; phenylephrine; α_1-adrenoceptors; glycerophosphoinositol 4-phosphate; phospholipase A_2/lysophospholipase; Na^+/Ca^{2+} exchanger; rat right ventricle

It is well known that the activated state of α_1-adrenoceptors promotes $PIns4,5P_2$† hydrolysis by PLC to generate DAG and $Ins1,4,5P_3$, the latter being responsible for Ca^{2+} release from sarcoplasmic reticulum with consequent increase in the cytoplasmic Ca^{2+} concentration [for reviews, see Refs. 1 and 2]. In mammalian heart, this signalling pathway has been implicated in the positive inotropic effect of α_1-adrenergic stimulation [1, 3]. However, there is no conclusive evidence that $Ins1,4,5P_3$ mediates the positive inotropic effect of α_1-adrenoceptor agonists, since $Ins1,4,5P_3$ does not seem to play a major role in the regulation of calcium movements in the mammalian heart [2–5]. Other mechanisms have been proposed to participate in the overall positive inotropic response to α_1-adrenergic stimulation, including an increase in myofibrillar responsiveness to Ca^{2+}, promotion of Ca^{2+} influx through voltage-dependent calcium channels, and alteration of Ca^{2+} fluxes through modifications of the Na^+/Ca^{2+} exchanger activity [6, 7]. Studying the regulation of the Na^+/Ca^{2+} exchanger by lipids [8], we observed that PIns4P and $PIns4,5P_2$ stimulated Na^+/Ca^{2+} exchange activity either in reconstituted proteoliposomes or enriched cardiac sarcolemmal vesicles [8, 9]. This observation was recently confirmed by Hilgemann and Ball [10], who showed that $PIns4,5P_2$ generated by lipid kinases in patches of cardiac membranes strongly activates the Na^+/Ca^{2+} exchanger, and by Berberian *et al.* [11], who showed that $PIns4,5P_2$ is involved in the MgATP stimulation of the Na^+/Ca^{2+} exchanger in cardiac sarcolemmal vesicles. In the course of our previous study on the effect of polyphosphoinositides on the cardiac Na^+/Ca^{2+} exchanger, we found that the deacylation products of polyphosphoinositides, GroPIns4P and $GroPIns4,5P_2$, are powerful inhibitors of the Na^+/Ca^{2+} exchanger in cardiac sarcolemmal vesicles [9].

It has been shown that GroPIns4P is produced in rat thyroid cells by a phosphoinositide-specific PLA_2 with lysophospholipase activity [12]. In addition, it has been

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† *Abbreviations:* $AACOCF_3$, arachidonyl trifluoromethyl ketone; DAG, diacylglycerol; GroPIns, glycerophosphoinositol; GroPIns4P, glycerophosphoinositol 4-phosphate; Ins, inositol; $Ins1P_1$, inositol 1-monophosphate; $Ins1,4P_2$, inositol 1,4-bisphosphate; $Ins1,4,5P_3$, inositol 1,4,5-trisphosphate; NA, noradrenaline; PLA_2, phospholipase A_2; PLC, phospholipase C; PIns4P, phosphatidylinositol 4-monophosphate; $PIns4,5P_2$, phosphatidylinositol 4,5-bisphosphate; and PSS, physiological salt solution.

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demonstrated that phosphoinositides can be metabolized by cytosolic PLA_2 in several tissues [13] and that these enzymes can be activated by α_1-adrenergic agonists [14, 15]. However, direct evidence for the α_1-adrenoceptor-mediated stimulation of PLA_2 is still lacking in the mammalian heart [4]. In this context, we investigated whether, in rat cardiac tissue homogenates, PLA_2/lysophospholipase activity producing glycerophosphoinositols from phosphoinositides is present and can be stimulated by NA. Furthermore, we investigated whether α_1-adrenergic agonists stimulate the formation of glycerophosphoinositols in rat right ventricle slices and electrically driven strips. The present results show that PLA_2/lysophospholipase activity is present in rat heart and that α_1-adrenergic agonists stimulate the formation of GroPIns and GroPIns4P in rat right ventricles. A preliminary report of this work has appeared in abstract form [16].

MATERIALS AND METHODS

Chemicals

(−)-Noradrenaline, phenylephrine, (±)-propranolol hydrochloride, and prazosin hydrochloride were obtained from Sigma. $AACOCF_3$ was from Calbiochem. [^{3}H]PIns4P, D-*myo*-[^{3}H]inositol, and $^{45}CaCl_2$ were purchased from NEN-DuPont. [^{3}H]$InsP_3$ assay kit (TRK 1000) was from Amersham. All other chemicals were from Sigma. All drugs were prepared in bidist. water except for noradrenaline and $AACOCF_3$, which were dissolved in 0.1% ascorbic acid and ethanol, respectively.

PLC and PLA_2/Lysophospholipase Activities in Homogenates of Rat Right Ventricles and Left Atria

PLC and PLA_2/lysophospholipase activities were measured in homogenates of cardiac tissue obtained from male Wistar rats (250–300 g). In some experiments (see Table 1), right ventricles or left atria (20–40 mg wet wt) were frozen in liquid nitrogen and then homogenized by mortar grinding in 0.4 mL of either a medium containing 50 mM HEPES/Tris buffer (pH 7.4) and 1 mM $CaCl_2$ (medium A), to evidence PLC activity, or a medium containing 50 mM Tris/maleate buffer (pH 8.0), 500 mM $Na_2S\,O_4$, and 1 mM EDTA [17] (medium B), to evidence PLA_2 activity. [^{3}H]PIns4P (5 nmol) (specific activity, 19.6 mCi/μmol) was dispersed in 0.2 mL of the corresponding medium (A or B). The suspension was vigorously shaken without sonication to avoid lipid degradation, and the reaction was started by adding tissue homogenates. The incubation was carried out at 37° for 30 min, the reaction was stopped by the addition of a mixture of $CHCl_3$/CH_3OH/concentrated HCl (2.0 mL, 200/400/5, v/v/v), and two phases were obtained by the addition of $CHCl_3$ (0.6 mL) and 0.1 N HCl (0.6 mL) [18]. The water-soluble [^{3}H]inositol-containing products of [^{3}H]PIns4P hydrolysis, [^{3}H]inositol phosphates and [^{3}H]glycerophosphoinositols, were separated by HPLC analysis as described below. PLC and PLA_2 activities were expressed as picomoles of Ins, Ins1P_1, Ins1,4P_2, GroPIns, and GroPIns4P released per milligram of tissue wet wt. Negligible hydrolysis of PIns4P occurred in the absence of tissue homogenates.

In other experiments (see Table 2), rat right ventricles were incubated in 10 mL of carbogen-saturated PSS (the same as used in contractile experiments) at 37° for 5 min in the absence and presence of 20 μM NA. At the end of incubation, the ventricles were pulverized in liquid nitrogen in the presence of 0.4 mL of a medium containing 50 mM HEPES/Tris buffer (pH 7.4) and 100 μM $CaCl_2$, to evidence both PLC and PLA_2 activities. Thereafter, the homogenates were incubated with [^{3}H]PIns4P as substrate and processed as described above. When used, 0.1 μM prazosin and 50 μM $AACOCF_3$ were incubated with the ventricles at 37° for 5 and 20 min, respectively, before NA treatment.

α_1-Adrenoceptor-Mediated Formation of Glycerophosphoinositols in Rat Right Ventricle Strips and Slices

Right ventricle strips (about 40 mg) from hearts of male Wistar rats (100–150 g) were placed vertically in an organ bath containing 1 mL of carbogen-saturated PSS and stimulated electrically at 0.1 Hz, and the tension was isometrically recorded as detailed previously [19]. The PSS was of the following composition (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 19, and glucose 5.4 (37° and pH 7.4). To evaluate the formation of phosphoinositide metabolites, the strips were labelled with 80 μCi/mL of D-*myo*-[^{3}H]inositol (specific activity, 21 Ci/mmol) for 3 hr, washed for 10 min with PSS, and then stimulated with 20 μM NA for 2 min in the presence of 1 μM propranolol and 10 mM LiCl. When used, 0.1 μM prazosin was incubated with the ventricle strips at 37° for 20 min before NA treatment. Thereafter, the strips were removed from the bath, quickly frozen in liquid nitrogen, and homogenized in a homogenizer (Polytron) in 0.5 mL of 0.1 N HCl. The water-soluble [^{3}H]inositol-labelled metabolites were then extracted from ventricle homogenates and separated by HPLC analysis as described below.

In right ventricle slices (0.5-mm thick), prepared with a tissue slicer in cold PSS (about 4°), the concentration- and time-dependent formation of glycerophosphoinositols was estimated. The slices were equilibrated in PSS for 30 min at 37° and then incubated with 40 μCi of D-*myo*-[^{3}H]inositol (specific activity, 21 Ci/mmol) in 3 mL of PSS for 2 hr. After a 10-min wash with PSS containing 5 mM D-*myo*-inositol, the slices were incubated either for 10 min with increasing concentrations (25–150 μM) of phenylephrine or for different periods of time (0.5–20 min) with 100 μM phenylephrine. At the end of incubation, the slices were quickly frozen in liquid nitrogen, homogenized, extracted, and assayed for the water-soluble [^{3}H]inositol-labelled metabolites by HPLC analysis as described below.

TABLE 1. Formation of [^{3}H]inositol phosphates and [^{3}H]glycerophosphoinositols from [^{3}H]PIns4P in rat cardiac tissue homogenates

	Medium A				Medium B			
	Ins	Ins1P_1	Ins1,4P_2	GroPIns4P	Ins	Ins1P_1	Ins1,4P_2	GroPIns4P
	(pmol/mg wet wt)							
Atria	14.5 ± 8.6	22.5 ± 0.9	16.0 ± 7.7	ND*	1.4 ± 0.6	1.2 ± 0.1	4.3 ± 1.0	3.1 ± 0.9
Ventricles	10.0 ± 4.2	17.4 ± 3.5	13.0 ± 2.9	ND*	0.9 ± 0.3	0.5 ± 0.1	ND*	0.5 ± 0.1

Rat cardiac tissue homogenates were incubated with [^{3}H]PIns4P in two different media (A and B) and [^{3}H]inositol-labelled metabolites were assayed as described in Materials and Methods. Medium A: 50 mM HEPES/Tris buffer (pH 7.4) and 1 mM $CaCl_2$. Medium B: 50 mM Tris/maleate buffer (pH 8.0), 500 mM $Na_2S\ O_4$, and 1 mM EDTA. The data are expressed as picomoles per milligram of tissue wet weight and are means ± SEM of at least 3 experiments.

*ND, not detectable.

Measurement of Inositol Phosphates and Glycerophosphoinositols

[^{3}H]Inositol phosphates and [^{3}H]glycerophosphoinositols were separated by HPLC analysis with a Partisphere 5 SAX column (Whatman) using a shallow discontinuous gradient of 0–1.0 M ammonium phosphate (pH 3.8) as detailed elsewhere [9]. Radioactivity in the eluate was quantified by collecting fractions followed by liquid scintillation counting. With tissue extracts, peak identification was made on the basis of co-elution with authentic, commercial ^{3}H-labelled Ins1P_1, Ins1,4P_2, Ins1,4,5P_3 (NEN-DuPont), and ^{3}H-labelled GroPIns, GroPIns4P, and GroPIns4,5P_2 prepared as in Luciani *et al.* [9] and, routinely, on the basis of the relative retention times to AMP, ADP, and ATP, used as internal standards. The identity of the putative GroPIns4P peak in aqueous extracts obtained from NA-treated ventricles was further investigated using periodate [20]. Long (36 hr in the dark, at room temperature) periodate oxidation was performed as described in [21] and the resulting products were analysed by HPLC. This treatment totally destroys only the GroPIns4P isomer, since its phosphates are in *para*-orientation [20]. In a sample of authentic [^{3}H]GroPIns4P, oxidized under identical conditions as a control, a complete loss of the radioactivity associated with its peak was obtained.

Effect of Noradrenaline on the Na^+/Ca^{2+} Exchange Activity of Isolated Rat Ventricular Myocytes

Rat ventricular myocytes, isolated as described elsewhere [22], were suspended in Ca^{2+}-free Joklik modified Eagle's medium (MEM) in 10 mM HEPES/Tris (pH 7.4) supplemented with 1% BSA. Cell suspensions (1.7–1.9 mg of cell protein) were loaded with sodium as described previously [22] and then incubated in the absence and presence of 20 μM NA at 37° for 10 min. At the end of incubation, cell suspensions were washed in NA-free medium and assayed for Na^+/Ca^{2+} exchange activity as $^{45}Ca^{2+}$ uptake dependent on intracellular Na^+ concentration at 30 and 60 sec as described previously [22].

Protein Determination

Proteins were assayed according to the method of Bensadoun and Weinstein [23], with BSA as standard.

Statistical Analysis

The statistical significance of the observed differences was analysed by the Student's two-tailed *t*-test for unpaired observations and by one-way ANOVA followed by posthoc comparison with the Bonferroni test, when three conditions were compared (Fig. 2). In all cases, differences were regarded as significant when $P < 0.05$.

RESULTS

PLC and PLA_2/Lysophospholipase Activities in Rat Heart Homogenates

To estimate PLC and PLA_2/lysophospholipase activities, homogenates of rat right ventricles and left atria were incubated with [^{3}H]PIns4P, and the formation of the corresponding water-soluble products, [^{3}H]inositol phosphates and [^{3}H]glycerophosphoinositols, was measured. When these experiments were performed in a medium usually employed to test PLC activity, only Ins, Ins1P_1, and Ins1,4P_2 were produced, while GroPIns4P was undetectable (Table 1, Medium A). When an assay condition (pH 8.0, no calcium, and high sodium) known to favour PLA_2/lysophospholipase activity [17] was employed, the levels of Ins, Ins1P_1, and Ins1,4P_2, both in atria and ventricles, were lower than those formed in medium A, while GroPIns4P was produced in percentages of 1.96 ± 0.32% (N = 3) and 1.07 ± 0.50% (N = 3) in homogenates of rat right ventricles and left atria, respectively (Table 1, Medium B). Similar results were obtained using [^{3}H]PIns4,5P_2 as the substrate. These findings indicate that PLA_2/lysophospholipase activity is present in rat heart.

To investigate whether PLC and PLA_2/lysophospholipase activities could be stimulated by α_1-adrenergic agonists, we evaluated the formation of [^{3}H]inositol phosphates and [^{3}H]glycerophosphoinositols from [^{3}H]PIns4P in homogenates prepared from either control or NA-treated rat right ventricles. For this purpose, we used a medium (pH 7.4 and low calcium) that meets the requests for the

TABLE 2. Effect of NA treatment of rat right ventricles on the formation of [^{3}H]inositol phosphates and [^{3}H]glycerophosphoinositols in homogenized ventricles incubated with [^{3}H]PIns4P

	Total (pmol/mg wet wt)						
	Ins	Ins1P_1	Ins1,4P_2		GroPIns	GroPIns4P	Total
Control	5.5	11.5	1.9	18.9	0.142	0.035	0.177
20 μM NA	10.0	21.0	5.4	36.5	0.253	0.151	0.404
Control + $AACOCF_3$	4.4	7.5	4.1	15.9	0.135	0.040	0.175
20 μM NA + $AACOCF_3$	11.1	21.2	5.4	37.7	0.176	0.010	0.186
20 μM NA + prazosin	4.1	8.1	5.7	18.0	0.131	0.050	0.181

Rat right ventricles were stimulated or not (control) for 5 min with 20 μM NA before tissue homogenates were made. The homogenates were then incubated with [^{3}H]PIns4P in a medium containing 50 mM HEPES/Tris buffer (pH 7.4) and 100 μM $CaCl_2$ as described in Materials and Methods. When indicated, ventricles were treated with or without 0.1 μM prazosin and 50 μM $AACOCF_3$ for 5 and 20 min, respectively, before addition of 20 μM NA. [^{3}H]inositol-labelled metabolites were assayed as described in Materials and Methods. The data are expressed as picomoles per milligram of tissue wet weight. Single representative experiments are shown. Two additional experiments for each condition gave qualitatively similar results.

expression of the activities of both PLC and PLA_2/lysophospholipase. Treatment of the ventricles with NA induced a 2-fold increase over control levels of either inositol derivatives (Ins, Ins1P_1, and Ins1,4P_2) or glycerophosphoinositols (GroPIns and GroPIns4P) in ventricle homogenates (Table 2). The increase of the inositol derivatives GroPIns and GroPIns4P was suppressed by exposure of the ventricles to the α_1-adrenergic antagonist prazosin prior to NA treatment (Table 2), while pretreatment of the ventricles with $AACOCF_3$, a selective inhibitor of 85–110 kDa PLA_2 [24], prevented only the NA-induced increase in the level of both GroPIns and GroPIns4P, leaving unaffected the level of inositol derivatives (Table 2).

α_1-Adrenoceptor-Mediated Formation of Glycerophosphoinositols in Rat Right Ventricle Strips and Slices

To investigate whether the positive inotropic response induced by α_1-adrenergic agonists is accompanied by formation of glycerophosphoinositols, rat right ventricular strips electrically driven at 0.1 Hz were used. In these preparations, a cumulative concentration–response curve to NA (not shown) in the presence of 1 μM propranolol gave an almost maximal increase in the force of contraction at 20 μM NA. When rat right ventricle strips prelabelled with D-*myo*-[^{3}H]inositol were exposed to 20 μM NA for 2 min in the presence of propranolol, an increase in the force of contraction of about 2-fold was accompanied by an increase in the level of the water-soluble [^{3}H]inositol-containing metabolites compared to the control. The HPLC profiles of water-soluble inositol metabolites in NA-treated strips (Fig. 1) showed that NA increased the level of Ins1P_1, Ins1,4P_2, Ins1,4,5P_3, and two compounds that were tentatively identified as GroPIns (peak nr 2) and GroPIns4P (peak nr 4) on the basis of the co-elution with authentic [^{3}H]GroPIns and [^{3}H]GroPIns4P, respectively, and the retention times relative to the internal standards (AMP, ADP, and ATP). A further investigation of the structure of GroPInsP species in NA-treated ventricles by means of long periodate oxidation (see Materials and Methods) showed a loss of 85 ± 7% (N = 3) of the radioactivity associated with the putative GroPIns4P, thus confirming that the majority of this peak is GroPIns4P. As shown in Fig. 2A, a 4.2- and 1.6-fold increase in the concentration of GroPIns and GroPIns4P, respectively, was found. An approximate 2-fold increase in the concentration of all the inositol phosphates was also observed (Fig. 2B), in agreement with the findings of Poggioli *et al.* [25] in isolated rat ventricles. The α_1-adrenergic antagonist prazosin prevented the increase in the formation of both glycerophosphoinositols and inositol phosphates induced by NA (Fig. 2). Similar results were obtained when phenylephrine (100 μM) was used instead of NA (not shown).

To determine whether the levels of GroPIns4P generated by NA stimulation were similar to those previously found effective in inhibiting the cardiac Na^+/Ca^{2+} exchanger [9], the concentration of GroPIns4P attained in rat right ventricular strips stimulated with NA was estimated. Since no direct method to evaluate the mass of GroPIns4P is presently available, the specific activity of [^{3}H]Ins1,4,5P_3 was calculated by measuring intracellular Ins1,4,5P_3 content by a specific receptor-binding assay kit (TRK 1000, Amersham). The specific activity obtained for Ins1,4,5P_3 was assumed for all the water-soluble [^{3}H]inositol-containing metabolites and thus used to calculate the molarity of GroPIns4P, on the basis of an estimated intracellular volume of 25 pL per cardiomyocyte [22]. The calculated concentration of GroPIns4P, 1.33 ± 0.12 μM (N = 6), was similar to that (1.6 μM) found to produce half-maximal inhibition of Na^+/Ca^{2+} exchange activity in cardiac sarcolemmal vesicles [9].

In order to evaluate the time–course and concentration dependency of GroPIns and GroPIns4P formation, rat right ventricle slices were used and stimulated with the selective α_1-adrenergic agonist phenylephrine. The concentration–response relationship of the production of glycerophosphoinositols and inositol phosphates induced by phenylephrine was determined 10 min after the addition of increasing concentrations of the drug (Fig. 3). The levels of GroPIns and GroPIns4P increased with phenylephrine in a concentration-dependent manner, reaching a maximal increase of 3.2- and 2.2-fold, respectively, at 100 μM phenylephrine

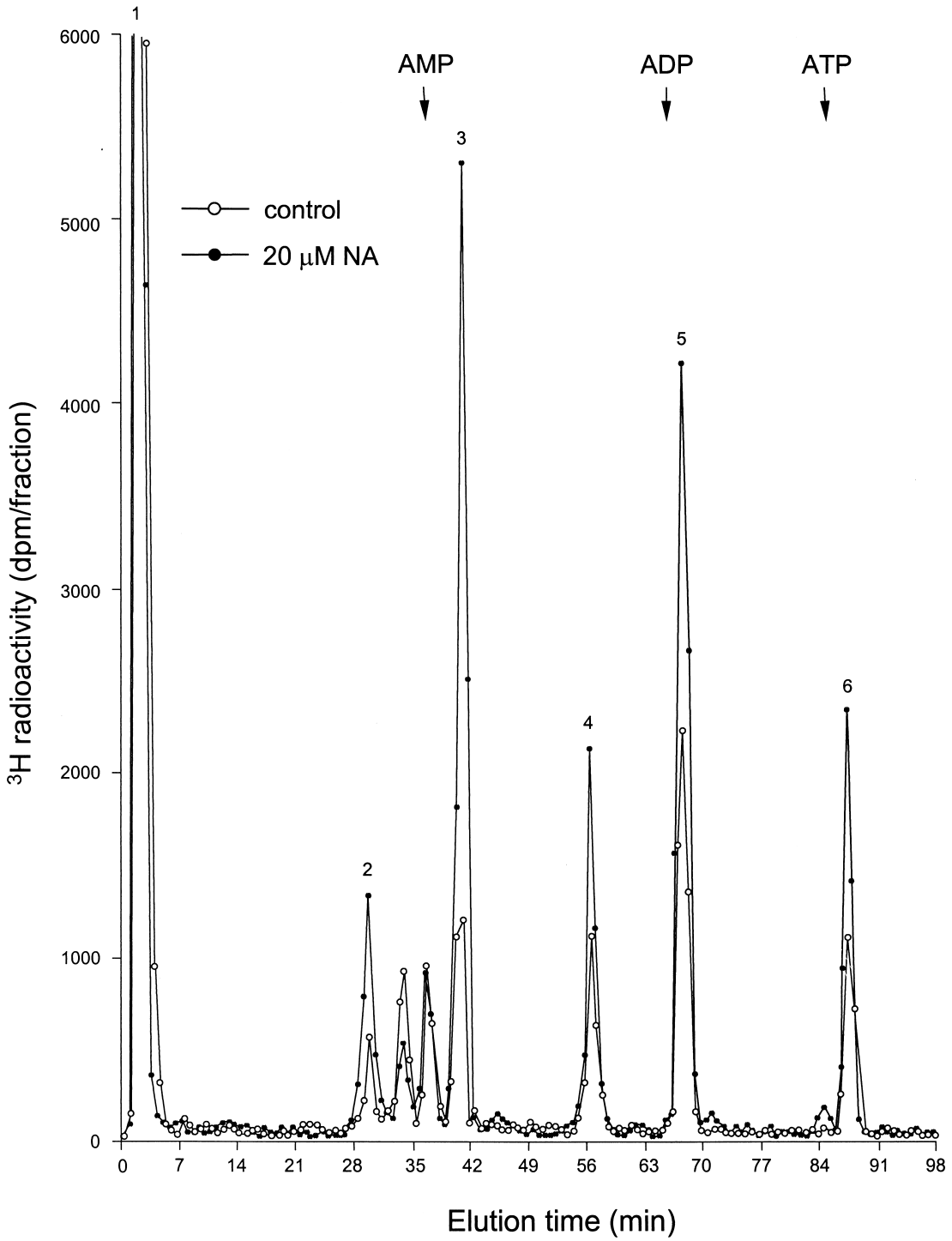

FIG. 1. HPLC elution patterns of water-soluble [^{3}H]inositol-containing metabolites in rat right ventricle strips prelabelled with D-*myo*-[^{3}H]inositol, stimulated electrically at 0.1 Hz, and then incubated for 2 min in the absence (control) and presence of 20 μM NA, in PSS containing 1 μM propranolol and 10 mM LiCl. Labelling, extraction, and HPLC analysis were performed as detailed under Materials and Methods. Numbers indicate the elution positions of the different metabolites, identified as in Materials and Methods: 1, Ins; 2, GroPIns; 3, Ins1P_1; 4, GroPIns4P; 5, Ins1,4P_2; 6, Ins1,4,5P_3.

(Fig. 3A). The level of Ins1P_1 reached a maximal increase of 3.6-fold at 150 μM phenylephrine, while no significant increase was observed for either Ins1,4P_2 or Ins1,4,5P_3 (Fig. 3B). As shown in Fig. 4A, the formation of GroPIns4P induced by 100 μM phenylephrine occurred in a time-dependent manner: it became appreciable at 5 min, peaked at 10 min (2.2-fold), and declined thereafter. GroPIns was undetectable up to 2 min, either in control or treated ventricle slices, and showed its highest increase (3.2-fold) at 10 min. The time dependency of Ins1P_1 formation

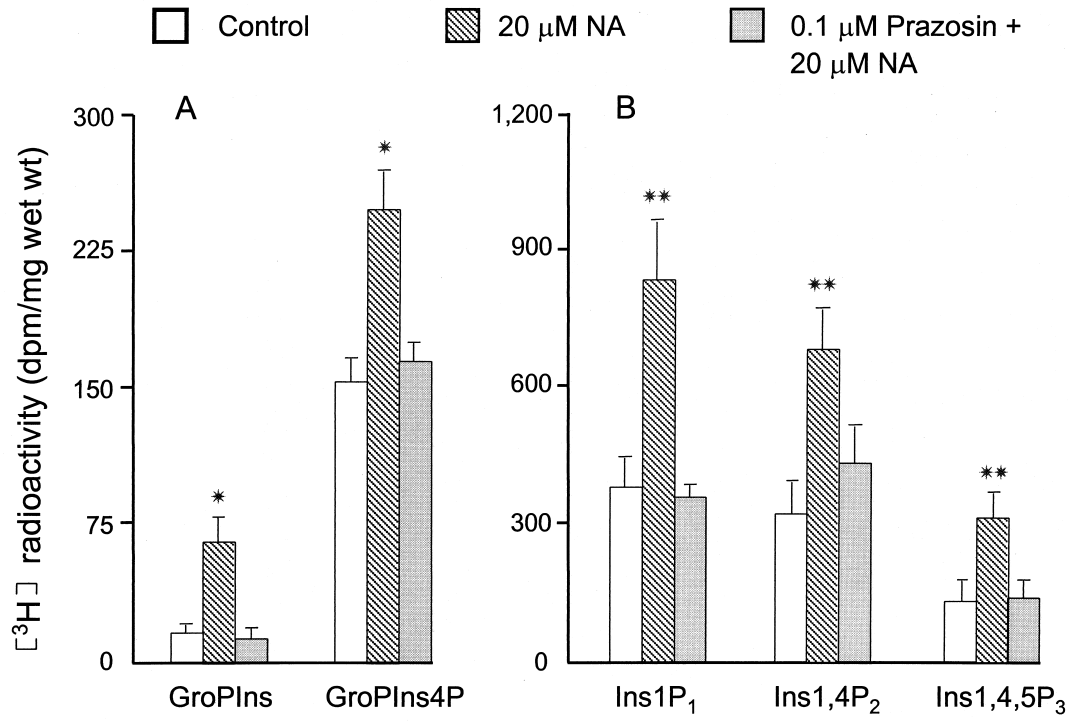

FIG. 2. Levels of [^{3}H]glycerophosphoinositols (A) and [^{3}H]inositol phosphates (B) in rat right ventricle strips prelabelled with D-*myo*-[^{3}H]inositol, stimulated electrically at 0.1 Hz, and then incubated for 2 min in the absence (control) and presence of 20 μM NA, in PSS containing 1 μM propranolol and 10 mM LiCl. When indicated, ventricular strips were incubated with 0.1 μM prazosin for 20 min before exposure to NA. GroPIns, GroPIns4P, Ins1P$_1$, Ins1,4P$_2$, and Ins1,4,5P$_3$ were assayed and identified as described in Materials and Methods. Data are means ± SEM of at least 3 experiments. *P < 0.002 and **P < 0.005, compared with the corresponding control values.

showed a maximal 3.3-fold increase after 10 min and declined thereafter, while the level of both Ins1,4P$_2$ and Ins1,4,5P$_3$ reached a maximal increase of 1.9- and 1.7-fold, respectively, after 20 min (Fig. 4B).

Experiments on Isolated Rat Ventricular Myocytes

The observation that α_1-adrenergic stimulation induces the formation of GroPIns4P in rat heart and that GroPIns4P inhibits Na^+/Ca^{2+} exchange activity in cardiac sarcolemmal vesicles [9] prompted us to investigate whether cardiac Na^+/Ca^{2+} exchange activity was inhibited by NA treatment. For this purpose, we used freshly isolated rat ventricular myocytes [22]. When the exchange activity was measured in these cells after a 10-min preincubation in the absence and presence of 20 μM NA, a 40% inhibition of the Na^+/Ca^{2+} exchange activity compared to the control was found (Fig. 5).

DISCUSSION

This study shows that PLA$_2$/lysophospholipase activity forming glycerophosphoinositols from inositol lipids is present in rat heart and that the stimulation of this enzyme by α_1-adrenergic agonists results in increased levels of GroPIns and GroPIns4P. The findings obtained with ventricle homogenates strongly suggest that the α_1-adrenoceptor-coupled enzyme might be the 85–110 kDa, *sn*-2-arachidonyl-specific cytosolic PLA$_2$ [26], since it is stimulated by micromolar concentrations of calcium and inhibited by AACOCF$_3$, a trifluoromethyl ketone analogue of arachidonyl acid, selective inhibitor of cytosolic PLA$_2$ [24]. These observations indicate that, in rat heart, α_1-adrenoceptors are coupled to activation not only of PLC but also of PLA$_2$, generating inositol phosphates and glycerophosphoinositols, respectively, from PIns4,5P$_2$. Although direct evidence for the α_1-adrenergic-coupled activation of PLA$_2$ has been reported in other tissues [14, 15], the present study shows for the first time the involvement of this pathway in rat heart. The finding that α_1-adrenergic agonists stimulate PLA$_2$, thus increasing the formation of GroPIns and GroPIns4P from polyphosphoinositides, suggests that this pathway could play a role in the positive inotropic response to α_1-adrenergic agonists. The experiments performed in electrically driven ventricle strips show that NA, at a concentration inducing a positive inotropic effect, increases the level of GroPIns and GroPIns4P. This effect is specifically mediated through α_1-adrenoceptor activation, since it is observed in the presence of the β-blocker propranolol and is inhibited by the α_1-adrenergic antagonist prazosin.

It is well established that the stimulation of α_1-adreno-

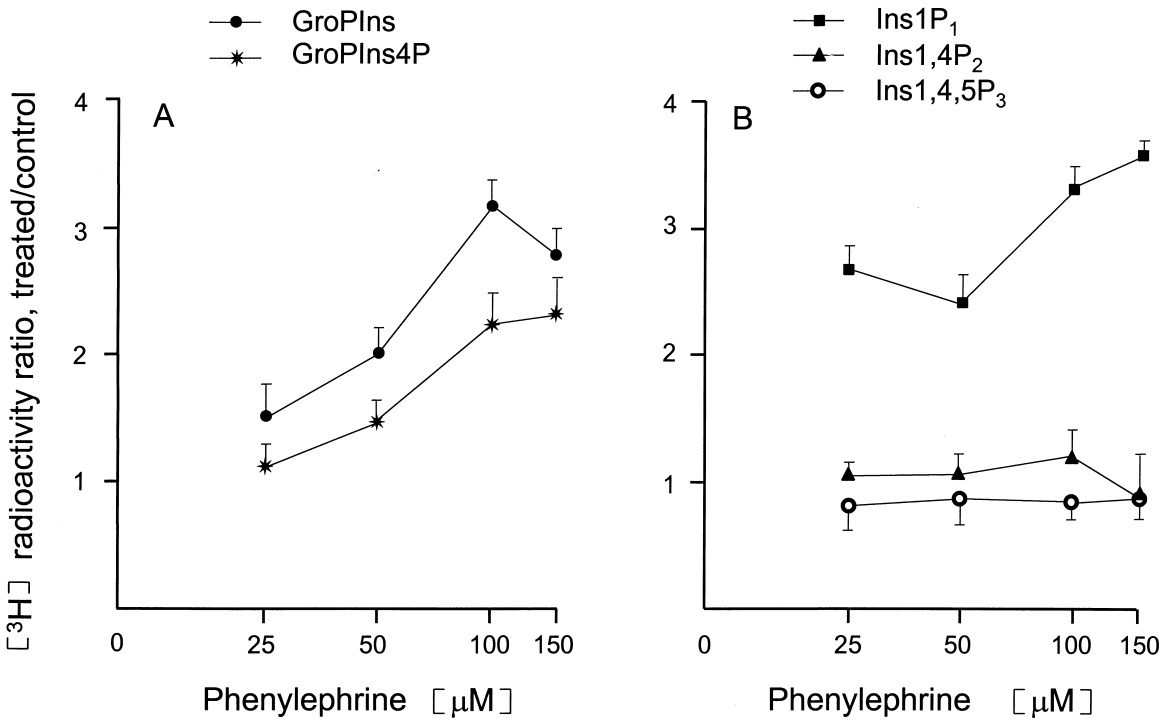

FIG. 3. Concentration–response curves for phenylephrine-induced formation of [^{3}H]glycerophosphoinositols (A) and [^{3}H]inositol phosphates (B) in rat right ventricle slices. Ventricle slices were incubated for 10 min in the absence (control) and presence (treated) of increasing concentrations of phenylephrine and assayed for GroPIns, GroPIns4P, Ins1P_1, Ins1,4P_2, and Ins1,4,5P_3 as described in Materials and Methods. Data are expressed as the ratio between the values of [^{3}H] radioactivity (dpm/mg protein) obtained in treated and control slices. Data are means ± SEM of 4 experiments. Basal values for GroPIns, GroPIns4P, Ins1P_1, Ins1,4P_2, and Ins1,4,5P_3 in control slices were 268 ± 50, 421 ± 34, 1289 ± 106, 3270 ± 251, and 3641 ± 222 dpm/mg protein (N = 15), respectively.

ceptors activates the breakdown of PIns4,5P_2, thus producing DAG and Ins1,4,5P_3, but the time–course of Ins1,4,5P_3 formation exhibits poor correlation with the positive inotropic effect of α_1-adrenergic agonists. In fact, Ins1,4,5P_3 is only transiently increased following α_1-adrenoceptor stimulation, whereas the positive inotropic effect is sustained [3, 5]. Thus, it would seem that Ins1,4,5P_3 might not be the major second messenger for the cardiac effects induced by α_1-adrenergic agonists. Recent studies have suggested that the positive inotropic response of the myocardium to α_1-adrenoceptor agonists might involve the other limb of the phosphoinositide pathway, sustained by DAG. In fact, DAG, producing prolonged activation of protein kinase C, can phosphorylate contractile proteins and/or calcium channels [27, 28], thus modulating cardiac contractility. In this study, the observed 2.4-fold increase in Ins1,4,5P_3 induced by NA in electrically driven rat right ventricles is very similar to that reported in the literature upon α_1-adrenergic stimulation in mammalian heart [3]. However, at variance with data in the literature [29], in rat ventricular slices a concentration and time dependency is found only for Ins1P_1 but not for Ins1,4,5P_3. This discrepancy is likely due to the absence in our incubation media of lithium, which preserves Ins1,4,5P_3 levels [30, 31].

Concerning the role of GroPIns4P, our previous finding that GroPIns4P is a powerful inhibitor of the Na^+/Ca^{2+} exchanger in cardiac sarcolemmal vesicles together with the observed stimulating effect of PIns4,5P_2 on the Na^+/Ca^{2+} exchanger [8, 9] led us to postulate that polyphosphoinositides may have a bidirectional regulatory role on the cardiac Na^+/Ca^{2+} exchanger [9]: an up-regulation by PIns4,5P_2 and a down-regulation by the product of PIns4,5P_2 hydrolysis, GroPIns4P, resulting from α_1-adrenoceptor-mediated activation of PLA_2. A role for PIns4,5P_2 as a signalling messenger in the regulation of the cardiac Na^+/Ca^{2+} exchanger [32] and in functional coupling to the Ins1,4,5P_3 receptor in neuronal cells [33] has recently been proposed. Furthermore, it has been shown that GroPIns4P is an inhibitor of phosphoinositide-specific PLC of guinea-pig uterus [34] and of adenylyl cyclase in rat thyroid cells transformed by the k-*ras* oncogene [12]. On the basis of this latter effect, GroPIns4P has been proposed as a second messenger in ras-induced transformation [35, but see 36].

Here, we show that the content of GroPIns and GroPIns4P increases upon α_1-adrenergic stimulation. The time–course of GroPIns4P formation induced by phenylephrine in rat right ventricle slices shows a substantial, late, and long-lasting increase in the levels of this metabolite. This observation does not allow us to establish a causal relationship between the increase in GroPIns4P and the onset of a positive inotropic effect induced by α_1-adrenergic agonists, but it may suggest a role for GroPIns4P in the

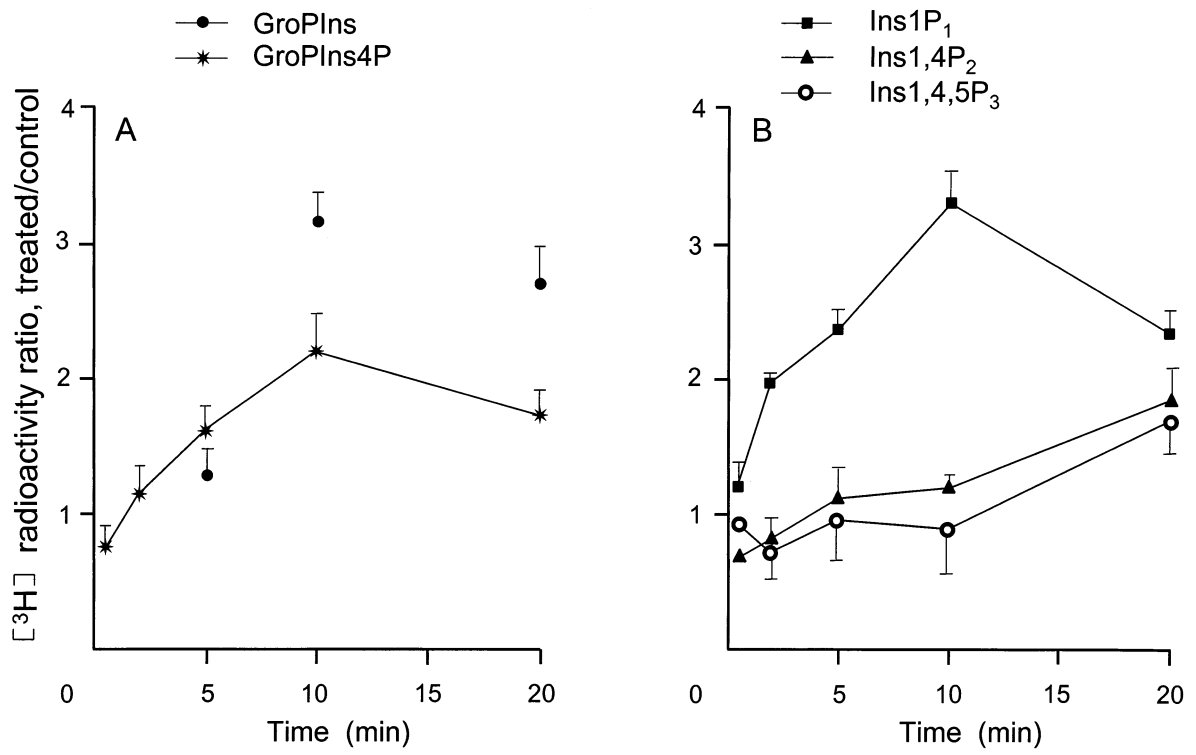

FIG. 4. Time–course of the formation of [^{3}H]glycerophosphoinositols (A) and [^{3}H]inositol phosphates (B) induced by 100 μM phenylephrine in rat right ventricle slices. Ventricle slices were incubated for different periods of time in the absence (control) and presence (treated) of 100 μM phenylephrine and assayed for GroPIns, GroPIns4P, Ins1P_1, Ins1,4P_2, and Ins1,4,5P_3 as described in Materials and Methods. Data are expressed as the ratio between the values of [^{3}H] radioactivity (dpm/mg protein) obtained in treated and control slices. Data are means ± SEM of 4 experiments. Basal values in control slices ranged between: 197 ± 28 and 353 ± 34 (GroPIns); 303 ± 53 and 562 ± 72 (GroPIns4P); 1017 ± 134 and 2664 ± 228 (Ins1P_1); 2284 ± 200 and 4802 ± 321 (Ins1,4P_2); 2449 ± 225 and 3647 ± 303 (Ins1,4,5P_3) dpm/mg protein (N = 4).

maintenance of the positive inotropic response to α_1-adrenergic stimulation in rat heart. The molecular mechanism underlying the positive inotropic effect of GroPIns4P could consist in an increase in intracellular Ca^{2+} concentration through inhibition of Ca^{2+} efflux via the Na^+/Ca^{2+} exchanger [37]. In fact, the estimated intracellular concentrations of GroPIns4P attained in rat right ventricles upon α_1-adrenergic stimulation are similar to those found to be active in inhibiting the cardiac sarcolemmal Na^+/Ca^{2+} exchanger [9]. This hypothesis is indirectly sustained by the observation that NA inhibits the Na^+/Ca^{2+} exchange activity of isolated rat ventricular myocytes (Fig. 5). Accordingly, a modification of the Na^+/Ca^{2+} exchange activity has been previously put forward to explain the positive inotropic effect induced by α_1-adrenergic agonists [6, 7].

Since the increase in GroPIns and GroPIns4P takes place over several minutes, we cannot dismiss the alternative possibility that the accumulation of GroPIns and GroPIns4P, produced by the PLA_2-catalyzed PIns4,5P_2 hydrolysis, may be unrelated to the positive inotropic response and is likely involved in the cytotoxicity (apoptosis) exerted by the sustained elevation of $[Ca^{2+}]_i$. Goldmann *et al.* [38] recently suggested that PLA_2 activation contributes to the toxicity exerted by chronic elevation of $[Ca^{2+}]_i$ in a human keratinocyte cell line.

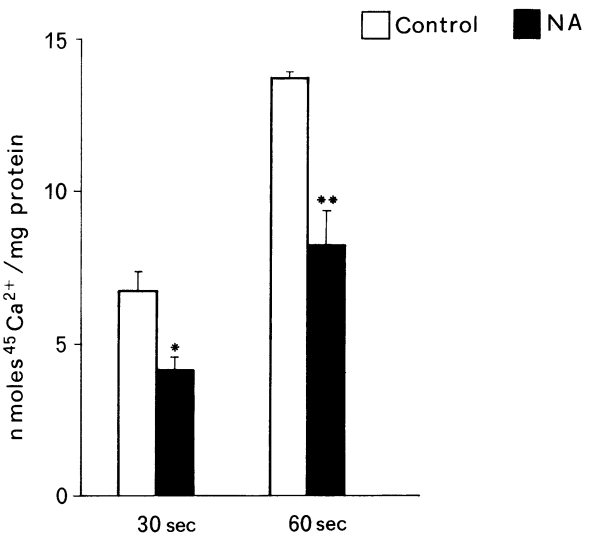

FIG. 5. Effect of NA treatment on Na^+/Ca^{2+} exchange activity in rat ventricle myocytes. Na^+/Ca^{2+} exchange activity was measured at 30 and 60 sec in cardiomyocytes preincubated for 10 min at 37° in the absence (control) and presence of 20 μM NA as $^{45}Ca^{2+}$ uptake dependent on intracellular Na^+ concentration, as described in Materials and Methods. Data are means ± SEM of 3 experiments. *P < 0.025 and **P < 0.025, compared with the corresponding control values.

In summary, we have demonstrated that, in rat right ventricles, α_1-adrenergic agonists activate, in addition to PLC, a PLA_2/lysophospholipase leading to the formation of GroPIns4P from $PIns4,5P_2$. This phosphoinositide metabolite may negatively regulate the Na^+/Ca^{2+} exchange activity with consequent reduction in Ca^{2+} efflux, thus contributing to the positive inotropic effect of α_1-adrenergic agonists. Therefore, our results are suggestive of a role for GroPIns4P as an intracellular messenger of a signal transduction pathway triggered by α_1-adrenoceptor-mediated stimulation of PLA_2, operating in concert with $Ins1,4,5P_3$ and DAG produced by the stimulation of PLC.

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