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DISTINCT α_1-ADRENERGIC AGONIST- AND ENDOTHELIN-1-EVOKED PHOSPHOINOSITIDE CYCLE RESPONSES IN CULTURED NEONATAL RAT CARDIOMYOCYTES

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Abstract Previously it was shown by us and others in cultured neonatal rat cardiomyocytes that the desensitization of the phenylephrine (PHE)- and endothelin-1 (ET-1)-mediated response of phospholipase C (PLC) was receptor-specific. The aim of this study was to characterize receptor-dependent specificities downstream of PLC. PHE (10^{-4} M) as well as ET-1 (10^{-8} M) stimulated the total [^{3}H]inositolphosphate ([^{3}H]InsP_n, predominantly [^{3}H]Ins(4)P) formation to about the same extent whereas Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 did not increase. Yet, ET-1 but not PHE stimulated Ins(1,3,4)P_3 and Ins(3,4)P_n formation. Activation of PLC in saponin-permeabilized cells by GTPγS- and Ca^{2+} gave predominantly the formation of Ins(1,4)P_2. The PHE- and ET-1-mediated increase of [^{3}H]1,2-diacylglycerol was significant after respectively 16 and 8 min. PHE but not ET-1 stimulated phosphorylation of a 30 kDa protein which was likely of myofibrillar origin. It is concluded that receptor-dependent specificities exist not only at the level of PLC but also downstream.

In heart inotropy, long term induction of hypertrophy and concomitant reprogramming of gene expression have been shown to be partially mediated by agonists such as α_1-adrenergic hormone and endothelin-1 (ET-1), that transduce their signals after receptor-GTP-binding protein (G-protein) coupling to the activation of phospholipase C-ß (PLC-ß)(1-3). PLC-ß on its turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), thereby producing the second messengers 1,2-diacylglycerol ((1,2)DAG), inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4). (1,2)DAG is known to activate members of the protein kinase C (PKC) family by binding to the regulatory domain of the enzymes resulting in translocation to plasmamembrane and intracellular sites such as

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Abbreviations: PHE, phenylephrine; ET-1, endothelin-1; PLC, phospholipase C; PLD, phospholipase D; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins, inositol; InsP_n, inositolphosphates; Ins(4)P, inositol 4-phosphate; Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; (1,2)DAG, 1,2-diacylglycerol; PKC, protein kinase C; G-protein, GTP-binding protein; HPLC, high performance liquid chromatography.

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cytoskeletal elements (4-7). The other product of PLC-ß action, Ins(1,4,5)P_3, is known to be involved in the release of Ca^{2+} from the non-mitochondrial Ca^{2+} pools thereby promoting the Ca^{2+}-induced influx of external Ca^{2+}, possibly in conjunction with Ins(1,3,4,5)P_4 (1-3). (1,2)DAG metabolism concerns either conversion to phosphatidic acid mediated by (1,2)DAG kinase and subsequent reincorporation into the phospholipids, or hydrolysis by (1,2)DAG lipase and presumably by nonspecific esterases to free fatty acids and glycerol (4). Ins(1,4,5)P_3 metabolism involves two metabolic pathways as well: successive dephosphorylation reactions finally resulting in free Ins, and phosphorylation by 3-kinase to Ins(1,3,4,5)P_4 also followed by dephosphorylation reactions (8,9). It is becoming more and more clear that the diversity of receptors linked with this signalling pathway and the heterogeneity of the downstream elements such as the G-proteins, PLC, and PKC, determine the receptor-specific responses of the signalling pathway to a large extent.

The first evidence for occurrence of receptor-dependent specificities in PtdIns cycle signalling in myocardium was previously obtained by us and others in studying the time-course and mutual influences of desensitization of the PHE- and ET-1-evoked total inositolphosphate ($InsP_n$) responses (10,11). In order to characterize receptor-dependent specificities downstream of PLC, in this study we followed the formation in time of second messengers and some of their products and the ultimate ^{32}P incorporation into cellular proteins upon stimulation of cardiomyocytes by PHE and ET-1.

Materials and Methods

Reagents PHE was obtained from Sigma (St. Louis MO, USA) and ET-1 was from Peninsula Laboratories (Belmont CA, USA). Culture dishes (4 well Multidish) were from Nunc (Roskilde, DK), while culture medium HAM F10 was from Gibco (UK); fetal calf serum, horse serum and penicillin/streptomycin were obtained from Boehringer Mannheim (Germany). Trypsin (type III) was from Sigma (St. Louis MO, USA). *Myo*-[2-^{3}H]inositol (17-19 Ci/mmol), [2-^{3}H]glycerol (1 Ci/mmol) and $^{32}P_i$ (carrier free) were obtained from Amersham International plc (Amersham, UK). En^{3}Hance was from Dupont, NEN products (Boston, USA) and scintillation cocktail Flo-scint IV was from Canberra Packard Benelux N.V./S.A. (The Netherlands).

Cell culture Primary cultures of neonatal ventricular myocytes were prepared from 1-2 day old Wistar rats as described before (10,11) using preplating (13) to further increase cardiomyocyte to non-cardiomyocyte ratio. Minor modifications were introduced, replacing the previously used double preplating step for a single preplating step on an increased surface (10 cm^2/rat heart). Cardiomyocytes were seeded in 1.8 cm^2 wells at 150 to 175x10^3 cells/cm^2 giving a confluent monolayer of spontaneously contracting cells after 24 hrs. The cells were kept at 37°C and 5% CO_2 in complete growth medium consisting of Ham F10 supplemented with 10% fetal calf serum, 10% horse serum, 100 U penicillin/ml and 100 µg streptomycin/ml. Growth medium was renewed 24 hrs after seeding and every 48 hrs thereafter. Experiments were performed 5 to 6 days after plating of the cells.

Inositolphoshate analysis Cardiomyocytes were labelled with 10 µCi *myo*-[2-^{3}H]Ins/ml for 48 hrs in Ins free complete growth medium. After washing and preincubation (15 minutes, 37°C) with incubation buffer (130 mM NaCl; 4.7 mM KCl; 1.3 mM $CaCl_2$; 0.44 mM NaH_2PO_4; 1.1 mM $MgSO_4$; 20 mM $NaHCO_3$; 0.2% glucose; 10 mM HEPES; pH 7.4, 37°C) the cells were stimulated with agonist in the presence of 10 mM LiCl. Incubations were stopped by rapidly washing the cells with ice-cold buffer. Water-soluble [^{3}H]Ins products were extracted by two subsequent extractions with $HClO_4$. The pooled $HClO_4$ fractions were neutralized with 2 M KOH; 1 M K_2CO_3. The remainder, water-insoluble products, was subsequently extracted with ice-cold CH_3OH:HCl (100:1 v/v) for 5 min at 4°C. The [^{3}H]$InsP_n$ isomers were separated by HPLC using a Partisil 10 SAX

column (Alltech, Deerfield, IL, USA) using gradient elution (2.0 M NH_4COOH/H_3PO_4, pH 3.7) (14) and an on-line ^3H detector (Radiometric, Flow-one beta A515 (Canberra Packard Benelux N.V./S.A.) and the amounts were expressed as % of total cellular [^{3}H]Ins (defined as the sum of water-soluble plus -insoluble [^{3}H]Ins containing products).

Permeabilization of cardiomyocytes prelabelled for 48 hrs with *myo*-[2-^{3}H]Ins, was performed by treatment with 100 μg saponin/ml in intracellular buffer (20 mM HEPES; 10 mM NaCl; 110 mM KCl; 1 mM KH_2PO_4; 4 mM $MgCl_2$; 1 mM EGTA; 3 mM ATP; 8 mM creatine phosphate; 6 U creatine kinase/ml; pH 7.0) for 5 min, at 37°C (15). The cells were rinsed three times with the latter buffer before incubation in fresh buffer containing 10 mM LiCl, Ca^{2+} (0 and 96 nM [Ca^{2+}_{free}]) and/or 30 μM GTPγS for 15 min at 37°C, whereafter the buffer was collected for estimation of water-soluble [^{3}H]InsP_n by HPLC. The remainder of [^{3}H]Ins products were extracted with ice-cold $HClO_4$ and CH_3OH:HCl (100:1 v/v) respectively. The $HClO_4$ extract was immediately neutralized. To be able to compare amounts of water-soluble [^{3}H]InsP_n found in buffer and $HClO_4$ extract, the collected buffer was acidified with $HClO_4$ as well, followed by neutralization with 2 M KOH and 1 M K_2CO_3.

1,2-Diacylglycerol analysis Cardiomyocytes were labelled with 2 μCi [2-^{3}H]glycerol/ml for 48 hrs in complete growth medium. The cells were rinsed, preincubated and stimulated as described above. After termination of the incubation the [^{3}H]glycerol containing lipids were extracted by two successive extractions with ice-cold CH_3OH:HCl (100:1 v/v). Water-soluble products were separated from lipids by phase-separation after addition of 1 volume of $CHCl_3$ and 0.5 volumes of 2.5 M HCl. The resulting organic phase was re-extracted once with 1 volume of $CHCl_3$:CH_3OH:0.6 M HCl (3:48:47 v/v/v). The [^{3}H]glycerol containing lipids were dried under N_2-gas followed by resuspension in $CHCl_3$:CH_3OH:H_2O (75:25:2 v/v/v). The analysis of the [^{3}H]glycerol containing (1,2)DAG was performed by thin layer chromatography as described before (16). A few modifications were introduced as follows; Silica gel 60 plates (MERCK, Darmstadt, Germany) were impregnated with 1% boric acid in CH_3OH and activated for 1.5-2 hrs at 110°C and $CHCl_3$:CH_3COCH_3 (96:4 v/v) was used as solvent system. The [^{3}H]glycerol lipids were fluorographically visualized after spraying with En^{3}Hance and subsequently scraped from the plates and counted by liquid scintillation. The amounts of [^{3}H](1,2)DAG were expressed as % of total cellular [^{3}H]glycerol (defined as the sum of water soluble plus water-insoluble [^{3}H]glycerol containing products).

Protein phosphorylation After washing the cells with P_i-free complete growth medium these were labelled with $^{32}P_i$ (100 μCi/ml, carrier free) in the same medium for 2 hrs. Thereafter, the cells were rinsed with incubation buffer and preincubated for 15 min at 37°C before stimulation with agonists. After removal of the medium, the cells were lysed in 150 μl Laemmli sample buffer and labelled proteins in 35 μl aliquots were separated on 10-20% gradient SDS PAAGE, and visualized by autoradiography as described (17).

Statistics Data were evaluated for statistical significance by the Students t-test and significance was set at probability of less then 0.05.

Results

Inositolphosphate isomers Earlier we have shown that stimulation of neonatal rat ventricular myocytes with either PHE or ET-1 results in the activation of the PtdIns-cycle in a dose dependent manner with both agonists, respectively at 10^{-4} M and 10^{-8} M, having almost the same maximal effect on the formation of total [^{3}H]InsP_n separated by chromatography on Dowex (10). In this study we separated the specific [^{3}H]InsP_n isomers by HPLC. The biologically active Ins(1,4,5)P_3 was found not to be elevated above control levels (Fig. 1) while Ins(1,3,4,5)P_4 was not detectable (not shown) even up to 30 min of stimulation by either agonist. Stimulation with PHE resulted in a moderate increase of Ins(1,4)P_2, setting off at 4 min after agonist addition and accomplishing a level of 0.23% after 30 min, which was significantly higher than control (Fig. 1). Yet, ET-1 evoked an immediate significant increase of Ins(1,4)P_2, resulting after 30 min in a higher level (0.47 %) comparing to PHE. On the other hand, PHE and ET-1 stimulation resulted in a similar bulk production of Ins(4)P (12.7 % vs 17.5 % respectively, $p > 0.05$) after 30 min. Only upon ET-1 stimulation, rapid

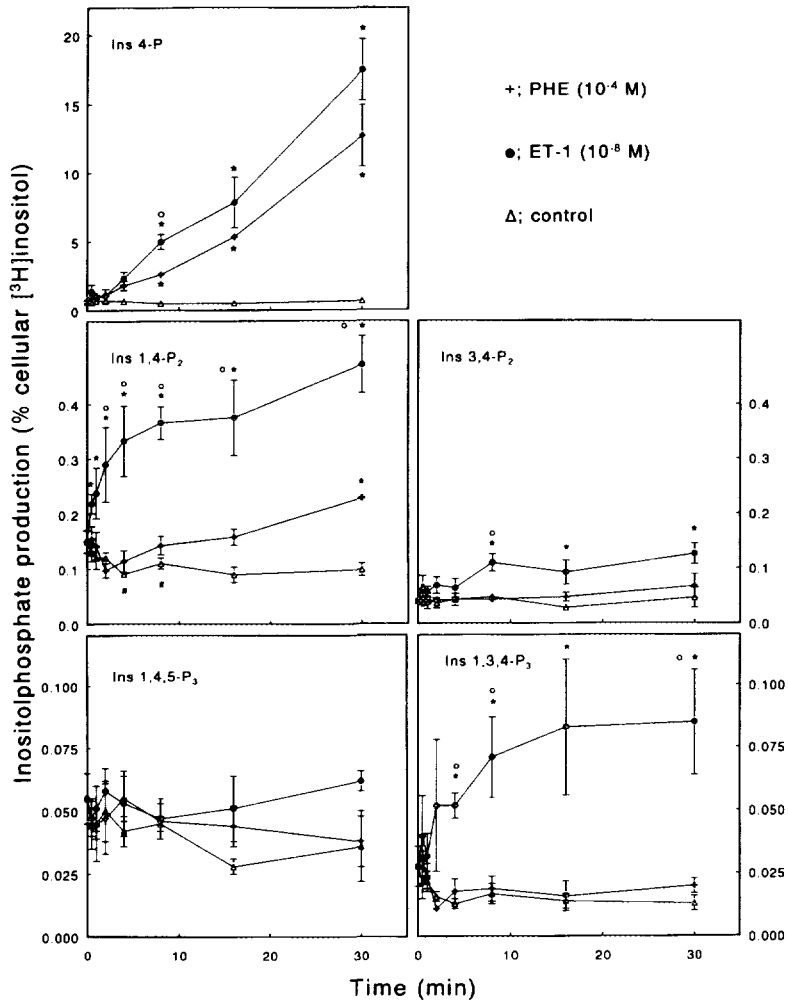

Figure 1. Time course of $InsP_n$ isomer formation in [^{3}H]Ins prelabelled cardiomyocytes after stimulation with PHE, ET-1 or vehicle. The results shown are the mean (± SEM) of 5-8 experiments performed with different cell batches. *, $p < 0.05$ compared to control; #, $p < 0.05$ versus 0 min control; °, $p < 0.05$ ET-1 versus PHE.

increases of dephosphorylation products of Ins(1,3,4,5)P_4, i.e. Ins(1,3,4)P_3 and Ins(3,4)P_2, were measured, which were significant compared to control already after about 5 min of agonist stimulation, resulting in levels of 0.09% and 0.13% respectively after 30 min (Fig. 1). The lack of elevation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 may indicate the occurrence of very small and local changes of the second messengers and/or extremely high activity of the 5-phosphatase. To circumvent the action of the latter enzyme in another experiment the [^{3}H]Ins-labelled cells were permeabilized by saponin and subsequently PLC was activated by GTPγS (and Ca^{2+}) as described (15). Assuming that the formed Ins(1,4,5)P_3 leaks immediately out of the permeable cells, it would therefore become less accessible to the 5-phosphatase and 3-kinase action as derived from another report (18). When PLC was

Table 1 Levels of $InsP_n$ isomers in the incubationbuffer and the acid extract of saponin permeabilized cardiomyocytes after stimulation by GTPγS and/or Ca^{2+}. The values represent the mean (± range/2) of duplicate measurements in one cell batch.

Stimuli		Inositolphosphate production (% of cellular [^{3}H]Inositol)			
		Incubation buffer		Cell extract	
GTPγS	*$[Ca^{2+}_{free}]$*	*Ins(4)P*	*$Ins(1,4)P_2$*	*Ins(4)P*	*$Ins(1,4)P_2$*
-	-	1.20(0.41)	0.92(0.12)	1.71(0.65)	0.23(0.02)
30 μM	-	3.43(1.46)	5.22(0.51)	2.50(0.24)	1.20(0.05)
-	96 nM	1.31(0.52)	2.13(0.62)	3.04(0.28)	0.98(0.00)
30 μM	96 nM	1.53(0.05)	7.98(0.91)	4.42(0.13)	1.67(0.17)

activated with GTPγS and Ca^{2+}, the bulk of $InsP_n$ found in the incubation buffer was, however, $Ins(1,4)P_2$ (8%) (Table 1), while neither $Ins(1,4,5)P_3$ nor $Ins(1,3,4)P_3$ were detectable (not shown). As expected, Ins(4)P was the predominant $InsP_n$ component found in the cell extract (Table 1).

1,2-Diacylglycerol The formation of (1,2)DAG was measured up till 16 minutes, a period of stimulation with time when the formation of $InsP_n$ still occurred at a linear rate (Fig. 1). After 16 min stimulation with PHE (10^{-4} M) (1,2)DAG levels raised significantly above control levels. ET-1 also evoked significant increase of the (1,2)DAG level above control that was reached after 8 min. Like the $Ins(1,4,5)P_3$ levels (Fig. 1), the control (1,2)DAG levels behaved irregularly after vehicle addition (Fig. 2). This may indicate that slight movements of the incubation buffer on top of the cells produces mechanical stretch which previously has been shown to lead to activation of the PtdIns cycle (19, 20).

Protein phosphorylation To further analyze receptor-dependent specificity downstream of PLC we investigated the protein phosphorylation pattern mediated by PHE (10^{-5} M) or ET-1 (10^{-8} M) in cardiomyocytes prelabelled with $^{32}P_i$. Stimulation of the cardiomyocytes with PHE resulted in rapid (1 min) phosphorylation of a 30 kDa protein, that became more pronounced

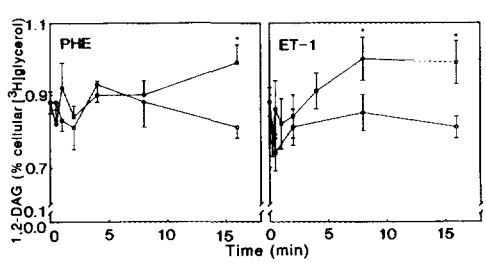

Figure 2. Time course of [^{3}H](1,2)DAG production of [^{3}H]glycerol prelabelled cardiomyocytes after stimulation (●) with PHE (10^{-4} M) and ET-1 (10^{-8} M) or after vehicle addition (○). The results shown are the mean (± SEM) of 6-8 experiments performed with different cell batches. *, $p < 0.05$ compared to control.

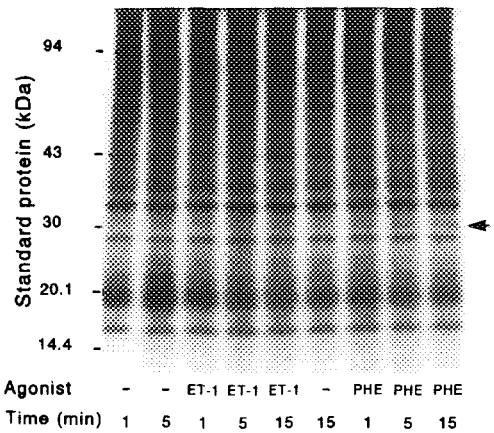

Figure 3. Autoradiogram of proteins labelled upon stimulation of $^{32}P_i$ prelabelled cardiomyocytes with PHE (10^{-5} M) or ET-1 (10^{-8} M). The experiment shown is representative for experiments carried out with 5 separate cell batches. The arrow indicates phosphorylation of a 30 kDa protein.

after 5 and 15 min. This in contrast to ET-1 stimulation that did not evoke phosphorylation of this protein (Fig. 3).

Discussion

In previous work we observed that the PLC activation is homologously desensitized after stimulation with ET-1 but not with PHE. Furthermore, the rate of desensitization of the $InsP_n$ response of the cardiomyocytes after addition of PHE was very slow compared to ET-1 (10,11). It was suggested that either the targets for regulation of the rate of desensitization are the receptors or that PHE and ET-1 use different isoforms of PLC-ß and/or G-protein. Therefore it was of interest to further characterize the receptor-mediated responses more downstream of PLC.

The second messenger, Ins(1,4,5)P_3, was not elevated above control (Fig. 1) while Ins(1,3,4,5)P_4 was not detectable suggesting that Ca^{2+} mobilization by these mediators is of limited or strictly compartmentalized importance in the spontaneously contracting cardiomyocytes. Looking at the receptor-dependent temporal differences in the appearance of (1,2)DAG (Fig. 2), the question arises whether this (1,2)DAG is solely originating from PtdIns(4,5)P_2 or from cross-talk with other (1,2)DAG producing signalling pathways such as phospholipase D (PLD) (2,3,21). The alliance of this two signalling pathways might be established by a PKC isoenzyme, possibly being dependent on the receptor activated (1,2)DAG release, or agonist-receptor coupled activation of $\beta\gamma$ subunits derived from G-proteins (22). Alternatively, the temporal differences in (1,2)DAG production could have its origin in the kinetics of different PLC-ß isoenzymes and/or PLD.

The receptor-dependent differences in the Ins(1,4,5)P_3 metabolism can be interpreted on other levels. If the PLC isoenzymes involved in transducing either the ET-1 or the PHE signal are not identical, the possibility of different kinetics or competition of the 5-phosphatase and the 3-kinase pathway can be supposed. In general, experiments to measure the metabolic destiny of Ins(1,4,5)P_3 performed with cell homogenates or permeabilized cells generally indicated that the 5-phosphatase pathway is predominant (23-25). Moreover, we should regard the consequences of different subcellular location of 5-phosphatase and 3-kinase on the Ins(1,4,5)P_3 metabolism (25). For instance, in liver and intestinal epithelial cells the bulk of the 5-phosphatase is restricted to the plasma membrane, while the 3-kinase is cytosolic and therefore its access to Ins(1,4,5)P_3 would not seem to be restricted. This would agree with our experiment performed with GTPγS stimulated G-protein-PLC in saponin-permeabilized cardiomyocytes showing no Ins(1,4,5)P_3 but predominantly the 5-phosphatase product Ins(1,4)P_2 in the extracellular buffer, while 3-kinase products were absent. Furthermore, the regulation of 5-phosphatase and 3-kinase activity may be critical. Receptor-induced increase of intracellular [Ca^{2+}_{free}] can increase the V_{max} of the 3-kinase (25,26), while the 5-phosphatase is insensitive to Ca^{2+}. Compartmentalization of the processes could create microenvironments that would come up to these requirements.

The receptor-specific protein phosphorylation may also be interpreted by the intervention of other signalling pathways, e.g. cross-talk to the ß-adrenergic signal transduction pathway. The 30 kDa protein that is phosphorylated upon PHE but not ET-1 stimulation (Fig. 3) appeared not to be membrane-bound or of cytoplasmic origin (results not shown) suggesting its association with the cytoskeleton or myofilaments. In fact, this protein may be identical to troponin I (30 kDa) which has been shown to be substrate for PKC *in vitro* in isolated myofibrils (27).

In conclusion, the results demonstrate that after maximal stimulation of PLC in cardiomyocytes with ET-1 or PHE several differences are present downstream of PLC. Distinct receptor-dependent responses can be noted concerning Ins(1,4,5)P_3 metabolism, temporal formation of (1,2)DAG and protein phosphorylation.

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