Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 366-371

DYNAMIC OF ALPHA-1-ADRENOCEPTOR MEDIATED DEGRADATION OF MEMBRANE PHOSPHOLIPIDS IN CULTURED RAT CARDIOMYOCYTES

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Received December 2, 1993

In many cell types, agonists can stimulate both phosphoinositide (PtdIns) and phosphatidylcholine (PC) hydrolysis by activating specific phospholipases. Using cultures of neonatal rat cardiomyocytes we have verified the existence of an α_1-adrenoceptor mediated hydrolysis of PtdIns and PC. PtdIns breakdown, evaluated as inositol phosphate production, occurred in the early phase of cell stimulation, while PC hydrolysis, evaluated as choline metabolite production, was evidenced at longer stimulation times. The appearance of a delayed peak of choline phosphate and the invariance of free choline in the intracellular water phase strongly suggest the involvement of a specific PC-phospholipase C, generating choline phosphate and diacylglycerol, the activator of protein kinase C. Since it is plausible that various metabolites of signal-induced degradation of membrane phospholipids may take part in long term physiological responses, PC breakdown could be involved in cellular mechanisms that require prolonged protein kinase C activation.

Although there is abundant evidence that many hormones and neurotransmitters cause some of their effects through the hydrolysis of inositol phospholipids (PtdIns) to inositol phosphates (IP_n) and 1,2-diacylglycerol (DAG) in their target cells, it is becoming clear that many of them also stimulate the breakdown of other phospholipids, particularly phosphatidylcholine (PC) (1).

PC can be hydrolyzed by phospholipase A_2, giving arachidonic acid which is subsequently metabolized to different eicosanoids, but also by phospholipase C (PLC) and D (PLD) (2,3). PC hydrolysis by the specific PLC yelds to DAG and choline phosphate (ChoP), while PLD generates phosphatidic acid (PA), which can be subsequently converted into DAG, and free choline (Cho) (4).

PtdIns hydrolysis yelds to a relatively small amount of DAG for a short period of time compared with PC hydrolysis. This may relate to the fact that the cellular

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0006-291X/94 $5.00

content of PC is higher than PtdIns (5). Furthermore, DAG derived from PC hydrolysis could produce different cellular effects from that resulting from PtdIns breakdown (6).

In many cell types, the signal activated formation of DAG is biphasic: it consists of an early peak, which is rapid and transient (and parallels the increase in IP_n concentration), followed by a later phase, which is slow in onset but is sustained over many minutes (7). Direct PC-PLC-mediated generation of DAG has now been suggested to occur in a number of cells, following stimulation by certain calcium mobilizing agonists such as bombesin, epinephrine, vasopressin and colecystokinin (8).

In previous works (9-10), using cultures of beating, neonatal rat ventricular cells, stimulated with an α_1-agonist, we demonstrated the existence of a receptor-mediated hydrolysis of PtdIns, generating IP_n and DAG. This PtdIns-derived DAG was demonstrated to be able to activate protein kinase C (PKC), which activity reached its maximum after short stimulation times, then declined very rapidly (11).

The aim of the present study is to verify the existence, in cultured cardiomyocytes, of an α_1-adrenoceptor mediated PC breakdown, and to determine the dynamic of the lipid-derived messengers produced by signal-activated phospholipases.

MATERIALS AND METHODS

Chemicals. Ham F10 was obtained from Gibco (Scotland), horse serum, fetal calf serum and trypsin were from Boehringer (Mannheim, Germany). Phenylephrine was purchased from Sigma (St. Louis, MO, USA); Dowex 1X8 and Dowex-50-WH^+ were from Bio Rad Laboratories (CA, USA), [2-^{3}H]-myoinositol (24.4 Ci/mmol) and [methyl-^{3}H]-choline (80 Ci/mmol) were obtained from NEN Products (Boston, Mass., USA). All chemicals and solvents were of analytical grade.

Cell cultures. Neonatal rat cardiomyocytes were isolated and grown as described (9-11). At confluence, cells were radiolabelled with [2-^{3}H]-myoinositol or [methyl-^{3}H]-choline (1 μCi/ml medium) for 48 hrs. Preliminary experiments demonstrated that equilibrium labelling of the inositol and choline containing lipids was achieved following a 48 hrs incubation.

Determination of PtdIns breakdown. Cells radiolabelled with [^{3}H]-myoinositol were stimulated with 30 μM phenylephrine (PhE) for different times (0-60 min), as reported in (12). Stimulations were stopped by rinsing with cold buffer (12); cells were scraped off in cold methanol:HCl (100:1 v/v) and the cellular lipid fraction separated according to Bligh and Dyer (13). The acqueous, upper phase derived from the Bligh and Dyer separation was used to evaluate the IP_n production at each stimulation time. IP_n were separated by anion exchange chromatography on Dowex 1X8 as described by Berridge et al. (14); IP_n were eluted all together, and the eluate was counted for radioactivity by liquid scintillation.

Determination of PC breakdown. Cells radiolabelled with [methyl-^{3}H]-choline were stimulated with 30 μM PhE for different times (0-60 min) (12). Stimulations were

stopped, cells scraped and lipids extracted as reported in the previous section. At each stimulation time the intracellular production of ChoP and Cho was determined by ion exchange chromatography on Dowex-50-WH$^+$ on the acqueous solution resulting from the lipid extraction, as described by Cook and Wakelam (15). Aliquots of each fraction were taken and the associated radioactivity determined by scintillating counting. Total [^{3}H]-choline metabolites released to the culture medium were quantitated by counting an aliquot of the medium mixed with scintillation fluid.

RESULTS

Preliminary experiments on radiolabelling of cell cultures for 48 hrs showed that the incorporation of radiolabelled precursors into the phospholipids of neonatal rat cardiomyocytes was highly specific. Ninetynine percent of the [^{3}H]-inositol was preferentially incorporated into the PtdIns fraction; 87% of the [^{3}H]-choline was preferentially incorporated into the PC fraction and 12% into the sphingomyelin. Thus, following treatment with the agonist, we were able to analyze the metabolites resulting from the hydrolysis of PtdIns and PC.

The time course of IP_n production in [^{3}H]-inositol prelabelled cardiomyocytes following α_1-adrenoceptor stimulation is shown in figure 1. As the radioactivity of the IP_n fraction largely depends on [^{3}H]-inositol uptake into the cells, the radioactivity of

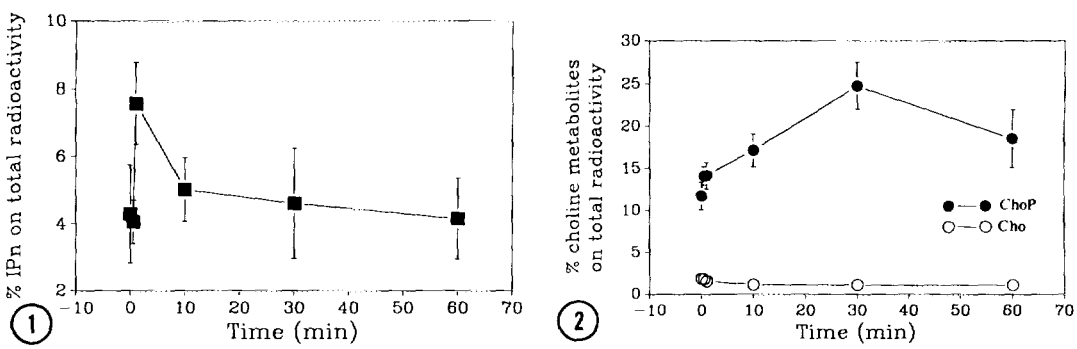

Figure 1. Time-course of IP_n production in [^{3}H]-inositol prelabelled cardiomyocytes following α_1-adrenoceptor stimulation.
Cells were stimulated with 30 µM PhE for different times and IP_n production was determined in the acqueous phase following lipid extraction. The radioactivity of the IP_n fraction is expressed as a percentage of the total uptake of [^{3}H]-inositol in each dish ($1.95 \pm 0.13 \times 10^5$ cpm/dish). Data are means ± S.D. of 5 independent experiments.

Figure 2. Time-course of ChoP and Cho production in [^{3}H]-choline prelabelled cardiomyocytes following α_1-adrenoceptor stimulation.
Cells were stimulated with 30 µM PhE for different times and the intracellular production of ChoP and Cho was determined in the acqueous phase following lipid extraction. The radioactivity of the ChoP and Cho fractions is expressed as a percentage of the total uptake of [^{3}H]-choline in each dish ($7.50 \pm 0.53 \times 10^5$ cpm/dish). Data are means ± S.D. of 5 independent experiments.

the IP_n fraction of each culture dish was expressed as a percentage of the total uptake of radioactivity of that dish (mean value of total uptake calculated in 5 independent experiments = $1.95 \pm 0.13 \times 10^5$ cpm/dish). IP_n production significantly increased after 1 min stimulation, declining toward basal value at longer stimulation times.

In order to gain insight on the enzyme activities induced by the agonist (i.e. PLC or PLD), cardiomyocytes were radiolabelled with [^{3}H]-choline, treated with PhE for different times, and the time course of choline metabolite production was analyzed. Figure 2 shows that when the cells were prelabelled with [^{3}H]-choline and treated with PhE, PC degradation was indicated by a time dependent accumulation of intracellular [^{3}H]-choline metabolites. PC hydrolysis continued through a 60 min incubation, and ChoP was the predominant PC metabolite released. As for the evaluation of IP_n production, Cho and ChoP production in each culture dish was expressed as a percentage of the total uptake of radioactivity of that dish (mean value of total uptake calculated in 5 independent experiments = $7.50 \pm 0.53 \times 10^5$ cpm/dish). In comparison to quiescent cells, ChoP production was not significant at short stimulation times (30 sec and 1 min), while it significantly increased at longer stimulation times. A peak of ChoP production was observed at 30 min stimulation. In contrast to ChoP, the intracellular level of Cho was very low and constant, independent of stimulation (fig. 2).

In addition, PhE stimulated an increase in the release of choline metabolites into the culture medium, beginning very early and continuing for at least 60 min (data not shown).

DISCUSSION

During the past decade, phospholipids have been shown to be precursors of second messengers for cell signalling, and the importance of PKC activation coupled to PtdIns hydrolysis is well appreciated (16). It is now clear that, in many cell types, PC hydrolysis also contributes to the generation of DAG in the relative later phases of cellular response (17).

Our previous data demonstrated that, in cultured cardiomyocytes, PtdIns were hydrolyzed upon treatment with the α_1-agonist PhE (12,18). Results here presented show that PtdIns breakdown, evaluated as IP_n production, occurs in the very early phase of cell stimulation. These findings are in agreement with data obtained in cultured adult rat cardiomyocytes (19).

Since Lindmar et al. (20) reported that the stimulation of perfused hearts "in vitro" with muscarinic agents causes the release of choline metabolites, and presented

indirect evidences that this is due to the hydrolysis of PC, we have investigated about the possibility of an α_1-adrenoceptor mediated hydrolysis of PC in cultured cardiomyocytes.

Our data reveale that PhE stimulates the hydrolysis of PC, as evidenced by the release of choline metabolites in both the intracellular acqueous phase and the culture medium.

The appearance of a delayed peak of ChoP production in the intracellular water phase strongly suggests the involvement of a specific PC-PLC in the later phases of cardiomyocyte stimulation. In fact, ChoP and DAG are the products of the activation of a PC specific PLC. In contrast to ChoP, intracellular Cho levels remained constant, independent of stimulation. Altogether these results give the first evidence that PhE is able to activate PC breakdown, involving a specific PLC and leading to DAG and ChoP production.

In addition, labelled water soluble metabolites of choline were found in the culture medium. Such an extracellular production of choline metabolites has already been reported in the case of several hormonal or pharmacological stimuli (21). Since we have not separated choline metabolites in the culture medium, we are at present unable to discriminate between the radioactivity associated with Cho or ChoP. Since intracellular and extracellular Cho pools rapidly equilibrate, the rise of radioactivity in the culture medium might explain the lack of evidence of an increase in Cho production in the intracellular fluid, due to the possible exit of Cho itself. Up to date, it is not possible to suggest an involvement of a specific PC-PLD, generating Cho and PA. Further studies, mainly the determination of PA production, are needed to completely clarify this hypothesis.

Our data clearly state that, as in many cell types (22), PC is implicated in cardiomyocyte response to α_1-agonists, and PLC-catalyzed PC hydrolysis begins in the later phases of the response. The final result of PC breakdown is the prolonged generation of DAG. In a previous work we analyzed the fatty acid composition of DAG generated following α_1-adrenoceptor stimulation of cultured cardiomyocytes for different times and we demonstrated that the first phase of DAG formation is due to the hydrolysis of PtdIns, while a second phase of DAG formation is most likely due to the hydrolysis of PC (23). Moreover, we previously demonstrated that, in our cell system, the early phases of PhE stimulation involve PKC activation (11). The elucidation of the temporal activation of PKC also in the later phases of cell stimulation could be vital for our understanding of the control of cellular responses of cardiomyocytes.

Since it is plausible that various metabolites of signal-induced degradation of membrane phospholipids may take part in sustained PKC activation, which is the prerequisite for long term physiological responses such as cell proliferation and differentiation, PC breakdown could be involved in cellular mechanism that require prolonged PKC activation. This is particularly important in cardiac myocytes "in vivo" since a specific PC-PLC has been demonstrated to exist and to contribute to electrophysiologic disfunction during myocardial ischemia (24).

ACKNOWLEDGMENT

This work was supported by grants from M.U.R.S.T. 60%.

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