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Calcium and the Endothelin-1 and α_1-Adrenergic Stimulated Phosphatidylinositol Cycle in Cultured Rat Cardiomyocytes

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H. A. A. VAN HEUGTEN, H. W. DE JONGE, K. BEZSTAROSTI AND J. M. J. LAMERS. Calcium and the Endothelin-1 and α_1-Adrenergic Stimulated Phosphatidylinositol Cycle in Cultured Rat Cardiomyocytes. *Journal of Molecular and Cellular Cardiology* (1994) **26**, 1081–1093. Cultured neonatal rat cardiac myocytes have been utilized as a model for the study of the effect of variations in cytoplasmic free Ca^{2+} on the activity of phospholipase C, a key enzyme in agonist-stimulated signal transduction through the phosphoinositide pathway. Cells prelabelled with [^{3}H]inositol were exposed to various agents in an attempt to modulate the cytoplasmic free Ca^{2+} concentration and the formation of [^{3}H]inositolphosphates (15–30 min) in the presence of Li^+ was taken as a measure of phospholipase C activity. Not the basal but the endothelin-1 (10^{-8} M) induced [^{3}H]inositolphosphate production (15 min) was stimulated 1.54- and 1.43-fold by A23187 (10 μM external Ca^{2+}) and 50 mM K^+ (1.3 mM external Ca^{2+}) treatment of cells, respectively. The phenylephrine (10^{-4} M) induced response was also stimulated (1.35-fold) by A23187, however it was 43% inhibited by high K^+. Ouabain (10 μM) treatment of cells did not affect either basal or agonist stimulated phosphoinositide turnover. On the other hand, total removal of external free Ca^{2+} by addition of 50 μM ethylene glycol bis(β-aminoethyl ether) (N,N,N',N'-tetraacetic acid strongly inhibited (75%) the endothelin-1 induced but not the basal phospholipase C activity. Endothelin-1 binding to its receptor was shown not to be inhibited by the absence of external Ca^{2+} while resynthesis of [^{3}H]phosphatidylinositol 4,5-bisphosphate was not rate-limiting under this condition. The lack of external Ca^{2+} eventually resulted in total standstill of the ET-1 induced PtdIns turnover after 30 min. Although not always as predicted, effects on basal and agonist-activated phospholipase C were observed too when cells were treated with low Ca^{2+} medium, Ca^{2+} entry blocker nifedipine (1 μM) or Ca^{2+}-channel agonist Bay K8644 (1 μM) but most of these effects were only seen after 90 min incubation. Fluorometric (fura-2) measurements showed that total removal of external free Ca^{2+} for a short period decreased, while short exposure to high K^+ increased cytoplasmic free Ca^{2+} but neither Ca^{2+} free buffer or nifedipine nor Bay K8644 had any effect. Furthermore, in saponin-permeabilized cardiomyocytes we could demonstrate that basal as well as GTPγS (30 μM) stimulated phospholipase C activity was strongly activated by free Ca^{2+} in the concentration range of 0.1–10 μM. We conclude that in the intact cardiomyocyte the signalling pathway through phospholipase C/phosphatidylinositol 4,5-bisphosphate, stimulated by agonist-receptor interaction that activates GTP-binding proteins as does GTPγS, is likely be a Ca^{2+} dependent process.

KEY WORDS: Ca^{2+}; Endothelin-1; α_1-Adrenergic agonist; Phosphatidylinositol cycle; Phospholipase C; Cardiomyocyte; Ca^{2+} overload.

Introduction

In heart, signal transduction through the phosphatidylinositol (PtdIns) pathway plays an important role in regulation of inotropy and the development of hypertrophy (Brown and Martinson, 1992; Morgan and Baker, 1991). Receptors for endothelin-1 (ET-1), angiotensin II and phenylephrine (PHE), were shown to be coupled to this signal transduction pathway. Activation of phospholipase C (PLC)

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by agonist binding to the GTP-binding protein (G-protein) coupled receptors results in generation of the second messengers 1,2-diacylglycerol [(1,2)DAG] and inositol 1,4,5-trisphosphate [Ins $(1,4,5)P_3$] by the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns$(4,5)P_2$]. Ins$(1,4,5)P_3$ releases Ca^{2+} from internal stores while this increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}_{free}]_i$) together with elevated (1,2)DAG levels activates protein kinase C, a key enzyme in regulation of cellular functions as, for example, in the transcriptional control during the development of hypertrophy (Morgan and Baker, 1991).

In various cell types it was shown that Ca^{2+} has profound effects on PLC activation either directly or by potentiating the receptor-mediated response (Abdel-latif, 1986; Cockcroft and Thomas, 1992). The direct effects of Ca^{2+} occur both in the physiological micromolar range as well as at high millimolar levels which are of less relevance for the intact cell. Potentiation of receptor-mediated stimulation of PLC by Ca^{2+} occurs at micromolar levels (Abdel-latif, 1986; Cockroft and Thomas, 1992). The myocardial studies available used two different approaches to show the Ca^{2+} dependence of PLC: isolated membrane preparations (Schwertz *et al.*, 1987; Schwertz and Halverson, 1989; Edes and Kranias, 1990; Renard and Poggioli, 1990) and saponin-permeabilized cells (McDonough *et al.*, 1988; Jones and Brown, 1988). In three of these studies the reported membrane-bound PLC activity was found to require Ca^{2+} in the absence of GTP analog, however these Ca^{2+} levels were well above the normal cytoplasmic range (Schwertz *et al.*, 1987; Schwertz and Halverson, 1989; Edes and Kranias, 1990). Other investigations demonstrated that only during stimulation of PLC by G-protein the Ca^{2+} sensitivity of the enzyme was in the physiological range (Renard and Poggioli, 1990; McDonough *et al.*, 1988; Jones and Brown, 1988). Therefore, it became important to obtain answers to the following questions for intact cardiomyocytes: (i) does an increase of cytoplasmic free Ca^{2+} stimulate basal PLC activity?; and (ii) does the elevation of $[Ca^{2+}_{free}]_i$ have a feed-forward stimulatory role in the receptor-mediated activation of PLC?

The $[Ca^{2+}_{free}]_i$ rises from 100 to several hundred nM during each cardiac cycle, so contractile-associated Ca^{2+} transients could normally induce PtdIns$(4,5)P_2$ hydrolysis. Mechanical loading of cultured cardiomyocytes results in the activation of PtdIns$(4,5)P_2$ hydrolysis and this effect may be caused by alterations of the $[Ca^{2+}_{free}]_i$ (Komura *et al.*, 1990). After ET-1 stimulation of cardiomyocytes Ca^{2+} was transiently increased (Vigne *et al.*, 1990; Hirata *et al.*, 1989) suggesting that PLC fluctuates accordingly. Another implication of regulation of PLC by Ca^{2+} is of pathophysiological relevance as during myocardial ischemia-reperfusion the development of intracellular Ca^{2+} overload might cause activation of PLC. The product of PLC activation Ins$(1,4,5)P_3$ on its turn might induce release of Ca^{2+} from the sarcoplasmic reticulum thereby aggravating the Ca^{2+}-overload which is believed to be causally related to myocardial injury (Lee and Allen, 1991). Indeed, several reports have shown that basal and α_1-adrenergic agonist-induced PtdIns$(4,5)P_2$ hydrolysis is increased during myocardial hypoxia or ischemia-reperfusion (Otani *et al.*, 1989; Mouton *et al.*, 1991; Heathers *et al.*, 1989).

We used intact cultured neonatal rat ventricular myocytes as a model system to study the effect of variations in $[Ca^{2+}_{free}]_i$ on the activity of PLC. It was shown that agonist (ET-1 or PHE)-induced generation of inositol phosphates ($InsP_n$) is modulated by protocols [depolarization by high K^+ concentration or Ca^{2+} chelation by ethylene glycol bis(β-aminoethyl ethe) N,N,N′,N′-tetraacetic acid (EGTA)] that were demonstrated by fura-2 fluorescence measurements, respectively to raise or lower cytosolic levels of Ca^{2+} in intact cultured neonatal rat ventricular cardiomyocytes. Ca^{2+} ionophore A23187 in the presence of micromolar Ca^{2+} was also shown to stimulate agonist-induced $InsP_n$ accumulation. Furthermore, in saponin-permeabilized cadiomyocytes Ca^{2+} concentrations of 0.1–10 μM were shown to stimulate strongly basal as well as G-protein activated phospholipase C activity. The cytoplasmic free Ca^{2+} concentration may play a regulatory role in the receptor-mediated PtdIns turnover particularly when Ca^{2+} increases due to the Ca^{2+} mobilizing action of Ins$(1,4,5)P_3$.

Materials and Methods

Reagents

Culture dishes (4-well multidish) were obtained from Nunc (Roskilde, DK). Cell culture medium Ham F10 was obtained from Gibco (UK), while fetal calf serum, horse serum and penicillin/streptomycin was from Boehringer Mannheim (Germany) as was Ca^{2+} ionophore A23187. Trypsin (type III) was from Sigma (St. Louis, USA) as were PHE and phosphoinositide standards. ET-1 was obtained from Peninsula Laboratories (Belmont CA, USA). Fura 2-AM was from Molecular Probes (Eugene,

OR, USA) while ionomycin was from Calbiochem (USA). Bay K1040 (nifedipine) was from Bayer (Leverkusen, Germany), Bay K8644 was obtained from Pharmuka Laboratories (Gennevilliers, France) while g-strophanthin (ouabain) was from MERCK (Darmstadt, Germany). *Myo*-[2-^{3}H]inositol (17.5 Ci/mMol) was from Amersham International PLC (Amersham, UK) and Dowex AG 1-X8 (200–400 mesh, formate form) was from Bio-Rad Laboratories (Richmond CA, USA) while En^{3}Hance was from NEN (Boston, USA).

Cell culture

Primary cultures of neonatal ventricular myocytes were prepared from 1–2-day-old Wistar rats as described before (Yagev *et al.*, 1984) using preplating (Blondel *et al.*, 1971) to increase further cardiomyocyte to non-myocyte ratio. Cardiomyocytes were seeded in 1.9 cm^2 wells at 150–175 $\times 10^3$ cells/cm^2 giving a confluent monolayer of spontaneously contracting cells after 24 h. The cells were maintained 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 h after seeding and every 48 h thereafter. Experiments were routinely performed 5 or 6 days after plating of the cells.

Measurement of water-soluble inositolphosphates and inositol-containing lipids

Cardiomyocytes were labelled with 2 μCi *myo*-[2-^{3}H]inositol/ml for 24 h in complete growth medium containing 3 μM inositol. Prior to performing the experiments, the cells 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 $NaHCO_3$; 11 mM glucose; 20 mM HEPES; pH 7.4, 37°C and aerated with 5% CO_2). Thereafter, the cells were incubated as described in the legends to the figures. Incubations were terminated by rapidly washing the cells with ice-cold buffer followed by two successive extractions with ice-cold 4% (w/v) $HClO_4$. Lipids were subsequently extracted by incubation with ice-cold methanol:12 M HCl (100:1). The $HClO_4$ extract, containing water-soluble products, was neutralized by addition of a solution of 2 M KOH and 1 M K_2CO_3. Lipids were extracted from the methanol:HCl fraction by phase-separation after addition of 1 volume of chloroform and 0.5 volumes of 2.5 M HCl. The resulting organic (lower) phase was re-extracted once with 1 volume of chloroform:methanol:0.6 M HCl (3:48:47 v/v/v) and used for lipid analysis as described below. The total cellular amount of *myo*-[2-^{3}H]inositol-containing compounds (defined as the sum of water-soluble inositol-containing products together with inositol-containing lipids) was constant during the experiments. The [^{3}H]inositol phosphates ([^{3}H]InsP_n) were separated from [^{3}H]inositol and [^{3}H]glycero-phosphoinositol by chromatography on Dowex AG 1-X8 as originally described by Berridge *et al.* (1982). [^{3}H]Inositol and [^{3}H]glycero-phosphoinositol were eluted with water and 5 mM Borax in 30 mM CHOONa respectively. Total [^{3}H]InsP_n was subsequently eluted with 1.0 M $CHOONH_4$ in 0.1 M HCOOH.

The [^{3}H]inositol-containing phospholipids PtdIns, phosphatidylinositol 4-monophosphate [PtdIns(4)P] and PtdIns(4,5)P_2 in the organic phase were analysed by thin layer chromatography on Silica gel 60 plates (MERCK) in chloroform:acetone:methanol:acetic acid:water (40:15:13:12:8 v/v/v/v/v) as described (Jolles *et al.*, 1981) and were visualized by fluorography after spraying with En^{3}Hance. Quantification of the separate inositol-containing lipids was carried out by scraping the spots off the plates and counting the scrapings in scintillation cocktail.

Inositol phosphate production in permeabilized cardiomyocytes

Cardiomyocytes were isolated and cultured as described above. After labelling with 2 μCi *myo*-[2-^{3}H]inositol/ml for 24 h the cells were washed with phosphate buffered saline (37°C) and subsequently permeabilized for 5 min with 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) with 100 μg saponin/ml. Hereafter, the cells were washed three times with intracellular buffer lacking saponin and fresh intracellular buffer containing 10 mM LiCl with Ca^{2+} and GTPγS as required was added. Permeabilized cells were incubated for 15 min at 37°C, buffer was collected and cells were extracted with $HClO_4$ and methanol/HCl as described above. As > 60% of InsPn was seen to be present in the buffer (not shown), both buffer and PCA extract of the cells were analysed by Dowex AG 1-X8 chromatography as described above.

Measurement of intracellular free Ca^{2+} concentration

Cardiomyocytes were grown on glass cover slips essentially as described above. After washing with incubation buffer (see above) the cells were loaded for 60 min at 37°C with 2 μM fura-2 AM in the presence of 2.5 mM probenicid. Subsequently, the cover slip was washed with incubation buffer and inserted in a holder tightly fitting into a quartz cuvette containing 2 ml incubation buffer. The cuvette was placed in a Perkin-Elmer LS-3B fluorescence spectrometer with a thermostated (37°C) cuvette holder. Fluorescence was continuously recorded at 510 nm using excitation wavelengths of 340 and 380 nm. The ratio of the fluorescence elicited at 340 nm ($F_{340\,nm}$) and 380 nm ($F_{380\,nm}$) was determined and taken as measure of the cytoplasmic free Ca^{2+} level. Calibration of the fluorescence signal was performed after each assay by addition of 2.5 μM ionomycin to obtain maximal ratio that ranged from 1.26 to 6.44. Hereafter, 10 mM EGTA was added to obtain the ratio at minimal Ca^{2+} saturation of the fluorescent dye (i.e. 0.93 ± 0.02, $n = 15$). Maximal ratio values were variable, therefore absolute Ca^{2+} concentrations could not be determined. Before starting the treatment, the cells were incubated for 15 min in buffer. During both periods $F_{340\,nm}$ and $F_{380\,nm}$ were determined several times and averaged.

Statistical analysis

Data were evaluated for statistical significance by one-way or multivariate analysis of variance where applicable.

Results

Effects of agents that modulate cytoplasmic free Ca^{2+} on basal PLC activity

To determined whether PtdIns(4,5)P_2 hydrolysis in unstimulated neonatal rat ventricular myocytes can be activated by an increase in $[Ca^{2+}_{free}]_i$, cells prelabelled with [^{3}H]inositol were exposed to the Ca^{2+} ionophore A23187 at different Ca^{2+} concentrations in the presence of LiCl (Table 1). Li^+ (10 mM) was routinely added in order to block inositol mono- and bisphosphatase and to take the accumulation of total [^{3}H]InsP_n (after incubation for 15 min) as a measure for PLC activity (van Heugten *et al.*, 1993; Meij and Lamers, 1989; Lamers *et al.*, 1992). Increasing the extracellular Ca^{2+} (10–100 μM) well above physiological intracellular levels in the presence of Ca^{2+} ionophore A23187 did not result in activation of InsP_n accumulation above basal values (Table 1). The possibility that the $[Ca^{2+}_{free}]_i$ was already optimal for PLC activity was investigated by total removal of external free [Ca^{2+}] through the addition of 50 μM EGTA to Ca^{2+} free medium. Even this drastic treatment of the cells did not significantly affect the basal InsP_n production (Table 1).

Effects of agents that increase cytoplasmic free Ca^{2+} on agonist-stimulated PLC activity

As it was reported (Schwertz and Halverson, 1989; Edes and Kranias, 1990; Renard and Pogglioli, 1990) that activation of the G-protein(s) coupled to

Table 1 Effect of agents modulating $[Ca^{2+}_{free}]_i$ on basal PLC activity in rat neonatal cardiomyocytes

Condition	[^{3}H]InsP_n levels	n
1.3 mM Ca^{2+}	1.03 ± 0.07	13
0 μM Ca^{2+}, 1 μM A23187	0.90 ± 0.06	6
1 μM Ca^{2+}, 1 μM A23187	1.10 ± 0.09	12
10 μM Ca^{2+}, 1 μM A23187	1.08 ± 0.08	11
100 μM Ca^{2+}, 1 μM A23187	1.10 ± 0.08	13
0 μM Ca^{2+}, 50 μM EGTA	0.91 ± 0.02	4

Cardiomyocytes were prelabelled for 24 h with *myo*-[2-^{3}H]inositol. Cells were washed with Ca^{2+} free incubation buffer and incubated for 15 min in the presence of 10 mM LiCl with buffer containing 1.3 mM Ca^{2+}, with buffer containing 1 μM Ca^{2+} ionophore A23187 and different Ca^{2+} concentrations as indicated, or with 50 μM EGTA in Ca^{2+} free buffer. Extraction and quantification of [^{3}H]InsP_n levels by Dowex chromatography were performed as described in Materials and Methods. [^{3}H]InsP_n levels are expressed as percentage of total cellular [2-^{3}H]inositol (defined as the sum of water-soluble inositol-containing products together with inositol-containing lipids). n refers to the number of experiments (mean ± S.E.).

PLC can increase Ca^{2+} sensitivity of PtdIns(4,5)P_2 hydrolysis, we incubated the cardiomyocytes with ET-1, a hormone known to activate phosphoinositide turnover in these cells (van Heugten *et al.*, 1993). The Ca^{2+} sensitivity of PtdIns turnover might be dependent on the agonist employed through the involvement of different G-protein or PLC isozymes so we performed parallel experiments in which cells were incubated with the α_1-agonist PHE (Meij and Lamers, 1989; Lamers *et al.*, 1992). Previously we demonstrated that the ET-1 and PHE stimulated [^{3}H]InsP_n production is almost linear during the first 30 min of incubation of the cardiomyocytes (van Heugten *et al.*, 1993; Meij and Lamers, 1989). ET-1 as well as PHE-evoked PtdIns(4,5)P_2 hydrolysis (15 min) could indeed be significantly stimulated by increasing $[Ca^{2+}_{free}]_i$ by using Ca^{2+}-ionophore A23187 in combination with 10 μM external Ca^{2+} [Fig. 1(a)] which is well above the normal intracellular level. ET-1 induced InsP_n accumulation was 1.54-fold increased while the PHE-coupled InsP_n production was 1.34-fold stimulated at 10 μM external Ca^{2+}. No significant stimulation was seen at 100 μM external Ca^{2+}.

Depolarization of cardiomyocytes with high extracellular K^+ is known to induce an increase in $[Ca^{2+}_{free}]_i$ (Shue *et al.*, 1986). Treatment of unstimulated cardiomyocytes with high K^+ medium did not have a significant effect on PLC activity [Fig. 1(b)]. In contrast, the ET-1 induced InsP_n accumulation was 1.43-fold stimulated by treatment with 50 mM K^+ while, unexpectedly, the PHE-evoked PtdIns turnover was 43% inhibited.

Inhibition of the Na^+/K^+ ATPase by ouabain is also known to increase $[Ca^{2+}_{free}]_i$ in cultured rat neonatal cardiomyocytes (Hallaq *et al.*, 1989). For this purpose the cells were preincubated for 10 min with 0, 0.1 and 10 μM ouabain, the latter two concentrations inhibiting the activity of Na^+/K^+ ATPase with 10 and 40% respectively (Xie *et al.*, 1989). Hereafter the cells were incubated for 15 min in the presence of 10 mM Li^+ to measure

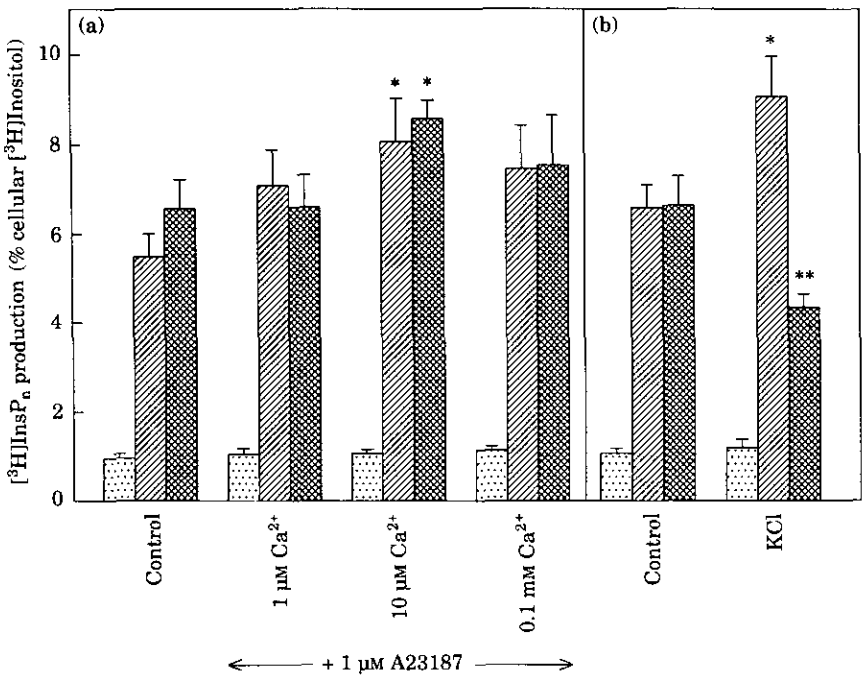

Figure 1 Effect of agents modulating $[Ca^{2+}_{free}]_i$ on basal or ET-1- and PHE-stimulated PLC activity. (a) After labelling for 24 h with *myo*-[2-^{3}H]inositol, cells were washed with Ca^{2+} free buffer and incubated for 15 min in the presence of 10 mM LiCl with buffer containing 1.3 mM Ca^{2+} ("control") or with buffer containing 1 μM Ca^{2+} ionophore A23187 and various Ca^{2+} concentrations as indicated. Subsequently cells were stimulated as described below. (b) After labelling for 24 h with *myo*-[2-^{3}H]inositol, cells were washed with normal buffer and subsequently incubated for 15 min in the presence of 10 mM LiCl in incubation buffer with 4.7 mM KCl ("control") or with 50 mM KCl ("KCl"). Subsequently cells were stimulated for 15 min by addition of buffer (▤), of ET-1 to 10^{-8} M (▨) or of PHE to 10^{-5} M (▩). Cells were extracted and InsP_n levels were determined as described in Materials and Methods. [^{3}H]InsP_n levels are expressed as percentage of total cellular [2-^{3}H]inositol (defined as the sum of water-soluble inositol-containing products together with inositol-containing lipids). Data are expressed as mean ± S.E. ($n = 8$ for buffer and $n = 12$ for agonist stimulations). *$P < 0.05$ and **$P < 0.005$ *v* control stimulation with the same agonist.

PLC activity. [^{3}H]InsP$_n$ productions (in % of cellular [^{3}H]inositol) were 0.91 ± 0.07, 0.94 ± 0.07 and 0.98 ± 0.06 ($n=7$) in the absence of agonist, 3.89 ± 0.07, 3.88 ± 0.43 and 3.54 ± 0.44 ($n=12$) in the presence of 10^{-8} M ET-1 and 5.02 ± 0.25, 4.71 ± 0.44 and 4.30 ± 0.30 ($n=12$) in the presence of 10^{-4} M PHE for 0, 0.1 and 10 μM ouabain respectively. Thus, neither basal nor agonist-induced PtdIns(4,5)P$_2$ hydrolysis was significantly altered by partially blocking Na^+/K^+-ATPase.

Effect of EGTA treatment of cells on ET-1 receptor-stimulated PLC activity

To determine whether agonist-induced activation of PLC requires any Ca^{2+} at all we incubated cardiomyocytes with ET-1 in buffer containing 1.3 mM Ca^{2+} or in Ca^{2+} free buffer with 50 μM EGTA. Part of the experiment was designed to evaluate the influence of EGTA on ET-1 receptor binding making use of the fact that ET-1 is a stable binder to its receptor (Bolger *et al.*, 1990). The presence of EGTA during the preincubation period where ET-1 but not Li^+ was present only led to a minor but not significant effect on binding of ET-1 to the receptor as judged by subsequent [^{3}H]InsP$_n$ accumulation during the incubation period where ET-1 (and EGTA) were removed and Li^+ was added to monitor PtdIns turnover [Fig. 2(a)]. However, EGTA being present during the incubation period of the experiment where ET-1 receptors were already occupied resulted in 75% inhibition of InsP$_n$ accumulation above the basal level that was not affected itself [Fig. 2(b)]. The possibility that resynthesis of PtdIns(4,5)P$_2$ through PtdIns 4- and 5-kinase was severely inhibited by EGTA leading to PtdIns(4,5)P$_2$ depletion was investigated by quantification of the cellular levels of the inositol-containing lipids of the experiment depicted in Figure 2. As shown in Table 2, the cellular [^{3}H]PtdIns, [^{3}H]PtdIns(4)P and [^{3}H]PtdIns(4,5)P$_2$ levels were even significantly higher after inhibition of [^{3}H]InsP$_n$ accumulation by EGTA as compared to the levels remaining after ET-1 induced PtdIns turnover suggesting that the resynthesis of PtdIns(4,5)P$_2$ was not rate-limiting. The only [^{3}H]inositol-containing lipid not affected by EGTA was [^{3}H]lyso-PtdIns, i.e not an intermediate of the PtdIns cycle. EGTA did not inhibit the ET-1 coupled PtdIns(4,5)P$_2$ hydrolysis by simply slowing down PtdIns turnover, as is shown in Figure 3. Even after preincubation with EGTA for 15 min, addition of ET-1 led to significant production of [^{3}H]InsP$_n$ during the first 30 min of stimulation. However, after this initial increase further [^{3}H]InsP$_n$ accumulation was totally blocked (Fig. 3) although

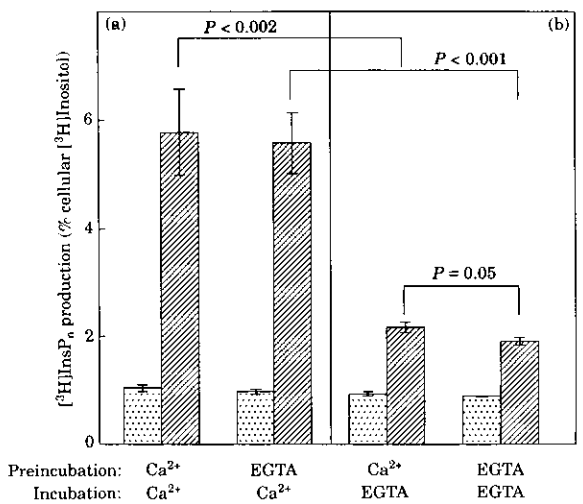

Figure 2 Effect of EGTA treatment on the basal and ET-1-stimulated PLC activity in cardiomyocytes. Cardiomyocytes, labelled for 24 h with *myo*-[2-^{3}H]inositol, were washed with Ca^{2+}-free buffer and subsequently preincubated for 15 min with (▨) or without (▩) 10^{-8} M ET-1 in buffer containing either 1.3 mM Ca^{2+} or 50 μM EGTA (in Ca^{2+}-free buffer) as indicated. Following this preincubation period Ca^{2+}, EGTA and unbound ET-1 were removed by washing with Ca^{2+}-free buffer. Hereafter PtdIns turnover was monitored by incubation for 15 min in the presence of 10 mM LiCl in 1.3 mM Ca^{2+} buffer or in Ca^{2+}-free buffer containing 50 μM EGTA as indicated in the Figure. Accumulated [^{3}H]InsP$_n$ was quantified as described in Materials and Methods. [^{3}H]InsP$_n$ levels are expressed as mean percentage ± range/2 and ± S.E. of the total amount of [2-^{3}H]inositol labelled products, defined as in Figure 1, for buffer ($n=2$) and ET-1 ($n=6$) experiments respectively. Significant differences are indicated in the Figure.

Table 2 Changes in concentration of [^{3}H]inositol-containing lipids after incubation of ET-1 preincubated cardiomyocytes with normal Ca^{2+} or EGTA in Ca^{2+} free buffer

	[^{3}H]Inositol-phospholipid content		
	1.3 mM Ca^{2+}	50 μM EGTA	*P*
PtdIns	69.5 ±0.4	73.4 ±0.4	<0.001
lysoPtdIns	2.83±0.11	2.80±0.17	>0.5
PtdIns(4)P	2.59±0.05	2.99±0.07	<0.002
PtdIns(4,5)P_2	2.36±0.09	2.66±0.04	<0.02

Cardiomyocytes were treated and stimulated exactly as described in the legend to Figure 2. After extraction of water-soluble products, lipids were extracted with methanol:12 M HCl (100:1, v/v) and analysed as described in Materials and Methods. No significant differences in [^{3}H]inositol-containing lipid content were detected between preincubation with ET-1 (10^{-8} M) in the presence of Ca^{2+} or in the presence of 50 μM EGTA in Ca^{2+} free buffer. Therefore, [^{3}H]inositol-phospholipid content after incubation with 10 mM Li^+ and 1.3 mM Ca^{2+} is taken from preincubation with Ca^{2+} together with data from preincubation with EGTA [i.e. Fig. 2(a)] while the phospholipid levels after incubation in the presence of 10 mM Li^+ and 50 μM EGTA in Ca^{2+} free buffer are accumulated data of preincubation with Ca^{2+} and with EGTA [Fig. 2(b)]. [^{3}H]Phosphoinositide levels are expressed as percentage of total cellular [^{3}H]inositol defined as in the legends to Figure 1 (mean ± S.E., $n=12$). Statistical significance is indicated by the *P* values.

[^{3}H]PtdIns(4,5)P_2 levels were not decreased (data not shown). As will be shown later, the velocity of ET-1 induced [^{3}H]InsP_n accumulation at normal extracellular Ca^{2+} (1.3 mM) only starts diminishing after about 60 min.

Effects of low Ca^{2+}, Ca^{2+} antagonist and agonist on basal and receptor-mediated PLC activity

The foregoing results on PLC were obtained incubating cells with Ca^{2+}-ionophore A23187, high K^+ buffer and EGTA, treatments that are expected to produce dramatic changes in $[Ca^{2+}_{free}]_i$ levels close to or in the non-physiological range. We asked ourselves whether or not the physiological range of $[Ca^{2+}_{free}]_i$ is optimal for PLC activity in cardiomyocytes. Therefore, we also analysed the effect of decreasing normal Ca^{2+} influx on basal as well as agonist-induced [^{3}H]InsP_n accumulation. The effect of these protocols could also be studied over a much longer period (up to 90 min), ET-1 evoked InsP_n accumulation was not significantly affected by low Ca^{2+} medium. Only 90 min of incubation of cardiomyocytes in low Ca^{2+} medium resulted in stimulation of basal PLC activity which finding was unexpected [Fig. 4(a)]. Treatment of cardiomyocytes with the dihydropyridine Ca^{2+}-channel blocker nifedipine (1 μM) reduced ET-1 induced [^{3}H]InsP_n accumulation (30 min) by 33% and did not have a significant effect on basal PtdIns turnover [Fig. 4(b)]. After 60 min no significant effect of nifedipine on PtdIns turnover prevailed. The influence of either low Ca^{2+} buffer or nifedipine on G-protein coupled PLC activity was not agonist dependent as data similar to those illustrated in Figure 4 were obtained after stimulation of the cells with 10^{-4} M PHE (data not shown).

Figure 5 shows that the ET-1 induced PtdIns turnover could be stimulated by increasing Ca^{2+} influx through the use of the Ca^{2+}-channel opener Bay K8644 but only after 90 min of incubation. Basal PtdIns turnover was seen to be increased by the use of Bay K8644 resulting in a doubling of InsP_n accumulation after 90 min of incubation.

Fluorometric measurement of changes in intracellular free Ca^{2+} concentration of cultured cardiomyocytes

To determine whether the protocols that were described above indeed had effect on $[Ca^{2+}_{free}]_i$, we set up a fluorometric assay on cardiomyocytes grown on glass cover slips using fura-2 AM. Due to the fact that cardiomyocytes became detached from the glass cover slip after prolonged incubation (60 min of loading with fura-2 AM and 40 to 50 min incubation in the fluorometer), no extended

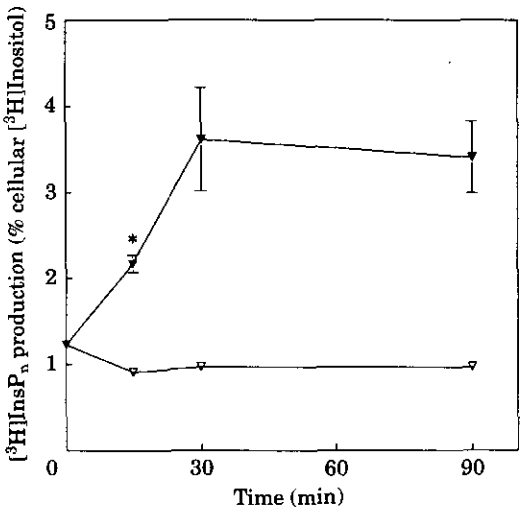

Figure 3 Time course of [^{3}H]InsP$_n$ accumulation after stimulation of cardiomyocytes with ET-1 in the presence of EGTA in Ca^{2+}-free buffer. Cells labelled with *myo*-[2-^{3}H]inositol were washed with Ca^{2+}-free buffer and incubated for 15 min in Ca^{2+}-free buffer containing 50 μM EGTA and 10 mM LiCl. Hereafter, buffer (▽) or ET-1 was added to 10^{-8} M (▼) and incubation was continued for the periods indicated. Extraction and quantification of [^{3}H]InsP$_n$ was performed as described in Materials and Methods. [^{3}H]InsP$_n$ levels are expressed as percentage of the total amount of [2-^{3}H]inositol labelled products as defined in Figure 1 (mean ± range/2 for buffer, $n=2$ and mean ± S.E., $n=6$ for ET-1). *$P<0.001$, ET-1 (15 min, $n=6$) v 0 min ($n=6$).

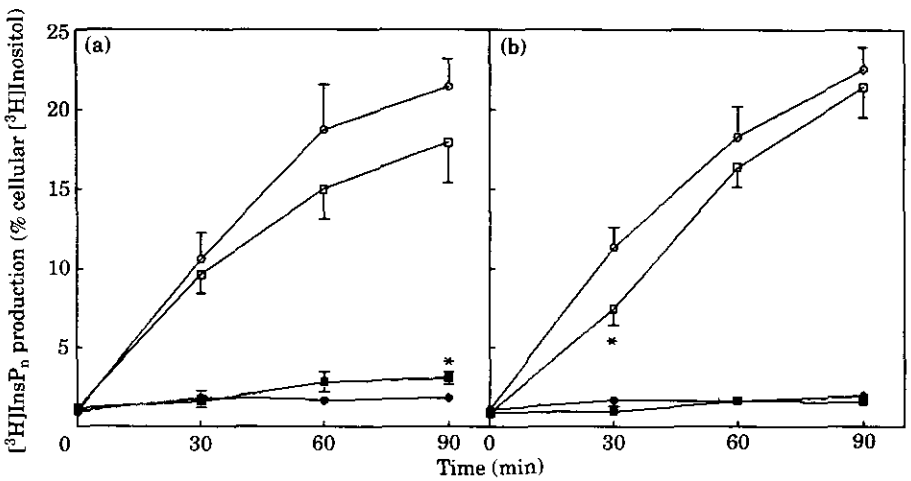

Figure 4 Effect of low Ca^{2+} or slow Ca^{2+}-channel blockade by nifedipine on the time-course of [^{3}H]InsP$_n$ production after stimulation of cardiomyocytes with ET-1. Cardiomyocytes were prelabelled with *myo*-[2-^{3}H]inositol for 24 h. (a) The cells were washed in Ca^{2+} free buffer and incubated for 15 min with 10 mM LiCl containing buffer with 1.3 mM Ca^{2+} (○,●) or without Ca^{2+} (□,■). Cells were then stimulated for the periods indicated by addition of buffer (●,■) or of ET-1 to 10^{-8} M (○,□). (b) Cells were washed and incubated for 15 min in normal buffer containing 10 mM LiCl with (□,■) or without (○,●) 1 μM nifedipine and were subsequently stimulated for the periods indicated by addition of buffer (●,■) or of ET-1 to 10^{-8} M (○,□). After incubation, the cells were extracted and [^{3}H]InsP$_n$ was quantified as described in Materials and Methods. [^{3}H]InsP$_n$ content is expressed as percentage of the total cellular content of [2-^{3}H]inositol defined as in the legends to Figure 1. Results are mean ± S.E. (a: $n=3$ for buffer and $n=6$ for ET-1; b: $n=4$ for buffer and $n=9$ for ET-1). *$P<0.05$ v similar incubation in the presence of Ca^{2+} (a) or in the absence of nifedipine (b).

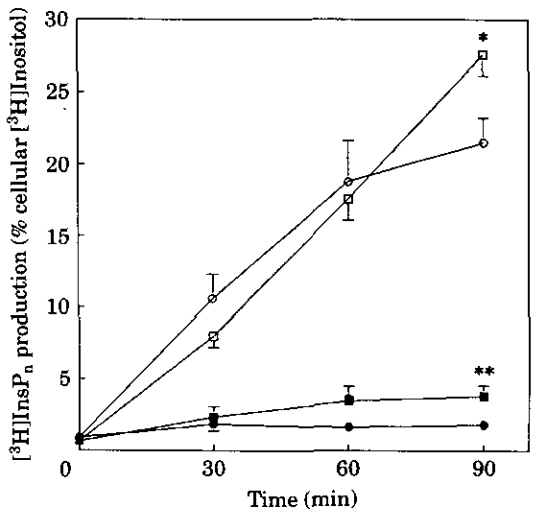

Figure 5 Influence of Ca^{2+}-channel opener Bay K8644 on the time course of $[^3H]InsP_n$ accumulation after stimulation of cardiomyocytes with ET-1. Cardiomyocytes were prelabelled for 24 h with *myo*-[2-^{3}H]inositol. Thereafter, the cells were incubated with incubation buffer containing 10 mM LiCl with (□,■) or without (○,●) 1 μM Bay K8644 for 15 min before addition of buffer (●,■) or ET-1 to 10^{-8} M (○,□) for the periods indicated. Extraction and quantification of $InsP_n$ was performed as described in Materials and Methods. $[^3H]InsP_n$ content is expressed as percentage of the total cellular content of [2-^{3}H]inositol as defined in legends to Figure 1. Results are mean ± S.E. ($n=6$ for ET-1 and $n=3$ for buffer). $^*P<0.02$ and $^{**}P<0.05$ *v* similar incubation in the absence of Bay K8644.

incubations could be performed to assess long-term effects on $[Ca^{2+}_{free}]_i$. As expected, Table 3 shows that the treatments that had the largest effect on agonist-induced $InsP_n$ accumulation also produced significant alterations in $[Ca^{2+}_{free}]_i$ as judged by the ratio of fluorescence elicited at 340 nm ($F_{340\,nm}$) and 380 nm ($F_{380\,nm}$). Addition of 50 μM EGTA, shown to decrease ET-1 induced $InsP_n$ accumulation (Figs 2 and 3) also significantly decreased $[Ca^{2+}_{free}]_i$, while a depolarization with 50 mM KCl that stimulated ET-1 induced and inhibited PHE mediated PLC activity (Fig. 1) led to an increase of $[Ca^{2+}_{free}]_i$. However, the presence of nifedipine that led to a significant decrease in ET-1 and PHE coupled $InsP_n$ accumulation after 30 min [Fig. 4(b)] did not show an effect on $[Ca^{2+}_{free}]_i$. Although low Ca^{2+} medium did not change ET-1 and PHE coupled $InsP_n$ production [Fig. 4(a)], a significant decrease in $[Ca^{2+}_{free}]_i$ was observed, but not of the same magnitude as after addition of EGTA. Bay K8644, not affecting agonist coupled $InsP_n$ production during the first 30 min (Fig. 5), did not influence $[Ca^{2+}_{free}]_i$ either.

Effect of intracellular free Ca^{2+} concentration on basal and GTPγS stimulated $InsP_n$ accumulation in permeabilized cardiomyocytes

The effect of free Ca^{2+} on the PLC activity was studied more directly using saponin-permeabilized cardiomyocytes as described by McDonough *et al.* (1988). Addition of an ATP regenerating systen was essential to stabilize the cells during permeabilization. The results (Fig. 6) show that after permeabilization of cardiomyocytes the basal and GTPγS (30 μM) stimulated PLC activity (15 min of incubation) was strongly activated by increasing $[Ca^{2+}_{free}]_i$ from 0.1 to 10 μM and even slightly more by increasing Ca^{2+} to 192 μM. Relative stimulation of PLC by GTPγS was largest in the nanomolar range of Ca^{2+}.

Discussion

We studied the effect of agents modulating $[Ca^{2+}_{free}]_i$ on basal as well as agonist-dependent PLC activity that is involved in the signal transduction through the PtdIns cycle in cardiomyocytes. In a variety of cell types it was shown that Ca^{2+} ionophore A23187 (Wakelam, 1983; Monaco, 1987; Godfrey and Putney, 1984) and depolarization by high K^+ (Gurwitz and Sokolowski, 1987; Haberman and Laux, 1986) activated $PtdIns(4,5)P_2$ breakdown by PLC. In saponin-permeabilized chick heart cells basal PLC activity was also shown to be Ca^{2+} dependent (McDonough *et al.*, 1988; Jones and Brown, 1988). In our study using intact rat

Table 3 Fluorometric measurement of $[Ca^{2+}_{free}]_i$ concentration of cultured cardiomyocytes

Cell treatment	$F_{340\,nm}/F_{380\,nm}$-ratio		
	Before	After	P (n)
Low Ca^{2+}	1.17±0.04	1.03±0.04	<0.05(6)
50 μM EGTA (no Ca^{2+})	1.03±0.04	0.93±0.01	<0.05(6)
50 mM KCl	1.14±0.05	1.41±0.06	<0.02(4)
1 μM Bay K8644	1.21±0.02	1.30±0.05	*NS (6)
1 μM Nifedipine	1.16±0.04	1.16±0.06	NS (4)

Cardiomyocytes were grown on glass cover slips and loaded for 60 min at 37°C with 2 μM fura-2 AM in the presence of 2.5 mM probenicid. The cover slip was inserted in a holder tightly fitting into a quartz cuvette containing 2 ml incubation buffer. Fluorescence was recorded at 510 nm using excitation wavelengths of 340 and 380 nm. The ratio of the fluorescence elicited at 340 nm ($F_{340\,nm}$) and 380 nm ($F_{380\,nm}$) can be taken as a measure of the $[Ca^{2+}_{free}]_i$. After each assay calibration of the fluorescence signal was performed by addition of 2.5 μM ionomycin to obtain maximal ratio followed by 10 mM EGTA to obtain the ratio at zero $[Ca^{2+}_{free}]_i$ (i.e. 0.93±0.02, n=15). Cells were incubated for 15 min in buffer ("Before") followed by 20 min incubation with various additions ("After") as indicated in the Table. Fluorescence ratios represent $[Ca^{2+}_{free}]_i$ during relaxation. Control cardiomyocytes contracted irregularly (8 to 15 times/min) which activity was completely blocked by low Ca^{2+} with or without 50 μM EGTA, 50 mM KCl and 1 μM nifedipine. Peak $F_{340\,nm}/F_{380\,nm}$ ratio during contraction in control was 1.45±0.09, n=8 (P<0.02 *vs* 1.19±0.02, n=8 during relaxation). Data are expressed as means±S.E. from four different cellbatches, n refers to the number of experiments while P values represent statistical significance between ratio before and after treatment; NS, not significant.

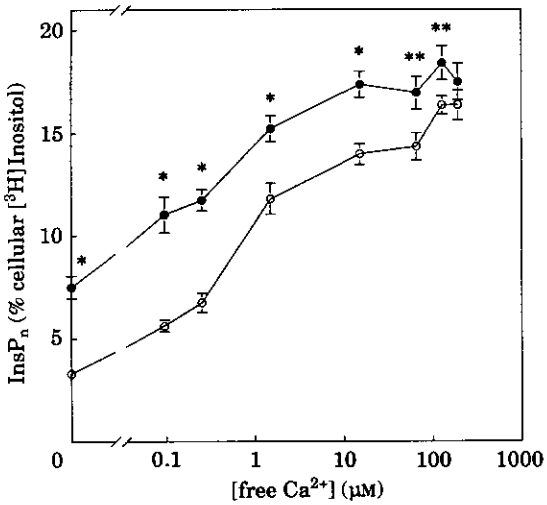

Figure 6 Effect of $[Ca^{2+}_{free}]_i$ on basal and GTPγS stimulated $InsP_n$ accumulation in permeabilized cardiomyocytes. Cardiomyocytes, prelabelled with *myo*-[2-^{3}H]inositol were washed with buffer and permeabilized for 5 min with 100 μg saponin/ml intracellular buffer as described in the experimental section. Hereafter, cells were washed with buffer without saponin and intracellular buffer was added. In addition to 10 mM LiCl this buffer contained Ca^{2+} as required with (●) or without (○) 30 μM GTPγS. Free Ca^{2+} in the buffer was calculated using the SPECS computer program described by Fabiato (1988). After 15 min incubation at 37°C the buffer was collected and the cells were extracted with perchloric acid and methanol/HCl as described in the experimental section. [^{3}H]$InsP_n$ content is the sum of inositolphosphates in buffer and perchloric acid extract and is expressed as percentage of the total cellular content of [2-^{3}H]inositol defined as in the legends to Fig. 1. Results are mean±S.E., n=12. *P<0.005 and **P<0.05 *v* incubation with the same Ca^{2+} concentration in the absence of GTPγS.

cardiomyocytes basal activity of PLC (30 min) could not be stimulated by either ionophore A23187 (external Ca^{2+} 1–100 μM) (Table 1) or high K^+ concentration (external Ca^{2+} 1.3 mM) (Fig. 1). Using fura-2 fluorescence it was shown that high Ca^{2+} induced increased $[Ca^{2+}_{free}]_i$ (Table 3). As A23187 is fluorescent no fura-2 measurements could be performed for this protocol. The basal $InsP_n$ production as well as $[Ca^{2+}_{free}]_i$ was also not affected after 30 min of incubation with Bay K8644 but unexpectedly, an increase in $InsP_n$ production was seen after 90 min incubation. On the other hand, total removal of external free Ca^{2+} through addition of 50 μM EGTA to Ca^{2+} free medium, Ca^{2+} free medium or nifedipine (1 μM) did not significantly affect the basal $InsP_n$ production (30 min) either (Table 1). Using fura-2 fluorescence it was shown that low external Ca^{2+} with or without EGTA decreased $[Ca^{2+}_{free}]_i$. Taken together these short-term effects suggest that changes in $[Ca^{2+}_{free}]_i$ do not affect basal PLC activity in intact cardiomyocytes. This conclusion is, however, not supported by experiments using permeabilized cadiomyocytes (Fig. 6) where basal PLC activity was seen to be strongly Ca^{2+} dependent in the range of 0.1 to 10 μM. It is possible that due to saponin-permeabilization the cells were depleted of PLC regulating factors. Although saponin treatment abolishes receptor activation of PLC, it is possible that some purinergic receptor activity remains.

It was reported that activation of PLC by G-proteins in rat heart membrane fragments lowers the concentration of Ca^{2+} necessary for optimal activity of PLC (Renard and Poggliolli, 1990; Jones and Brown, 1988). Likewise, we observed that relative stimulation of PLC by GTPγS was largest in the nanomolar range of Ca^{2+} in saponin-permeabilized rat cardiomyocytes (Fig. 6). Activation of G proteins by either ET-1 or PHE in intact cardiomyocytes indeed revealed Ca^{2+}-sensitivity of PLC activity. Using Ca^{2+} ionophore A23187 at different Ca^{2+} concentrations only showed significant stimulation of [^{3}H]$InsP_n$ accumulation at 10 μM external Ca^{2+} resulting in 54 and 34% increases in PLC activity induced by ET-1 and PHE, respectively. Whether PLC or G-proteins were activated by Ca^{2+} can not be deduced from these experiments. The absence of stimulation by A23187 at 100 μM external Ca^{2+} in intact cardiomyocytes (Fig. 1) might arise from a possible cell necrotic effect of the combined addition of 100 μM Ca^{2+} and ionophore A23187 as high Ca^{2+} alone did not show an inhibitory effect at 100 μM and higher using permeabilized cells (Fig. 6). On the other hand, secondary effects of Ca^{2+} overload (e.g. increases in intracellular Na^+ or K^+ concentrations) might also be detrimental for PLC activity.

The Ca^{2+}-channel agonist Bay K8644 induced a slight increase in PLC activity, but only after 90 min incubation with agonist (Fig. 5). Sei *et al.* (1991) also showed that Bay K8644 had no effect on PHE-induced PtdIns turnover during 60 min of incubation of neonatal rat ventricular myocytes.

Although the Na^+/K^+ ATPase inhibitor ouabain (0.05–5 μM) was reported to have positive inotropic activity and to increase $[Ca^{2+}_{free}]_i$ in cultured rat neonatal cardiomyocytes (Hallaq *et al.*, 1989), the receptor-mediated PtdIns response was not affected as established in the present study. Using the same model system prolonged exposure to ouabain although at high level (1 mM), was shown to increase Na^+ and to decrease K^+ and Mg^{2+} concentrations in the cytoplasm together with a decrease in ATP content (Morris *et al.*, 1989). The complexity of cellular responses to ouabain makes it difficult to evaluate the $InsP_n$ accumulation data in the light of elevated $[Ca^{2+}_{free}]_i$.

Besides increasing $[Ca^{2+}_{free}]_i$ (Table 3), depolarization of the membrane by high K^+ concentrations is known to change G-protein expression after prolonged exposure (Foster *et al.*, 1990). Incubation of cardiomyocytes in high K^+ buffer stimulated ET-1 induced PtdIns cycle activity at the same time inhibiting PHE-coupled [^{3}H]$InsP_n$ accumulation (Fig. 1). These results, together with stimulation of [^{3}H]$InsP_n$ accumulation by A23187 at 10 μM Ca^{2+} suggest that the $[Ca^{2+}_{free}]_i$ was not the only factor involved in high K^+-induced changes in PtdIns cycle activity at least in the case of PHE. Either G-protein activity or membrane electrical depolarization might be additional factors regulating the PtdIns pathway.

Regulation of PtdIns cycle activity by changes in $[Ca^{2+}_{free}]_i$ as described above suggests that the signal transduction pathway through PLC and PtdIns(4,5)P_2 might be absolutely dependent on the presence of Ca^{2+} in intact cells as was also detected for permeabilized cardiomyocytes (Fig. 6). Indeed, total removal of extracellular free Ca^{2+} by including 50 μM EGTA in the Ca^{2+} free incubation buffer markedly inhibited the ET-1 induced $InsP_n$ accumulation (Fig. 3). Under these conditions the fluorescence ratio was decreased to that detected at zero intracellular free Ca^{2+} (see also the legends to Table 3), ET-1 binding to its receptor was shown not to be inhibited by EGTA (Fig. 2) while resynthesis of PtdIns(4.5)P_2 was not rate-limiting in the absence of external Ca^{2+} (Table 2). The latter finding is in agreement with our previous observation that rat heart sarcolemmal PtdIns 4- and 5-

kinases are Ca^{2+} independent enzymes which are inhibited by micromolar Ca^{2+} concentrations (Mesaeli *et al.*, 1992). The absence of free Ca^{2+} in the cell eventually results in the total standstill of ET-1 induced PtdIns turnover. Whether the Ca^{2+} that remained bound to membrane phospholipids or G-proteins/PLC is responsible for the initial but attenuated activity of PLC remains to be elucidated. The simple withdrawal of extracellular Ca^{2+} did not significantly affect [^{3}H]InsP_n accumulation during a 90 min incubation with ET-1 or PHE [Fig. 4(a)] suggesting that the residual Ca^{2+} that was present under these conditions (Table 3) was sufficient to give normal PLC activation by the agonists. Ca^{2+}-influx inhibition by addition of the Ca^{2+}-channel blocker nifedipine (1 μM) led to significant inhibition of ET-1 and PHE-induced PtdIns cycle activity (33 and 30%, respectively [Fig. 4(b)] after 15 min preincubation and 30 min stimulation in the presence of nifedipine). However, incubation of the cells with nifedipine for 20 min did not result in a change in $[Ca^{2+}_{free}]_i$ (Table 3). Prolonged incubation with nifedipine led to complete recovery of PLC activity [Fig. 4(b)]. Although the incubations were carried out in the dark, instability of the dihydropyridine might be the cause of the late reversal of the nifedipine inhibition. On the other hand, Wetzel *et al.* (1991) demonstrated that neonatal rat ventricular myocytes are relatively deficient in slow Ca^{2+}-channel activity when compared to adult cardiomyocytes.

At this point it is important to stress the fact that fura-2 measurements were performed on unstimulated cells to determine whether the experimental protocols were having any effect on $[Ca^{2+}_{free}]_i$, largely explaining the effects on basal PLC activity. However, we have to be cautious in interpreting the results from the experiments where cardiomyocytes were stimulated with ET-1, as stimulation with this agonist might lead to increased $[Ca^{2+}_{free}]_i$, as described before (Vigne *et al.*, 1990).

In conclusion, the present study shows that the signalling pathway through PLC/PtdIns(4,5)P_2 activated by agonist-receptor interaction is likely to be a Ca^{2+}-dependent process in the intact cardiomyocyte as was the case for permeabilized cardiomyocytes. On the other hand, basal PLC activity in intact cardiomyocytes seems not to be sensitive to changes in the $[Ca^{2+}_{free}]_i$, although after saponin-treatment of the cardiomyocytes the basal PLC activity also became Ca^{2+}-dependent. The activation of PLC by an increase in $[Ca^{2+}_{free}]_i$ level may follow upon Ins(1,4,5)P_3-induced release of Ca^{2+} from the sarcoplasmic reticulum. Ca^{2+} potentiation of agonist-mediated PLC activation may also play a role in aggravating cellular Ca^{2+} overload developing during myocardial ischemia-reperfusion (Lee and Allen, 1991; Otani *et al.*, 1989; Mouton *et al.*, 1991; Heathers *et al.*, 1989).

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