Endothelin-Induced Response of the Phosphatidylinositol Cycle in Cultured Cardiomyocytes Exposed to Substrate-Free Hypoxia-Reoxygenation[a]

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INTRODUCTION

Endothelin-1 (ET-1), an endothelium-derived peptide, has a potent and sustained vasoconstrictor effect.[1] ET-1 also has both positive inotropic and chronotropic effects on the heart[2] and was reported to induce cardiac hypertrophy.[3] The release of ET-1 is increased during hypoxia and myocardial infarction leads to increased plasma levels of ET-1.[4] Moreover, hypoxia increases the number of myocardial ET-1 binding sites[5] as well as α_1-adrenergic receptors, the latter accompanied by an increase in phospholipase C (PLC) activity in response to receptor stimulation.[6,7] The signaling pathway activated by ET-1 also involves activation of PLC[8,9] resulting in inositol 1,4,5-trisphosphate ($I(1,4,5)P_3$)–induced Ca^{2+} mobilization and protein kinase C (PKC) activation.[10] Activation of PKC was shown to aggravate hypoxic myocardial injury presumably by stimulation of Na^+/H^+ exchange leading to Ca^{2+} overload by Na^+/Ca^{2+} exchange.[11] Another acute effect of activation by ET-1 is enhanced Ca^{2+} entry through the T-type Ca^{2+} channels.[12]

The possibility that PLC activation, through increased ET-1 and receptor levels during hypoxia, leads to increased hypoxia-reoxygenation injury prompted us to investigate the activity of PLC in cultured cardiomyocytes after ET-1 stimulation during normoxia and glucose-free hypoxia-reoxygenation.

[a] This work was supported by Grant 89.221 from the Netherland Heart Foundation.

METHODS

Cell Culture

Primary cultures of neonatal ventricular myocytes were prepared from 1–2 day-old Wistar rats as described before.[9]

Analysis of Water-Soluble Inositol Phosphates

Cardiomyocytes, 4 days after plating, were labeled with 2 μCi *myo*-[2-^{3}H]inositol/ml for 48 h. Before performing the experiments, myocytes were washed 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 $NaHCO_3$; 20 mM HEPES; pH 7.4, 37°C that was gassed with either 95% air/5% CO_2 or 95% N_2/5% CO_2). Thereafter, cells were incubated as described in TABLES 1 and 2. Incubations were terminated by washing the cells followed by extraction with 4% perchloric acid and CH_3OH:HCl (100:1 vol/vol), respectively. Inositol phosphates (IP_n) were quantified by chromatography on Dowex AG 1-X8 as described before.[9] The total of cellular [^{3}H]inositol was defined as the sum of water-soluble inositol-containing products together with inositol lipids.

RESULTS AND DISCUSSION

As previously shown[9] exposure of cardiac myocytes to ET-1 (10^{-8} M) results in activation of PLC. Exposure of the cells to ET-1 for only 15 min led to persistent stimulation of IP_n production, which declined to a low level after 4 h (TABLE 1), still above unstimulated cells. The decline was not caused by deterioration of cell viability as maximal stimulatability remained high over this period (TABLE 1). Not only was the

TABLE 1. Long-Lasting but Desensitized Activation of PLC after Short (15 min) Exposure of Cardiomyocytes to ET-1

Addition During		[^{3}H]IP_n Level (% of cellular [^{3}H]inositol)	
Preincubation 0–15 min	Incubation 15–270 min	30–60 min	240–270 min
Buffer	ET-1	25.6 ± 6.9	34.9 ± 0.2
ET-1	Buffer	13.0 ± 3.8	2.6 ± 0.3
ET-1	ET-1	19.9 ± 7.9	11.8 ± 0.3

Cardiomyocytes, prelabeled with *myo*-[2-^{3}H]inositol, were preincubated with buffer or with ET-1 (10^{-8} M) for 15 min in the absence of Li^+ followed by extensive washing of the cells. At different intervals after the onset of this initial incubation cells were challenged with LiCl (10 mM) in the absence or presence of ET-1 (10^{-8} M). Incubation in the presence of Li^+ was always for 30 min. After incubation, the cells were extracted and [^{3}H]IP_n was quantified. Further details are described in *Materials and Methods*. Results are mean ± range/2 for two experiments. Note that the presented data are already corrected for unstimulated [^{3}H]IP_n levels (4.23 ± 0.48).

TABLE 2. Partial Inhibition of ET-1 Induced IP_n Accumulation by Hypoxia but not by Reoxygenation

	[^{3}H]IP_n Level (% of cellular [^{3}H]inositol)			
	Basal		ET-1 (10^{-8} M) Stimulated	
	Normoxia	Hypoxia	Normoxia	Hypoxia
30 min	2.50 ± 0.42	2.71 ± 0.38	21.55 ± 2.65	16.03 ± 3.01
60 min	2.59 ± 0.38	2.85 ± 0.30	21.58 ± 3.95	13.06 ± 2.91
90 min	2.37 ± 0.25	2.82 ± 0.23	20.39 ± 2.71	9.70 ± 2.38*
Reoxygenated	1.60 ± 0.13	2.97 ± 0.41**	5.54 ± 1.49	8.25 ± 1.05

Cardiomyocytes, prelabeled with *myo*-[2-^{3}H]inositol, were incubated in incubation buffer (see *Methods*) at 37°C in 95% air/5% CO_2 (normoxia) or in 95% N_2/5% CO_2 (hypoxia) for the periods indicated in the table. Then ET-1 was added to 10^{-8} M in the presence of 10 mM LiCl and incubation proceeded under the same conditions for 15 min. For reoxygenation, hypoxic buffer was replaced with fresh normoxic buffer and the incubation was continued for 15 min whereafter ET-1 and LiCl were added as described above. To mimic the reoxygenation protocol, buffer exchange was also performed after 90 min of normoxia. Further details are described in *Methods*. Data are presented as mean ± SEM, $n = 7$–10, * $p < 0.02$ and ** $p < 0.005$ versus normoxia.

ET-1 response desensitized but the responsiveness of cells to a second dose of ET-1 was also diminished, remaining so up to 4 h.[9] These results show that brief exposure of cardiomyocytes to ET-1 can lead to long-lasting but desensitized activation of PLC.

To evaluate the ET-1–evoked IP_n production during glucose-free hypoxia-reoxygenation, we established a model of cultured cardiomyocytes exposed to 95% N_2/5% CO_2 for periods up to 90 min followed by 30-min reoxygenation by buffer-change in 95% air/5% CO_2. The severity of hypoxia-reoxygenation was characterized before by monitoring ATP depletion and lactate dehydrogenase (LDH) leakage during reoxygenation and these results (not shown) correlate nicely with earlier reports.[13,14] Basal activity of PLC was not stimulated during 90-min hypoxia (TABLE 2). This contrasts with earlier data[15] where phosphatidylinositol (PI), PI(4)P and PI(4,5)P_2 were decreased in mass suggesting PLC activation, but IP_n was not measured. Stimulatability of the cardiomyocytes with ET-1 (10^{-8} M) stayed relatively constant during a 90-min normoxic period (TABLE 2). However, after buffer change to mimic the reoxygenation protocol, the ET-1 response was unexpectedly diminished. Hypoxia gradually decreased the stimulatability of cardiomyocytes with ET-1 to 38% after 90 min. Reoxygenation led to a slight but not significant increase in ET-1 response relative to normoxia.

These results show that ET-1 release from the coronary endothelium, as for example, induced by tissue hypoxia,[4] can give long-lasting signal transduction in the myocardial cells at a low level. During the hypoxic period the ET-1–evoked response is further decreased by a decline in responsiveness of the cells. ET-1–induced increases in intracellular free Ca^{2+} by stimulation of Na^+/H^+ exchange, opening of T-type channels, and I(1,4,5)P_3-induced Ca^{2+} mobilization may contribute to development of Ca^{2+} overload, which is generally thought to be causally related to development of irreversible cell injury.

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