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EVIDENCE FOR A LACK OF INOSITOL - (1,4,5)TRISPHOSPHATE KINASE ACTIVITY IN NOREPINEPHRINE-PERFUSED RAT HEARTS

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SUMMARY : The products of the phosphatidylinositol turnover pathway in norepinephrine-perfused hearts have been examined using high performance liquid chromatography. Inositol-1 phosphate, inositol-(1,4)bisphosphate and inositol-(1,4,5)-trisphosphate were all increased in response to norepinephrine stimulation. However, at perfusion times from 5 sec to 20 min there was no appearance of inositol-(1,3,4,5)tetrakisphosphate or inositol-(1,3,4)trisphosphate. This suggests the absence of the inositol-(1,4,5)trisphosphate phosphorylation/dephosphorylation pathway in heart and demonstrates that this secondary pathway is not essential to the functioning of the phosphatidylinositol turnover cycle.

The primary event in the mechanism of action of many different hormones and neurotransmitters involves receptor-mediated stimulation of the breakdown of plasma membrane inositol phospholipids. This so-called phosphatidylinositol (PI) turnover pathway generates two second messengers inositol-(1,4,5)trisphosphate (Ins-(1,4,5)P_3) and sn1,2-diacylglycerol (DAG). DAG stimulates the membrane-bound

Abbreviations :
Ins-(1,4,5)P_3, inositol-(1,4,5)trisphosphate; Ins-(1,4)P_2, inositol-(1,4)bisphosphate; Ins-1P, inositol-1phosphate; Ins-(1,3,4,5)P_4, inositol-(1,3,4,5) tetrakisphosphate; DAG, sn 1,2-diacylglycerol; GPI, glycerophosphoinositol; GPIP, glycerophosphoinositol-4 phosphate; $GPIP_2$, glycerophosphoinositol-(4,5)bisphosphate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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phospholipid-dependent, Ca^{2+}-dependent protein kinase C while Ins-(1,4,5)P_3, releases Ca^{2+} from endoplasmic reticulum stores [1]. In tissues so far investigated, removal of Ins-(1,4,5)P_3 occurs by two different pathways; hydrolysis to Ins-(1,4)P_2 and phosphorylation to Ins-(1,3,4,5)P_4 [2,3,4,5]. In some tissues higher phosphorylation products have been reported [6]. Ins-(1,3,4,5)P_4 is subsequently dephosphorylated to Ins-(1,3,4)P_3 which is relatively inactive in releasing Ca^{2+} compared with the (1,4,5)-isomer [7]. The physiological significance of this extra loop of the PI-turnover pathway is unknown in any mammalian cell system but, in sea urchin eggs Ins-(1,3,4,5)P_4 has been shown to enhance Ca^{2+} entry into the cell [8]. An alternative possibility is that phosphorylation provides a second pathway of elimination of the active -(1,4,5)-isomer, in addition to dephosphorylation, thereby providing extra flexibility in the system. Whatever its importance, this phosphorylation/dephosphorylation pathway has now been described in a wide range of tissues including brain, liver, pancreas, adrenal glomerulosa cells, parotid gland [9,10,5,11] and is generally considered an integral part of the PI-turnover pathway.

Phosphatidylinositol turnover in heart is stimulated by both muscarinic cholinergic agonists and alpha-adrenergic agonists but the details of the pathway have not been studied in detail [12]. We have made a study of the products of the PI-turnover pathway in isolated rat hearts and we present evidence that neither Ins-(1,3,4,5)P_4 nor Ins-(1,3,4)P_3 is formed prior to or during perfusion with norepinephrine.

MATERIALS AND METHODS

Inositol phosphate production in isolated-perfused hearts

Adult, male, Sprague-Dawley rats weighing 200-300g were killed by decapitation. The chest was opened immediately and an

ice slurry placed over the heart. Chilled hearts were removed, rinsed clear of blood and then perfused via the dorsal aorta according to the method of Langendorff. The perfusing solution was Krebs' medium buffered to pH7.4 with Hepes buffer (20mM)(KH) and was constantly gassed with 95% O_2, 5% CO_2 and maintained at 37°C. Perfusion was continued in a non-recirculating manner for 15 min or until the perfusate was free of blood. After this time, hearts were perfused for 2 hr with KH containing 1.5 μCi/ml [^{3}H]inositol, to label inositol phospholipids. Following the labeling period, the perfusion was continued for a further 15 min with KH containing 5 mM non-radioactive inositol, 100 nM atropine, 100 nM propranolol and 10 mM LiCl. Norepinephrine was then added and the perfusion continued for the times indicated on the figures and was terminated by dropping the heart into liquid nitrogen.

Separation of inositol phosphates

Frozen hearts were homogenized in 10 ml ice cold 10% TCA containing 5 mM EDTA using a Polytron homogenizer followed by sonication. Following centrifugation, the supernatant was extracted three times with 10 ml of ether and then lyophyllized. The residue was resuspended in 1 ml of water and used in subsequent high performance liquid chromatographic analysis.

High performance liquid chromatography was achieved essentially as described by Irvine et al [13].The chromatographic column was a Whatman partisil SAX column packed by Waters for use in a radial compression system. The inositol phosphates were eluted using a linear gradient of 0-1.75 M ammonium formate buffered to pH 3.7 with orthophosphoric acid. The flow rate was 1 ml/min and fractions were collected at half minute intervals. A standard solution of ATP, ADP and AMP was added to each sample prior to injection. The nature of the [^{3}H]inositol-labeled material was checked by including [^{32}P]-labeled Ins-IP, Ins-(1,4)P_2 and Ins-(1,4,5)P_3 prepared from erythrocytes [14]. Commercially available [^{3}H]labeled Ins-(1,4,5)P_3 was also used as standard. Glycerophosphoinositols were identified by comparison with standards prepared from [^{32}P]-labeled erythrocytes [14]. Chromatography was performed on a Waters model 441 liquid chromatography system.

Inositol phosphate accumulation in adrenal glomerulosa cells.

Rat adrenal glomerulosa cells were prepared as described elsewhere [15]. Inositol phospholipids were labeled by incubating cells for 18 hr with [^{3}H]inositol (20μCi/ml) in Hepes-buffered medium 199 at 37°C. Subsequently, 5 mM non-radioactive inositol was added and the cells were incubated for a further 15 min before being harvested by centrifugation and washed twice in medium 199 containing 5 mM inositol and 10 mM LiCl. [^{3}H]-Labeled cells (2-5x10^6/ml) were incubated with 100 nM angiotentin II for the times indicated on the figure. Inositol phosphates were extracted as described above except that 1 ml 10% TCA was used.

Preparation of Inositol-(1,3,4,5)P_4

A crude preparation of Ins-(1,4,5)P_3 kinase was made from rat brain as described elsewhere [9]. [^{3}H]-labeled Ins-(1,4,5)P_3 (10μCi, 10^{-5}M) was incubated with brain extract (1-2 mg protein) in a final volume of 0.3 ml containing 5 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol and 50 mM tris HCl pH 7.3. Incubation was carried out at 37°C for 10 min and terminated by adding TCA as described above. High performance liquid chromatography showed the

appearance of material eluting at 78-80 min. Control incubations either with enzyme added after TCA or incubated in the absence of ATP did not contain any [^{3}H]labeled material with a longer retention time than Ins-(1,4,5)P_3.

Materials

[^{3}H]-myo-inositol, specific activity 10-20Ci/mmol was purchased from the Radiochemical Centre, Amersham UK. [^{32}P]Phosphate was purchased from the Australian Atomic Energy Commission, Lucas Heights, N.S.W. [^{3}H]-myo-inositol-(1,4,5)trisphosphate (Amersham) was a gift from Dr. M.E. Dunlop, Royal Melbourne Hospital, Victoria.

Norepinephrine bitartrate was obtained from the Sigma Chemical Co., Missouri. Propranolol was supplied by Imperial Chemical Industries and atropine sulfate by David Bull Laboratories. Angiotensin II was Hypertensin, Ciba.

RESULTS

Inositol phosphates in norepinephrine-perfused rat hearts

Isolated rat hearts were perfused with [^{3}H]inositol to label inositol phospholipids and subsequently perfused with norepinephrine (3×10^{-5}M) for various times up to 20 min. All experiments were performed in the presence of 100 nM atropine and 100 nM propranolol to prevent stimulation of muscarinic acetylcholine receptors or β-adrenoceptors by release of endogenous neurotransmitters or by norepinephrine in the perfusate. TCA-soluble, [^{3}H]inositol-labeled products were examined using high performance liquid chromatography as described under "Methods". Experiments were performed at the following norepinephrine perfusion times : zero, 5 sec, 15 sec, 30 sec, 2 min, 5 min and 20 min. Experiments at each time point were performed at least twice and those at 15 sec and 20 min were performed three times. Typical profiles obtained at zero time, 15 sec and 20 min are shown in figure 1. Qualitatively similar profiles were observed at all perfusion times. Peak heights (especially InsP and $InsP_2$) increased with increasing perfusion time, but no differences were detected in the nature of the products formed betwen zero time and 20 min perfusion with norepinephrine. Comparison with [^{32}P]-labeled standards allowed

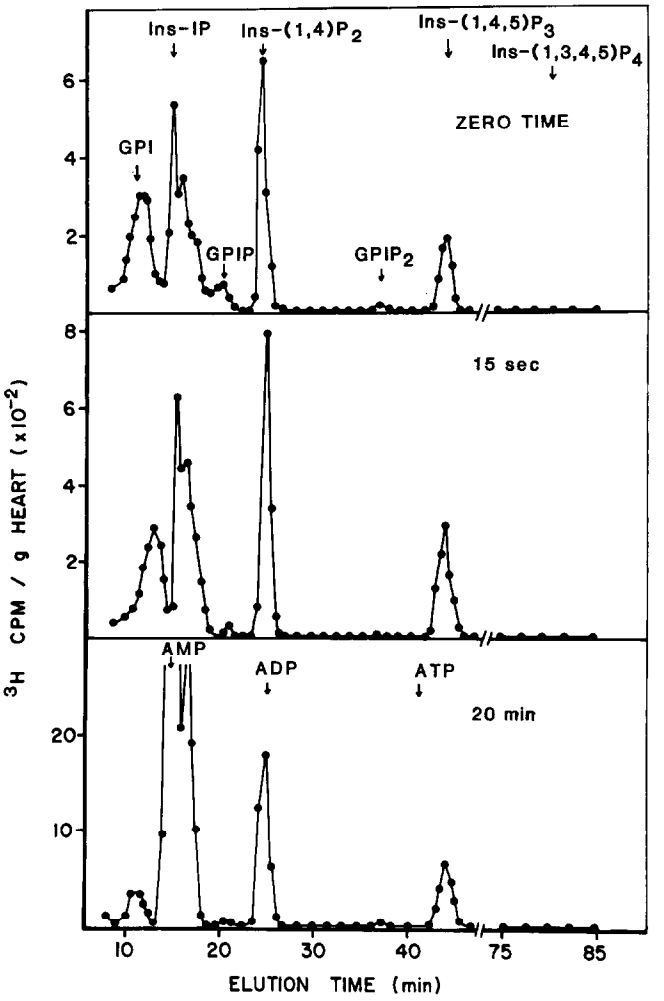

FIGURE 1 . Inositol phosphate accumulation in norepinephrine-perfused [^{3}H]inositol-labeled rat hearts. High performance liquid chromatography of TCA-soluble products was performed as described under "Methods". Elution times of standard inositol phosphates and glycerophosphoinositols as well as AMP, ADP and ATP are indicated. GPI, glycerophosphoinositol, GPIP, glycerophosphoinositol-4 phosphate; $GPIP_2$, glycerophosphoinositol-(4,5) bisphosphate. Times indicated refer to norepinephrine perfusion times. Zero time means the heart was not perfused with norepinephrine. Shown are the profiles obtained in a typical experiment. Similar results were obtained with perfusion times, 5 sec, 30 sec, 2 min, 5 min. Experiments were performed 3 times at 15 sec and 20 min and twice at each of the other perfusion times.

the identification of three of the peaks as Ins-1P, Ins-$(1,4)P_2$ and Ins-$(1,4,5)P_3$. The Ins-IP peak was immediately followed by another peak which was likely Ins-4P [16] formed by

dephosphorylation of Ins-(1,4)P. The appearance of this isomer indicates that a large percentage of InsP formed in hearts arises from dephosphorylation of higher inositol phosphates. Ins-1P could be derived either by dephosphorylation of $InsP_2$ and $InsP_3$ or by phosphodiesterase cleavage of phosphatidylinositol. However, the profiles observed in heart differed markedly from those reported in other tissues (2,4,5). In the profiles obtained in heart tissue, no peak corresponding to Ins-(1,3,4,5)P_4, the immediate phosphorylation product of Ins-(1,4,5)P_3, was detected. Also the breakdown product of Ins-(1,3,4,5)P_4, Ins-(1,3,4)P_3 was not detectable nor was Ins-(3,4)P_2, a breakdown product of Ins-(1,3,4)P_3 (17). Given a limit of detection of 20 cpm, each of these products, if present at significant concentrations, would have been readily detected. Average peak heights of Ins-(1,4,5)P_3 ranged between 150-300 cpm and, in other tissues, this isomer represents a minor component relative to Ins-(1,3,4)P_3 (2,4,5). In none of the experiments performed in heart was there any peak of greater than 20 cpm at the position of Ins-(3,4)P_2, Ins-(1,3,4)P_3 or Ins-(1,3,4,5)P_4. The inclusion of LiCl in the perfusate would be expected to enhance the accumulation of Ins-(1,3,4)P_3 which is degraded by the 1'phosphatase [17].

Unlike the situation observed in heart, the profile of inositol phosphates generated in angiotensin II-stimulated adrenal glomerulosa cells is similar to that reported in other tissues (5,4). Profiles obtained in adrenal cells stimulated with angiotensin II for 15 sec and 20 min are shown in figure 2 for comparison with the heart profiles. Ins-1P, Ins-4P, Ins-(1,4)P_2 and Ins-(1,4,5)P_3 were observed, as in heart, but peaks corresponding to Ins-(1,3,4,5)P_4, Ins-(1,3,4)P_3 and Ins-(3,4)P_2 were also readily detectable. At both 15 sec and 30

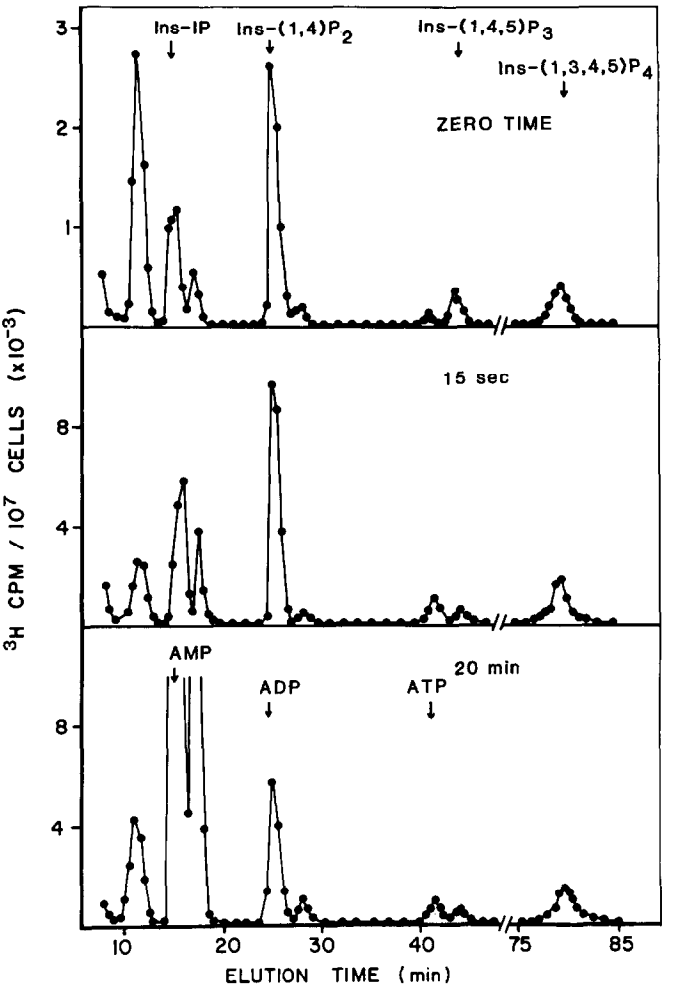

FIGURE 2 . Inositol phosphate accumulation in angiotensin II-stimulated, [^{3}H]inositol-labeled rat adrenal glomerulosa cells. High performance liquid chromatography of TCA-soluble products was performed as described under "Methods". Elution times of standard inositol phosphates, AMP, ADP and ATP are indicated. Indicated times refer to duration of angiotensin II stimulation. Zero time refers to a preparation not exposed to angiotensin II. Shown are typical profiles. The experiment was performed three times with similar results.

min angiotensin II stimulation the peak of Ins-(1,3,4)P_3 was 2-3 fold higher than the Ins-(1,4,5)P_3 peak.

Other minor peaks in the chromatographic profiles obtained in heart tissue were identified as glycerophosphoinositol, glycerophosphoinositol-4P and glycerophosphoinositol-(4,5)P_2 by

comparison with [^{32}P]-labeled standards. These compounds were not increased by norepinephrine perfusion.

DISCUSSION

While extensive studies have been made of phosphatidylinositol turnover in many different tissues, few detailed studies in heart tissue have been reported. Most of these studies have reported measurements of total inositol phosphate content in isolated ventricular myocytes and have shown a relatively small stimulation by both α-adrenergic agonists and muscarinic cholinergic agonists [18]. The present studies performed in isolated, perfused hearts show that levels of $InsP_3$, $InsP_2$, and InsP are all stimulated by perfusion with norepinephrine. Furthermore, the active Ca^{2+}-releasing compound Ins-(1,4,5)P_3 was clearly formed in heart in agreement with an initial cleavage of phosphatidylinositol-(1,4)bisphosphate as described in other tissues [16,19]. However, while Ins-(1,4,5)P_3 is clearly formed in heart, its physiological significance is less obvious. Ins-(1,4,5)P_3 has been reported to be active [20], inactive [21] and weak [22] in releasing Ca^{2+} from cardiac sarcoplasmic reticulum. Thus, Ins-(1,4,5)P_3 may not be an important factor in raising myocardial Ca^{2+} concentrations, leading to the question of a possible alternative role for this compound in heart.

Unlike the situation in other tissues, in heart $InsP_3$ appeared to be entirely comprised of the -(1,4,5)-isomer. There was no appearance of the -(1,3,4)-isomer at any norepinephrine perfusion time up to 20 min; nor was there any indication of Ins-(1,3,4,5)P_4, the phosphorylation product of Ins-(1,4,5)P_3. This result suggests a lack of Ins-(1,4,5)P_3 kinase activity in rat heart. In parallel experiments, both Ins-(1,3,4)P_3 and

Ins-(1,3,4,5)P_4 were detected in adrenal glomerulosa cells demonstrating the ability of our chromatographic system to detect these compounds. Lack of demonstrable Ins-(1,4,5)P_3 kinase activity in heart might be due to a lack of the enzyme itself, the presence of inhibitory factors or the absence of stimulators. In some tissues, Ca^{2+} acts as a stimulator of Ins-(1,4,5)P_3 kinase activity (5,23), but this is not a universal finding [4]. In any case, the cytosolic Ca^{2+} concentration in heart should be sufficient to permit kinase activity [24]. It is alsso possible that the Ins-(1,4,5)P_3 kinase pathway occurs in heart but degradation of the products is so rapid that they are not detected. However, such an explanation is unlikely, because Ins-(1,3,4,5)P_4 is degraded by the same 5' phosphatase which degrades Ins-(1,4,5)P_3 and Ins-(1,3,4)P_3 is degraded by the 1' phosphatase which is inhibited by LiCl (17). LiCl was included in the perfusate and this would be expected to increase the accumulation of Ins-(1,3,4)P_3.

The functional significance of the Ins-(1,4,5)P_3 kinase pathway is not yet understood. Therefore it is not possible to evaluate the importance of the lack of this pathway in heart. If Ins-(1,3,4,5)P_4 acts to stimulate entry of extracellular Ca^{2+}, our results may indicate that α1-adrenoceptor stimulation in heart promotes Ca^{2+} entry via an alterntive pathway. Such a suggestion would agree with data showing that protein kinase C activates a sarcolemmal protein involved in controlling the slow, inward Ca^{2+} current [25]. Alternatively, it is possible that the relative inactivity of Ins-(1,4,5)P_3 in releasing Ca^{2+} in heart means that a single pathway of removal is sufficient, thereby removing the need for the phosphorylation/ dephosphorylation cycle. Whatever its functional significance, the lack of this secondary pathway in heart shows that it is not an essential

component of the PI-turnover pathway. These findings may help to provide some insight into the importance of this Ins-(1,4,5)P_3 phosphorylation/dephosphorylation pathway in other tissues.

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