Phospholipase Cδ$_1$ does not mediate Ca^{2+} responses in neonatal rat cardiomyocytes

Elizabeth A. Woodcock[a,*], Christopher J. Mitchell[b], Trevor J. Biden[b]

[a] *Cellular Biochemistry Laboratory, Baker Heart Research Institute, P.O. Box 6492, St Kilda Rd Central, Melbourne, Vic. 8008, Australia*
[b] *Garvan Institute of Medical Research, Sydney, NSW, Australia*

Received 7 April 2003; revised 22 May 2003; accepted 23 May 2003

First published online 3 June 2003

Edited by Veli-Pekka Lehto

Abstract Phospholipase C (PLC) activation in neonatal rat ventricular cardiomyocytes (NRVM) generates inositol(1,4,5)-trisphosphate (Ins(1,4,5)P_3) in response to elevations in Ca^{2+} or inositol(1,4)bisphosphate in response to G protein stimulation. Overexpression of PLCδ$_1$ increased total [^{3}H]inositol phosphate (InsP) content and elevated [^{3}H]Ins(1,4,5)P_3, but failed to increase [^{3}H]InsP responses to the Ca^{2+} ionophore A23187. Antisense PLCδ$_1$ expression reduced endogenous PLCδ$_1$ content but did not decrease the A23187 response. In permeabilized NRVM, [^{3}H]InsP responses to elevated Ca^{2+} were not inhibited by Ins(1,4,5)P_3, even at concentrations 1000-fold greater than required for selective inhibition of PLCδ$_1$. Taken together these data provide evidence that PLCδ$_1$ does not mediate the InsP response to elevated Ca^{2+} in NRVM.

Key words: Inositol phosphate; Cardiomyocyte; Phospholipase Cδ$_1$; Adenovirus

1. Introduction

Receptor stimulation of phosphatidylinositol (PtdIns)-specific phospholipase C (PLC) causes generation of *sn*-1,2-diacylglycerol, which is able to activate protein kinase C isoforms [1], and inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3), which releases Ca^{2+} from intracellular stores [2]. Studies from our laboratory using intact heart and isolated cardiomyocytes have shown, however, that unlike responses in other cell types, α_1-adrenergic stimulation generates principally inositol(1,4)bisphosphate (Ins(1,4)P_2) which has no Ca^{2+}-mobilizing function [3,4]. In marked contrast, increased intracellular Ca^{2+} itself stimulates robust Ins(1,4,5)P_3 responses [4]. Ins(1,4,5)P_3 generation in the heart is arrhythmogenic [5–10], although the mechanisms involved have not been fully explained. Ins(1,4,5)P_3 receptors are expressed at low levels in ventricular myocardium [11], but it is possible that activation of these receptors causes localized Ca^{2+} release. Even local changes in Ca^{2+} could lead to perturbations in Ca^{2+} homeostasis [12] in cells whose Ca^{2+} levels are otherwise finely regulated during each depolarization/contraction cycle by mechanisms that do not involve Ins(1,4,5)P_3, e.g. voltage-dependent Ca^{2+} influx, the ryanodine receptor system and Na^+/Ca^{2+} exchange. The finding that increased intracellular Ca^{2+} itself can initiate an Ins(1,4,5)P_3 response increases the likelihood that disturbances of Ca^{2+} homeostasis will be self-perpetuating, leading to arrhythmias and sudden cardiac death. We therefore sought to identify the mechanisms responsible for Ca^{2+}-activated Ins(1,4,5)P_3 generation in cardiomyocytes.

The well-known classes of mammalian PtdIns-specific PLC enzymes are β, γ and δ, and a new class PLCε has recently been identified [13,14]. All PLC isozymes are activated by Ca^{2+} in vitro but of these, PLCδ$_1$ is the most sensitive to Ca^{2+} and is considered the most likely to be activated by increases in Ca^{2+} alone over the concentration range encountered in cardiomyocytes [15–17].

In the current study we investigated the role of PLCδ$_1$ in mediating Ca^{2+}-induced inositol phosphate (InsP) responses in neonatal rat cardiomyocytes (NRVM).

2. Materials and methods

2.1. Culture of neonatal cardiomyocytes

Neonatal cardiomyocyte (NRVM) cultures were prepared from 1–3-day-old Sprague–Dawley rat pups as previously described [4]. NRVM were isolated by repeated trypsin digestion with gentle mechanical dispersion, pre-plated twice for 30 min each to remove non-myocytes and left to attach for 18 h in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, 0.1 mM bromodeoxyuridine (BrdU), 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate onto uncoated dishes, at a typical seeding density of 800 cells/mm^2. Medium was then replaced with a defined serum-free medium consisting of DMEM, 10 μg/ml human insulin, 10 μg/ml bovine apo-transferrin, 0.1 mM BrdU, 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate and 125 μg/ml fungizone. BrdU was omitted after 3 days. NRVM were treated with adenovirus and labeled with [^{3}H]inositol in defined inositol-free, serum-free medium for 48 h prior to experiments. Wells were washed thoroughly with non-radioactive medium before use.

2.2. Preparation of adenoviruses and infection of NRVM

Rat PLCδ$_1$ cloned into the *Not*I site of pZipNeo and the rat PLCγ$_1$ plasmid were gifts from Dr. S.G. Rhee [18]. The *Not*I restriction fragment corresponding to PLCδ$_1$ was subcloned into the plasmid pxcx3.CMV in the sense and antisense orientations. These constructs were cotransfected into HEK293 cells with pJM17 (modified adenovirus backbone) and cultures were overlaid with agarose. Following recombination, individual plaques were selected and purified through sequential rounds of infection and agarose overlay. High titer viral stocks were purified on CsCl gradients.

Adenoviruses were used at a multiplicity of infection of 100 plaque-forming units (pfu)/cell. As described in other cell types [19], overexpressed PLC enzymes were active as shown by increased PLC activity in extracts prepared from infected NRVM.

2.3. Protein analysis

NRVM infected with adenoviruses were washed twice in ice-cold phosphate-buffered saline, followed by the addition of an ice-cold lysis

*Corresponding author. Fax: (61)-3-8532 1100.
E-mail address: liz.woodcock@baker.edu.au (E.A. Woodcock).

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00608-2

buffer at pH 7.7 containing the following constituents (in mM): Tris–HCl 20, NaCl 150, EDTA 2, EGTA 2, β-glycerophosphate 20, sodium orthovanadate 1, with 10% glycerol and 0.5% Nonidet P-40, and 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 5 μg/ml aprotinin and 10 μg/ml leupeptin as protease inhibitors. Wells were scraped and the DNA sheared using a 27-gauge needle. Lysates were cleared by centrifugation and protein content of the supernatants measured by the Bradford method [20] with bovine serum albumin as standard and using a dye reagent from Bio-Rad.

Proteins (40 μg) from whole-cell lysates were separated on 7.5% denaturing polyacrylamide gels [21] and electrophoretically transferred to nitrocellulose membranes from Schleicher and Schuell (Dassel, Germany). Nitrocellulose membranes were stained with Ponceau-S (Sigma) to confirm equal protein loading per lane and equal transfer efficiency, then blocked for 1 h in 5% non-fat dry milk and probed with appropriate antibodies. Enhanced chemiluminescence detection was carried out according to the manufacturer's instructions (Amersham Biosciences).

Mouse monoclonal anti-PLCδ$_1$ antibody was from Upstate Biotechnology (Lake Placid, NY, USA) and used at a final concentration of 0.4 μg/ml.

2.4. Generation and extraction of [^{3}H]InsPs

[^{3}H]Inositol-labeled (48 h, 10 μCi/ml) NRVM were washed with unlabeled medium and pretreated with 10 mM LiCl in DMEM for 10 min prior to the addition of Ca^{2+} ionophore (A23187, 10 μM). For experiments involving permeabilized cells, [^{3}H]inositol-labeled NRVM were permeabilized by treatment with saponin (50 μg/ml) for 4 min at 37°C in 'intracellular' salts medium containing an ATP-generating system, followed by four washes in 'intracellular' medium. 'Intracellular' medium contained (in mM): KCl, 110; NaCl, 10; KH_2PO_4, 1; $MgSO_4$, 5; EGTA, 1; HEPES, 20; ATP, 2; creatine phosphate, 10; with 20 U creatine phosphokinase (Sigma). $CaCl_2$ was added to provide a free Ca^{2+} of 0.1 μM (basal) or 10 μM (Ca^{2+}-activated). $CaCl_2$ additions were calculated using a Ca^{2+}–EGTA titration curve. Fresh 'intracellular' medium containing 10 mM LiCl was added for 10 min, prior to increases in Ca^{2+}. After stimulation, [^{3}H]InsPs were extracted with ice-cold 5% trichloroacetic acid (TCA)/2.5 mM EDTA/5 mM sodium phytate and the supernatants subsequently treated with a 1:1 mixture of 1,1,2-trichlorotrifluoroethane:tri-*n*-octylamine to remove remaining TCA. The aqueous phase containing [^{3}H]InsPs was prepared for high performance liquid chromatography to separate individual isomers [22], or was subjected to chromatography on Dowex 1 columns for total [^{3}H]InsP measurement [23].

2.5. Inositol polyphosphate 1-phosphatase

Inositol polyphosphate 1-phosphatase (INPP) activity was assayed using 10 μM [^{3}H]Ins(1,4)P_2 (10 cpm/pmol) as substrate in 100 mM KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 1 mM glucose 6-phosphate and 50 mM HEPES, pH 7.4. Reactions were carried out at 37°C for 5 min and terminated by the addition of 50 mM ammonium formate, 200 mM formic acid. [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(4)P_1 were separated on Dowex 1 (formate) columns [24,25] and [^{3}H]InsP_1 measured by liquid scintillation counting.

2.6. Materials

Fetal calf serum used during NRVM isolation was specially selected for low endotoxin and obtained from the Commonwealth Serum Laboratories (Parkville, Australia). DMEM, HEPES, and other materials for the preparation of cell culture solutions and media were cell culture grade, obtained from Sigma and dissolved in milli-Q H_2O. [^{3}H]Inositol (18.00 Ci/mmol) was obtained from Amersham Biosciences, [^{3}H]inositol(1,4)bisphosphate (INS(1,4)P_2, 6.5 Ci/mmol) was from New England Nuclear (Boston, MA, USA), Ins(1,4,5)P_3 (sodium salt) was obtained through Sigma, A23187 (free acid) was supplied by Calbiochem. Other reagents were obtained from Sigma or BDH/AnalaR and were of analytical reagent grade.

2.7. Treatment of data

Differences between treatment groups were assessed by unpaired Student's *t*-test or one-way analysis of variance with Tukey's test for multiple comparisons, and accepted as statistically significant at $P < 0.05$. Unless otherwise noted in figure legends, results shown are from representative experiments performed in triplicate, which were repeated independently three times.

3. Results

3.1. Overexpressed PLCδ$_1$ increases Ins(1,4,5)P_3 and total InsPs

PLCδ isoforms bind both PIP_2 and Ins(1,4,5)P_3 with high affinity, and the association with PIP_2 would be expected to target PLCδ to the sarcolemma in the absence of other stimuli. We examined the effects of overexpressed PLCδ$_1$ on [^{3}H]InsP content of NRVM. NRVM were labeled with [^{3}H]inositol and treated for 48 h with control virus (Ad-MX NRVM) or with virus encoding PLCδ$_1$ (Ad-PLCδ$_1$ NRVM).

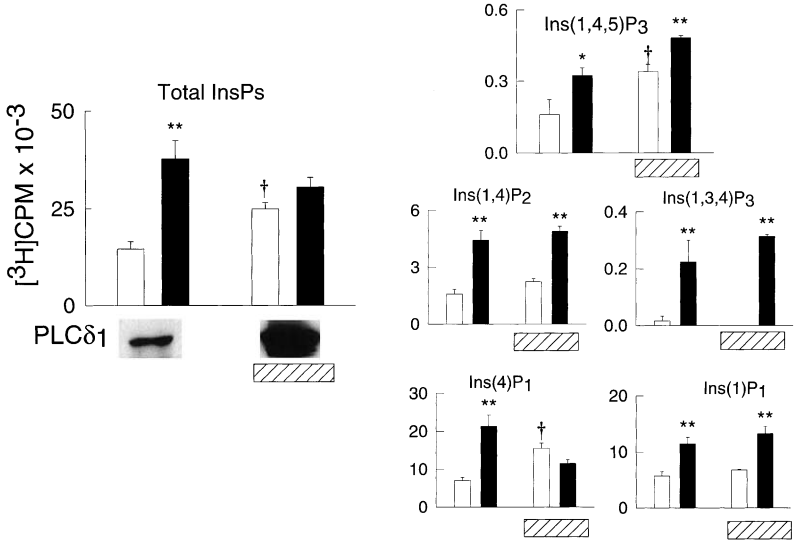

Fig. 1. Overexpressed PLCδ$_1$ does not enhance InsP responses to Ca^{2+}. [^{3}H]Inositol-labeled Ad-MX NRVM and Ad-PLCδ$_1$ NRVM were treated with 10 μM A23187 in the presence of 10 mM LiCl for 20 min. [^{3}H]InsPs were extracted and individual isomers separated and quantified. Open bars, vehicle control; filled bars, A23187; striped boxes indicate PLCδ$_1$ overexpression. Results are [^{3}H]InsPs, mean ± S.E.M. of triplicate values from one of three experiments. $^{**}P < 0.01$ relative to no additions; $^{\dagger}P < 0.01$ relative to Ad-MX NRVM. PLCδ$_1$ expression in the NRVM is indicated.

[^{3}H]InsPs were extracted and quantified. Total [^{3}H]InsP content was higher in Ad-PLCδ$_1$ NRVM than in Ad-MX NRVM indicating that the overexpressed enzyme was active and could access substrate. This increase was primarily due to heightened Ins(4)P$_1$, that accumulates in the presence of Li$^+$. Ins(4)P$_1$ is the major final dephosphorylation product of Ins(1,4)P$_2$ and Ins(1,4,5)P$_3$. [^{3}H]Ins(1,4,5)P$_3$ was also increased by PLCδ$_1$ overexpression most likely primarily reflecting binding to its PH domain (Fig. 1).

3.2. Overexpression of PLCδ$_1$ reduces the [^{3}H]InsP response to elevated Ca^{2+}

The PLCδ isoforms of PtdIns-specific PLC are more Ca^{2+}-sensitive than the β and γ isoforms and are therefore likely mediators of Ca^{2+}-induced InsP responses. To establish whether PLCδ$_1$ mediates Ca^{2+}-induced InsP responses in NRVM, we measured [^{3}H]InsP responses to increased Ca^{2+} in [^{3}H]inositol-labeled Ad-MX and Ad-PLCδ$_1$ NRVM. NRVM were stimulated with the Ca^{2+} ionophore A23187 (10 μM) in the presence of LiCl for 20 min. Addition of 10 μM A23187 to Ad-MX NRVM caused an increase in [^{3}H]InsP content, involving increases in all of the InsP isomers detected in the cells, as shown in Fig. 1. In marked contrast, addition of A23187 failed to significantly increase total [^{3}H]InsPs over unstimulated levels in cells overexpressing PLCδ$_1$ (Fig. 1). However, even though there was no significant increase in [^{3}H]InsPs, significant rises in [^{3}H]Ins(1,4,5)P$_3$, [^{3}H]Ins(1,3,4)P$_3$, [^{3}H]Ins(1,4)P$_2$ and [^{3}H]Ins(1/3)P$_1$ were observed. This was balanced by a reduction in Ins(4)P$_1$ most likely reflecting Ca^{2+} inhibition of its generation from Ins(1,4)P$_2$. INPP, the enzyme responsible for hydrolyzing to Ins(4)P$_1$, is inhibited by Ca^{2+} [26].

To further investigate the possible role of PLCδ$_1$ in mediating Ca^{2+}-induced [^{3}H]InsP responses in NRVM, we expressed full length antisense PLCδ$_1$ (Ad-anti-PLCδ$_1$ NRVM) and measured [^{3}H]InsP responses to 10 μM A23187. Expression of antisense PLCδ$_1$ (100 pfu/cell) reduced the expression of PLCδ$_1$ in NRVM but did not alter the [^{3}H]InsP response to A23187 (Fig. 2).

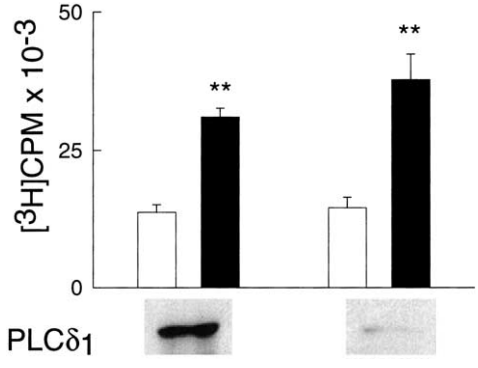

Fig. 2. Decreasing the expression of PLCδ$_1$ using antisense constructs does not reduce [^{3}H]InsP responses to A23187. [^{3}H]Inositol-labeled Ad-MX NRVM or Ad-anti-PLCδ$_1$ NRVM were incubated for 20 min with A23187 (10 μM) in the presence of LiCl. [^{3}H]InsPs were extracted and quantified. Open bars, vehicle control; filled bars, A23187. Results are total [^{3}H]InsPs, mean ± S.E.M. of triplicate samples from one of three experiments. $^{**}P < 0.01$ relative to vehicle control. Expression of PLCδ$_1$ is shown. Similar changes were observed in three experiments.

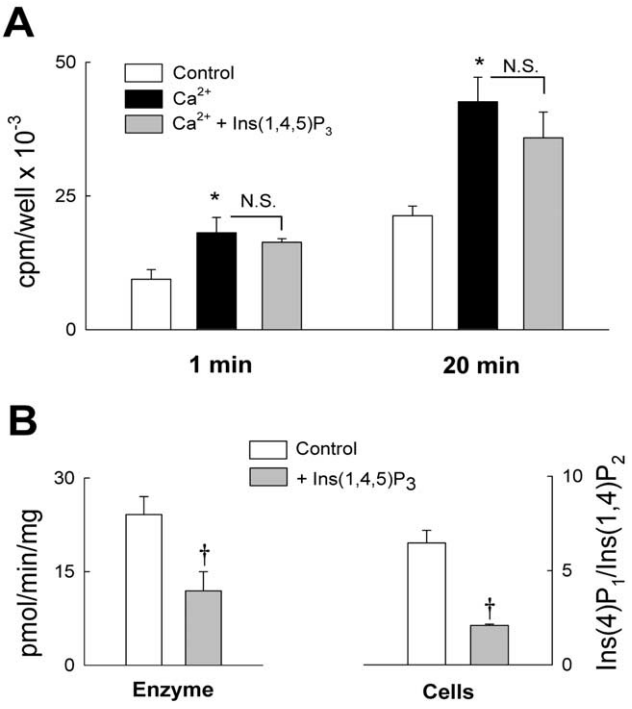

Fig. 3. Ins(1,4,5)P$_3$ does not inhibit the [^{3}H]InsP response to heightened Ca^{2+} in permeabilized NRVM. A: [^{3}H]Inositol-labeled NRVM were permeabilized and maintained in 'intracellular' medium with Ca^{2+} buffered to 0.1 μM. Total [^{3}H]InsP responses were initiated by increasing Ca^{2+} to 10 μM for 1 or 20 min, in the absence or presence of 300 μM Ins(1,4,5)P$_3$ as indicated. B: Inhibition of INPP activity by 300 μM Ins(1,4,5)P$_3$. Left panel: Inhibition of the recombinant INPP enzyme activity measured using 10 μM [^{3}H]Ins(1,4)P$_2$ as substrate. Right panel: Inhibition of INPP activity in [^{3}H]inositol-labeled permeabilized cells measured from the ratio of the product [^{3}H]Ins(4)P$_1$ to the substrate [^{3}H]Ins(1,4)P$_2$. Values shown are mean ± S.E.M., $n = 3$. $^*P < 0.05$ relative to no additions; $^\dagger P < 0.01$ relative to no Ins(1,4,5)P$_3$.

3.3. Ins(1,4,5)P$_3$ does not inhibit Ca^{2+}-stimulated [^{3}H]InsP responses in permeabilized NRVM

Altering the level of expression of PLCδ$_1$ did not provide evidence to support a major role for PLCδ$_1$ in Ca^{2+} responses in NRVM. We next sought evidence of a role for PLCδ$_1$ at physiological expression levels in non-transfected NRVM. The activity of PLCδ but not the other isoforms of PLC is inhibited by Ins(1,4,5)P$_3$ with an EC$_{50}$ of 100–300 nM [27–29]. To further investigate the role of PLCδ$_1$ in Ca^{2+}-mediated responses in NRVM, we examined the effect of high concentrations of Ins(1,4,5)P$_3$ on [^{3}H]InsP responses to elevations in Ca^{2+} in permeabilized cells. [^{3}H]Inositol-labeled NRVM were permeabilized, and maintained in 'intracellular' medium with Ca^{2+} buffered at 0.1 μM. [^{3}H]InsP responses were initiated by increasing Ca^{2+} to 10 μM for 1 or 20 min. [^{3}H]InsPs were extracted and quantified. Heightened Ca^{2+} caused increases in [^{3}H]InsPs at both time points and Ins(1,4,5)P$_3$, even at 300 μM, did not inhibit the Ca^{2+}-induced response (Fig. 3A). This argues against a major involvement of PLCδ isoforms.

To ensure that the Ins(1,4,5)P$_3$ added to the medium could access intracellular targets, we examined the effect of Ins(1,4,5)P$_3$ on another intracellular enzyme, INPP, which hydrolyzes Ins(1,4)P$_2$ to Ins(4)P$_1$. We first demonstrated that recombinant INPP is inhibited by high concentrations of Ins(1,4,5)P$_3$. Recombinant INPP was incubated with and

without 300 μM Ins(1,4,5)P_3 and activity measured as described in Section 2. Ins(1,4,5)P_3 caused an inhibition of enzyme activity as shown in Fig. 3B, right panel. Then we examined effects of Ins(1,4,5)P_3 on INPP activity in permeabilized NRVM, under similar conditions to those used in the studies of Ca^{2+}-activated [^{3}H]InsP responses. [^{3}H]Inositol-labeled NRVM were permeabilized and maintained in 'intracellular' medium, as described above. Addition of 300 μM Ins(1,4,5)P_3 to the medium reduced INPP activity in permeabilized NRVM, as demonstrated by the selective reduction in the ratio of the enzyme product [^{3}H]Ins(4)P_1 to its substrate [^{3}H]Ins(1,4)P_2. The effectiveness of Ins(1,4,5)P_3 in inhibiting INPP activity in permeabilized NRVM shows that Ins(1,4,5)P_3 added to the extracellular medium can access intracellular enzymes and would therefore be expected to inhibit PLCδ$_1$.

4. Discussion

We have recently reported two distinct pathways of InsP generation in NRVM, one involving Ins(1,4,5)P_3 generation in response to elevated Ca^{2+} and the other principally involving generation of Ins(1,4)P_2 in response to α$_1$-adrenergic receptor activation [4]. Additionally we showed that the α$_1$-adrenergic receptor response is mediated primarily by PLCβ$_1$ [23]. In the current study, we sought to identify the PLC isoform responsible for the Ca^{2+} response. Of the classes of PtdIns-specific PLCs, PLC-δ isoforms are particularly sensitive to activation by Ca^{2+} in the absence of other stimuli [15] and thus it seemed likely that PLCδ$_1$ was responsible for the Ca^{2+}-mediated generation of Ins(1,4,5)P_3 in NRVM. In an attempt to confirm this, we manipulated PLCδ$_1$ content in NRVM using adenoviruses encoding sense or antisense PLCδ$_1$ sequences.

Adenoviral overexpression of PLCδ$_1$ increased basal [^{3}H]InsP content, indicating that the expressed PLCδ$_1$ was catalytically active. Overexpressed PLCδ$_1$ also heightened Ins(1,4,5)P_3 content, in keeping with binding by its PH domain which has a high affinity for Ins(1,4,5)P_3. Despite the fact that overexpressed PLCδ$_1$ was catalytically active and localized appropriately, responses to the Ca^{2+} ionophore A23187 were reduced, rather than increased, in PLCδ$_1$-overexpressing cells. Increasing expression levels had failed to provide evidence that PLCδ$_1$ mediated Ca^{2+} responses in NRVM. We next sought evidence for involvement of endogenous PLCδ$_1$. In the first series of experiments endogenous expression of PLCδ$_1$ was reduced using adenoviruses encoding antisense PLCδ$_1$. The finding that Ca^{2+}-activated InsP responses were not reduced argued against a requirement for PLCδ$_1$ (Fig. 2). As discussed above Ins(1,4,5)P_3 binds PLCδ isoforms with high affinity and inhibits their activity. In experiments using permeabilized NRVM we showed that responses to heightened Ca^{2+} were not inhibited by Ins(1,4,5)P_3 at concentrations 1000 times higher than required for inhibition of PLCδ$_1$ [27]. The lack of inhibitory effect of Ins(1,4,5)P_3 argues against involvement of PLCδ$_1$.

Taken together studies using overexpression, reduced expression and selective inhibition of PLCδ$_1$ have failed to provide evidence that PLCδ$_1$ mediates Ca^{2+}-induced InsP responses in NRVM.

Acknowledgements: This work was supported by the Australian National Health and Medical Research Council, by the National Heart Foundation of Australia and by the Ramaciotti Foundation. We thank Bronwyn Kenney for technical assistance.

References

[1] Newton, A.C. (1995) Trends Biochem. Sci. 20, 2–3.
[2] Berridge, M. (1993) Nature 361, 315–325.
[3] Woodcock, E., Suss, M. and Anderson, K. (1995) Circ. Res. 76, 252–260.
[4] Matkovich, S.J. and Woodcock, E.A. (2000) J. Biol. Chem. 275, 10845–10850.
[5] Du, X.-J., Anderson, K., Jacobsen, A., Woodcock, E. and Dart, A. (1995) Circulation 91, 2712–2716.
[6] Jacobsen, A.N., Du, X.J., Lambert, K.A., Dart, A.M. and Woodcock, E.A. (1996) Circulation 93, 23–26.
[7] Jacobsen, A.N., Du, X.J., Dart, A.M. and Woodcock, E.A. (1997) Am. J. Physiol. 42, H1119–H1125.
[8] Woodcock, E.A., Reyes, N., Jacobsen, A.N. and Du, X.J. (1999) Circulation 99, 823–828.
[9] Felzen, B., Shilkrut, M., Less, H., Saparov, I., Coleman, R., Robinson, R., Berke, G. and Binah, O. (1998) Circ. Res. 82, 438–450.
[10] Bian, J.S., Zhang, W.M., Xia, Q. and Wong, T.M. (1998) J. Mol. Cell. Cardiol. 30, 2103–2110.
[11] Moschella, M.C. and Marks, A.R. (1993) J. Cell Biol. 120, 1137–1146.
[12] Lipp, P., Laine, M., Tovey, S.C., Burrell, K.M., Berridge, M.J., Li, W. and Bootman, M.D. (2000) Curr. Biol. 10, 939–942.
[13] Rhee, S.G. and Bae, Y.S. (1997) J. Biol. Chem. 272, 15045–15048.
[14] Kelley, G.G., Reks, S.E., Ondrako, J.M. and Smrcka, A.V. (2001) EMBO J. 20, 743–754.
[15] Allen, V., Swigart, P., Cheung, R., Cockcroft, S. and Katan, M. (1997) Biochem. J. 327, 545–552.
[16] Lomasney, J.W., Cheng, H.F., Roffler, S.R. and King, K. (1999) J. Biol. Chem. 274, 21995–22001.
[17] Kim, Y.H., Park, T.J., Lee, Y.H., Baek, K.J., Suh, P.G., Ryu, S.H. and Kim, K.T. (1999) J. Biol. Chem. 274, 26127–26134.
[18] Suh, P.G., Ryu, S.H., Moon, K.H., Suh, H.W. and Rhee, S.G. (1988) Cell 54, 161–169.
[19] Gasa, R., Trinh, K.Y., Yu, K., Wilkie, T.M. and Newgard, C.B. (1999) Diabetes 48, 1035–1044.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Cannon-Carlson, S. and Tang, J. (1997) Anal. Biochem. 246, 146–148.
[22] Woodcock, E. (1997) Mol. Cell. Biochem. 172, 121–127.
[23] Arthur, J.F., Matkovich, S.J., Mitchell, C.J., Biden, T.J. and Woodcock, E.A. (2001) J. Biol. Chem. 276, 37341–37346.
[24] York, J.D., Veile, R.A., Doniskeller, H. and Majerus, P.W. (1993) Proc. Natl. Acad. Sci. USA 90, 5833–5837.
[25] Woodcock, E.A., Wang, B.H., Arthur, J.F., Lennard, A., Matkovich, S.J., Du, X.-J., Brown, J.H. and Hannan, R.D. (2002) J. Biol. Chem. 277, 22734–22742.
[26] Inhorn, R. and Majerus, P. (1987) J. Biol. Chem. 262, 15946–15952.
[27] Lemmon, M.A., Ferguson, K.M., Obrien, R., Sigler, P.B. and Schlessinger, J. (1995) Proc. Natl. Acad. Sci. USA 92, 10472–10476.
[28] Cifuentes, M.E., Delaney, T. and Rebecchi, M.J. (1994) J. Biol. Chem. 269, 1945–1948.
[29] Garcia, P. et al. (1995) Biochemistry 34, 1622–16234.