Metabolism of inositol phosphates in α_1 -adrenoceptor-stimulated and homogenized cardiac myocytes of adult rats

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Previous studies demonstrated the accumulation of inositol mono- and poly-phosphates in carbamoylcholine-stimulated cultured cardiac ventricular myocytes of adult rats [Berg, Guse & Gercken (1989) *Biochim. Biophys. Acta* 1010, 100–107]. Stimulation with noradrenaline (50 μM) in the presence of propranolol (10 μM) led to a time-dependent pattern of inositol mono- and poly-phosphates in cultured cardiac-ventricular myocytes. $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ increased in a rapid initial phase. The degradation products of $\text{Ins}(1,4,5)\text{P}_3$, namely $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(4)\text{P}$, accumulated between 1 and 15 min. $\text{Ins}(1,3,4,5)\text{P}_4$ was rapidly dephosphorylated to $\text{Ins}(1,3,4)\text{P}_3$, which was then metabolized to $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}(3,4)\text{P}_2$. The last two InsP_2 isomers and their degradation products, $\text{Ins}(1)\text{P}$ and $\text{Ins}(3)\text{P}$ (determined as an enantiomeric mixture), increased at extended stimulation periods. To confirm the pathway of $\text{Ins}(1,3,4,5)\text{P}_4$ degradation, homogenates of isolated ventricular myocytes were incubated with $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$. The subsequent products were $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(3,4)\text{P}_2$, $\text{Ins}(1,3)\text{P}_2$ and InsP . The effect of noradrenaline was antagonized by prazosin (0.1 μM), but not by yohimbine (0.1 μM), indicating phosphoinositidase activation via the α_1 -adrenoceptor.

INTRODUCTION

In mammalian heart, phosphoinositidase was shown to be activated via the muscarinic cholinergic receptor [1,2], the α_1 -adrenoceptor [2,3,4] and the purinoceptor P_2 [5]. Although some of the inositol mono- and poly-phosphate isomers were found in response to agonist stimulation in rat heart [1,4,6], a study on the whole set of metabolic products of the subsequent metabolism of $\text{Ins}(1,4,5)\text{P}_3$ as described for other cells is still lacking.

Consequently we have addressed several specific points raised by our and others' studies [1,6]: (1) the time courses of individual inositol mono- and poly-phosphate isomers in α_1 -adrenoceptor-stimulated cultured cardiac myocytes, with special interest in InsP , InsP_2 and InsP_3 isomers; (2) the subsequent metabolism of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ and $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ through distinct pathways in homogenized cardiac myocytes; (3) the effects of the α_1 - and α_2 -adrenoceptor antagonists prazosin and yohimbine on α_1 -adrenoceptor-induced inositol phosphate metabolism in cultured cardiac myocytes.

EXPERIMENTAL

Materials

(–)-Noradrenaline, propranolol, prazosin, saponin, yohimbine and phosphocreatine were purchased from Sigma, St. Louis, MO, U.S.A. *myo*- $[^3\text{H}]\text{Inositol}$ (sp. radioactivity 80–120 Ci/mmol) was from Amersham-Buchler, Braunschweig, Germany. The $[^3\text{H}]\text{inositol}$ phosphates used as standards and substrates were obtained from NEN, Dreieich, Germany. Creatine kinase was from Boehringer, Mannheim, Germany. All other materials were essentially the same as described elsewhere [1]. Filtered water from a Milli-Q water-purification

system (Millipore–Waters, Eschborn, Germany) was used throughout.

Cell culture, labelling and stimulation experiments

The isolation procedure for ventricular myocytes, labelling with *myo*- $[^3\text{H}]\text{inositol}$ of the cultured cells and sample preparation for h.p.l.c. were carried out as described previously [1]. Before stimulation with noradrenaline (final concn. 50 μM), the cultured myocytes were incubated for 10 min with LiCl (10 mM) and propranolol (10 μM) and, when drug antagonism was examined, with prazosin (0.1 μM) or yohimbine (0.1 μM).

Preparation of homogenized myocytes

Myocytes were isolated as described previously [1]. Instead of plating the cell suspension on culture dishes, the cells from one heart were suspended in 2 ml of an intracellular buffer (KCl 110 mM, NaCl 10 mM, KH_2PO_4 1 mM, Hepes 20 mM, MgCl_2 4 mM, EGTA 1 mM, Na_3ATP 3 mM, phosphocreatine 8 mM, creatine kinase 6 units/ml, saponin 0.2 mg/ml, pH 7.0) and immediately placed on ice. Homogenates were then prepared by ten strokes of a glass/Teflon homogenizer. The resulting suspension was centrifuged (Eppendorf centrifuge, 12000 rev./min for 10 min at 4 °C), and the supernatant was used to study $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ metabolism.

Degradation of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$ by a myocyte homogenate

The reaction mixture consisted of 100 μl of homogenate, 25 μl of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ or $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (3.7 kBq) and 250 μl of the intracellular buffer described above, to give a total volume of 375 μl . The reaction was performed at 37 °C. To terminate the reaction, 75 μl portions were

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Fig. 1. Time course of $\text{Ins}(1,4,5)\text{P}_3$ and its degradation products in response to α_1 -adrenergic stimulation in cultured cardiac myocytes

Cultured prelabelled cardiac myocytes were preincubated for 10 min with LiCl (10 mM) and propranolol (10 μM). Then buffer without (●) or with (○) noradrenaline (final concn. 50 μM) was added for the times indicated. Neutralized HClO_4 extracts were analysed for inositol polyphosphates on Partisil 10 SAX and for inositol monophosphates on LiChrosorb NH_2 . Values are presented as means $\pm$ S.D. ($n = 3-6$). Significant differences from control values are marked by * $P \leq 0.01$ and ** $P \leq 0.05$).

removed from the mixture and mixed with 25 μl of ice-cold HClO_4 . The HClO_4 extracts were neutralized and stored as described previously [1].

Separation of inositol phosphates by h.p.l.c.

Neutralized HClO_4 extracts (0.8–1 ml) were analysed either on a Partisil 10 SAX column (Whatman, Clifton, NJ, U.S.A.; 250 mm $\times$ 4.6 mm) for inositol polyphosphates, or on a LiChrosorb NH_2 column (Chromatographie Service, Eschweiler, Germany; particle size 5 μm ; 300 mm $\times$ 4 mm) for inositol monophosphates. A step gradient was used on Partisil 10 SAX to separate at least three isomers of InsP_2 and InsP_3 as described by Dean & Moyer [7]. The gradient was increased to 0.9 M- $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 3.8) to elute InsP_4 from the column (fraction size 0.5–1 ml). In detail, the following gradient was used: 0 min, 100% water (A); 30 min, 9% B (0.9 M- $\text{NH}_4\text{H}_2\text{PO}_4$, adjusted to pH 3.8 with

Fig. 2. Time course of $\text{Ins}(1,3,4,5)\text{P}_4$ and its degradation products in response to α_1 -adrenergic stimulation in cultured cardiac myocytes

For experimental conditions see Fig. 1.

H_3PO_4); 32 min, 22% B; 62 min, 31% B; 64 min, 56% B; 94 min, 58% B; 125 min, 100% B; 135 min, 100% B; 145 min, 0% B; 165 min, 0% B. Inositol monophosphates were separated on a LiChrosorb NH_2 column by a linear gradient from water to 12.5 mM- $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 3.35, adjusted with H_3PO_4) in 50 min as described by Morgan *et al.* [8].

The individual isomers were identified by comparison with radiolabelled standards or by their elution positions relative to the labelled standards. Enantiomers such as

Ins(1)*P* and Ins(3)*P* were not separated, and therefore results are given for the enantiomeric mixture.

Liquid-scintillation counting and data processing were carried out as described previously [1].

RESULTS

Inositol phosphate accumulation in cultured cardiac myocytes in response to α_1 -adrenergic stimulation

As shown in Fig. 1, Ins(1,4,5)*P*₃ increased linearly in 20 s to its maximum. Between 20 s and 15 min a slow decline to about 60 % of the maximal stimulation value was observed. Significant increases in Ins(1,4)*P*₂, the major Ins*P*₂ isomer in cardiac myocytes, occurred already 10 s after addition of noradrenaline. This rapid increase was followed by a steady-state phase from 1 min to at least 15 min (last measurement point, Fig. 1). Ins(4)*P*, which was recently shown to be the only degradation product of Ins(1,4)*P*₂ [9], increased nearly linearly within 15 min (Fig. 1).

The time course of Ins(1,3,4,5)*P*₄ resembled, at least in the initial phase, that of Ins(1,4,5)*P*₃ (Fig. 2). The time point of maximal stimulation was shifted 10 s to the right compared with Ins(1,4,5)*P*₃. The early rise was followed by a decrease within 5 min nearly to control values. Ins(1,3,4)*P*₃, which is formed by Ins(1,3,4,5)*P*₄ dephosphorylation, showed an initial rise like Ins(1,4,5)*P*₃ and Ins(1,3,4,5)*P*₄, but the curve was once more shifted to the right (time point of maximal response 1 min; Fig. 2). Two minor Ins*P*₂ isomers, Ins(3,4)*P*₂ and Ins(1,3)*P*₂, were formed in different amounts and showed different kinetics. Ins(3,4)*P*₂ increased slowly within 15 min, whereas Ins(1,3)*P*₂ increased in 1 min and afterwards remained nearly constant (Fig. 2). The amount of Ins(3,4)*P*₂ was about 3-fold higher than Ins(1,3)*P*₂ after 15 min stimulation. The enantiomeric mixture of Ins(3)*P* and Ins(1)*P* increased in a constant rate within 15 min (Fig. 2).

The α_1 -adrenoceptor-induced Ins(1,4,5)*P*₃ release and subsequent metabolism were antagonized by the α_1 -antagonist prazosin (0.1 μ M). The α_2 -adrenoceptor antagonist yohimbine (0.1 μ M) had only little, but not significant, inhibitory effect. When the antagonists were given in the absence of noradrenaline, no significant effects on inositol phosphate accumulation were observed (Table 1).

Degradation of Ins(1,3,4,5)*P*₄ and Ins(1,4,5)*P*₃ by a myocyte homogenate

The dephosphorylation of [³H]Ins(1,3,4,5)*P*₄ by a myocyte homogenate started rapidly within the first 3 min and was accompanied by a concomitant increase in Ins(1,3,4)*P*₃. After 3 min about 39 % of the initial radioactivity still remained in Ins(1,3,4,5)*P*₄, and about 55 % was found in Ins(1,3,4)*P*₃. Between 3 and 15 min Ins(1,3,4,5)*P*₄ and Ins(1,3,4)*P*₃ decreased at a constant rate, while Ins(3,4)*P*₂, Ins(1,3)*P*₂ and Ins*P* increased. Ins(3,4)*P*₂ was the major Ins*P*₂ isomer (95 % of total Ins*P*₂ after 15 min), whereas Ins(1,3)*P*₂ was detected only in small amounts. Ins(1,4,5)*P*₃ or its degradation products were not found in this experiment.

When [³H]Ins(1,4,5)*P*₃ was added to the homogenate, it was metabolized in two ways. The main pathway was the dephosphorylation to Ins(1,4)*P*₂. After 1 min about 26 %, and after 3 min about 70 %, of the initial radioactivity was found in Ins(1,4)*P*₂. The minor path-

Table 1. Effect of prazosin and yohimbine on α_1 -adrenoceptor-induced Ins(1,4,5)*P*₃ and Ins*P*₄ accumulation in LiCl-pretreated cultured cardiac myocytes

Prelabelled cultured myocytes were preincubated for 10 min with LiCl (10 mM), propranolol (10 μ M) and prazosin (0.1 μ M) or yohimbine (0.1 μ M). Then buffer with noradrenaline (final concn. 50 μ M) or without noradrenaline (control) was added for 1 min. Neutralized HClO₄ extracts were analysed as described in the Experimental section. Values are presented as means $\pm$ S.D. ($n = 3-5$).

Additions	Accumulation (% of control)	
	Ins(1,4,5) <i>P</i> ₃	Ins(1,3,4,5) <i>P</i> ₄
Control	100 $\pm$ 11	100 $\pm$ 24
Noradrenaline	1853 $\pm$ 313	2618 $\pm$ 383
Noradrenaline + prazosin	417 $\pm$ 26	241 $\pm$ 115
Control + prazosin	126 $\pm$ 67	150 $\pm$ 61
Noradrenaline + yohimbine	1548 $\pm$ 357	2127 $\pm$ 401
Control + yohimbine	100 $\pm$ 5	162 $\pm$ 49

way was phosphorylation to Ins(1,3,4,5)*P*₄ and catabolism to Ins(3,4)*P*₂ and Ins(1,3)*P*₂. Only 3 % (after 1 min) and 5 % (after 3 min) of the initial radioactivity was found in these compounds. Accumulation of Ins*P* and inositol started already 3 min after addition of [³H]-Ins(1,4,5)*P*₃. In contrast, only small amounts of Ins*P* and inositol were found at extended incubation periods when [³H]Ins(1,3,4,5)*P*₄ was metabolized.

DISCUSSION

Recent studies investigated the presence of the Ins*P*₃/Ins*P*₄ pathway in mammalian heart and in cultured embryonic-chick heart cells [1,6,11]. Although Woodcock *et al.* [12] assumed that this pathway was not existent in rat heart, Poggioli *et al.* [6] and Jones *et al.* [11] detected Ins(1,3,4,5)*P*₄ and Ins(1,3,4)*P*₃ in their heart preparations. Our recent observations [1,4] and the finding of additional Ins*P*₂ isomers, which are exclusively formed by Ins(1,3,4,5)*P*₄ degradation, clearly demonstrate the presence of both pathways of Ins(1,4,5)*P*₃ metabolism. Furthermore, the presence of different Ins*P* isomers, shown here for the first time in cardiac myocytes, confirmed our supposition.

Any attempts to find Ins*P*₅ and Ins*P*₆ by increasing the NH₄H₂PO₄ gradient to 2.0 M were not successful (results not shown). The reason might be that Ins*P*₅ and Ins*P*₆ represent a different pool which does not incorporate [³H]inositol during a labelling period of 6 h. Further experiments need to be performed to decide whether or not Ins*P*₅ and Ins*P*₆ are present in rat heart.

Recent publications on the physiological role of inositol phosphates suggested that the positive inotropic effect of both α_1 -agonists and carbamoylcholine may be mediated by an increase in Ins(1,4,5)*P*₃ and/or Ins(1,3,4,5)*P*₄ [3,13]. Commonly it was suggested that the increase in force of contraction was triggered by Ins(1,4,5)*P*₃-induced Ca²⁺ release [14]. Moreover, Ins(1,3,4,5)*P*₄ was recently proposed to regulate the Ca²⁺ entry across the plasma membrane directly into the

Ins(1,4,5) P_3 -sensitive pool [15]. In addition, Ins(1,3,4,5) P_4 was shown to inhibit the dephosphorylation of Ins(1,4,5) P_3 to Ins(1,4) P_2 in permeabilized hepatocytes [16]. In both ways Ins(1,3,4,5) P_4 may contribute to a prolonged Ca^{2+} release from the Ins(1,4,5) P_3 pool. But recently Korth *et al.* [17] showed that an increase in intracellular Ca^{2+} in ventricular myocytes was caused by carbamoylcholine, but not by the α_1 -agonist methoxamine. These findings would argue against the same mechanism of positive inotropy, because we observed the accumulation of Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 with both agonists [1].

Another proposed mechanism of Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 action, the Ca^{2+} sensitization of the contractile fibres, was recently investigated by Brückner *et al.* [13], who demonstrated that both Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 at a concentration of 0.1 mM led to an increase in Ca^{2+} -sensitivity in skinned pig cardiac muscle fibres.

Furthermore, it was shown that Ins(1,4,5) P_3 , Ins P_4 , Ins(2,4,5) P_3 and Ins(1,3,4,5,6) P_5 interact with aldolases from different tissues [18]. In this context, inositol phosphates may also play a role as specific effectors of other cellular proteins, e.g. enzymes or regulator proteins.

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