PIP2 as local second messenger: a critical re-evaluation

Rheenen, Jacobus Emiel van

Chapter 2

PtdIns(4,5)P_2 depletion is essential for stress-induced apoptosis
submitted

PtdIns(4,5)P_2 depletion is essential for stress-induced apoptosis

Jonathan R. Halstead[1], Jacco van Rheenen[2], Mireille Snel[1], Kees Jalink[2] and Nullin Divecha[1*]

From the Division of Cellular Biochemistry[1] and the Division of Cell Biology[2], The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, The Netherlands.

Stress-induced apoptosis is believed to contribute to a number of human diseases however, little is known about the signalling events that regulate this process. Reports demonstrating that the phosphoinositide, phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2) inhibits caspase activation and promotes pro-survival signals has led to the hypothesis that PtdIns(4,5)P_2 suppresses apoptosis. However, to date it has not been demonstrated that apoptotic stimuli negatively regulate PtdIns(4,5)P_2 levels. Here, we show that both hydrogen peroxide (H_2O_2) and UV-irradiation, two apoptotic stress stimuli, cause irreversible depletion of PtdIns(4,5)P_2 in a caspase-independent manner. Depletion of PtdIns(4,5)P_2 is essential, as ectopic expression of phosphatidylinositol 4-phosphate 5-kinase (PIP 5-K), a lipid kinase which synthesises PtdIns(4,5)P_2 *in vivo*, rescues cells from H_2O_2-induced apoptosis. We find that H_2O_2 inhibits PIP 5-K activity and simultaneously induces the translocation of PIP 5-K away from its substrate at the plasma membrane. These observations identify PtdIns(4,5)P_2 as an essential regulator of stress-induced apoptosis and establishes PIP 5-K as a target for control by stress stimuli.

Introduction

Stress-induced apoptosis (or programmed cell death) is linked to the aetiology of a number of pathological conditions. For example, both the onset of Alzheimer's disease and cardiac infarction have been linked to apoptosis induced by reactive oxygen species, such as H_2O_2 (Andersen, 2004; Zhao, 2004).

A key survival pathway in cells inhibiting multiple components of the apoptotic machinery is the PKB/Akt signalling cascade. PKB is activated by PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 which clearly implicates these 3-phosphorylated phosphoinositides in the promotion of cell survival (Alessi, 1996; Stokoe, 1997; Scheid 2003). In contrast, the role of the phosphoinositide, PtdIns(4,5)P_2, in promoting cell survival remains less well defined. PtdIns(4,5)P_2 regulates a wide range of cellular processes, including ion channel activation, actin cytoskeleton remodelling and vesicular trafficking (Yin, 2003; Itoh, 2004; Hilgemann, 2004). PtdIns(4,5)P_2 is also hydrolysed by

phospholipase C (PLC) generating the second messengers, diacylglycerol (DAG) which promotes cellular proliferation (via protein kinase C activity) and IP_3 which regulates intracellular calcium release. PtdIns(4,5)P_2, is synthesised *in vivo* by the phosphorylation of PtdIns4P on the 5 position of the inositol head group by the PIP 5-K family of lipid kinases. The PIP 5-K family is encoded by four genes, which generate a number of different protein products that appear to have non-redundant functions (Loijens, J.C, 1996; Ishihara, 1998). We have shown that in response to H_2O_2, PIP 5-K synthesises PtdIns(3,4,5)P_3 via its PtdIns(3,4)P_2 5-kinase activity (Halstead, 2001). This data implicates PIP 5-K in cell survival, as activation of the anti-apoptotic protein kinase, PKB/Akt is PtdIns(3,4,5)P_3-dependent. A more direct role for PIP 5-K and PtdIns(4,5)P_2 in the regulation of cell survival has been suggested as PtdIns(4,5)P_2 can inhibit the activation of caspases 8, 9 and 3 (Mejillano, 2001; Azuma, 2000). A possible link between caspase activation and PtdIns(4,5)P_2 levels comes from the observation that human PIP 5-Kα (the homologue of murine PIP 5-Kβ) is cleaved and inactivated by caspases (Mejillano, 2001). In this model of apoptotic control, activated caspases cleave and inhibit PIP 5-K thereby blocking PtdIns(4,5)P_2 synthesis and triggering further caspase activation.

In cardiomyocytes, a role for PtdIns(4,5)P_2 depletion has been suggested in promoting apoptosis. Expression of a constitutively active version of the alpha subunit of Gq causes PtdIns(4,5)P_2 depletion and apoptosis, that is associated with a concomitant decrease in PKB/Akt signalling (Althoefer, 1997; Adams, 1998; Howes, 2003). In line with the concept that depleting PtdIns(4,5)P_2 levels is an apoptotic event, expression of the PtdIns(4,5)P_2 phosphatase, inositol polyphosphate 5-phosphatase IV, causes apoptosis and inhibits PKB activation in HEK293 cell lines (Kisseleva, 2002). Together, these data strongly suggest that PtdIns(4,5)P_2 is an important survival factor in cells and implicate PIP 5-K as a target for inactivation during apoptosis. If this hypothesis were true, it would be predicted that PtdIns(4,5)P_2 levels would decrease during apoptosis. However, apoptotic stimuli have never been shown to negatively regulate PtdIns(4,5)P_2 levels. Using a combination of biochemical and confocal imaging techniques, this paper addresses the issue of PtdIns(4,5)P_2 in apoptotic regulation. We demonstrate that apoptotic stress stimuli, such as H_2O_2 and UV-irradiation, cause PtdIns(4,5)P_2 depletion and that this event is essential for apoptosis and occurs independently of caspase activation. Moreover, we show that during stress-induced apoptosis, PIP 5-K activity is inhibited by a novel dual mechanism which serves to attenuate PtdIns(4,5)P_2 synthesis at the plasma membrane.

Results

Sustained PtdIns(4,5)P_2 depletion leads to apoptosis.

As a first step towards clarifying the role of PtdIns(4,5)P_2 in apoptosis, we used a constitutively active version of the alpha subunit of Gq (Gαq*) ectopically expressed in HeLa cells to deplete PtdIns(4,5)P_2 levels. The GFP fusion of the PH domain of PLCδ1 (GFP-PH^{PLC}) was used as an *in vivo* PtdIns(4,5)P_2 probe (Varnai, 1998; Stauffer, 1998). GFP-PH^{PLC} is concentrated at the plasma membrane in control cells (not expressing Gαq*)(Figure1A).

In HeLa cells expressing Gαq*, GFP-PH^{PLC} was spread diffusely throughout the cell (with an apparent membrane-cytosolic ratio of 1:1) (Figure 1B), indicating that plasma membrane PtdIns(4,5)P_2 levels were

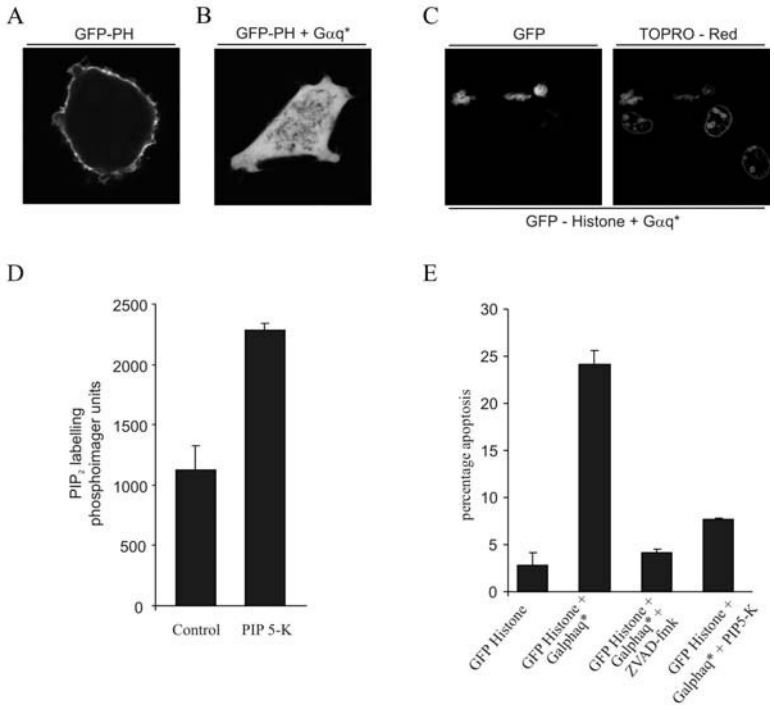

Figure 1, Expression of Gαq* in HeLa cells depletes PtdIns(4,5)P$_2$. HeLa cells were grown overnight on cover slips and then transfected with GFP-PH^{PLC} alone or together with Gαq*. GFP-PH^{PLC} localisation was then examined by confocal microscopy 24 hours after transfection. Shown are representative images of the PtdIns(4,5)P_2 probe transfected alone (A) or co-transfected with Gαq* (B). (C) HeLa cells were grown overnight on glass cover slips and then transfected with GFP-Histone or with both GFP-Histone and Gαq*. 36 hrs after transfection cells were fixed, permeabilised and stained with TOPRO-Red to visualise nuclei. Cells were then analysed via confocal imaging. Shown are representative images of GFP and TOPRO-Red channels from cells expressing both GFP-Histone and Gαq*. (D) PtdIns(4,5)P_2 labelling is increased in cells expressing murine PIP 5-Kα. HeLa cells were transfected with either GFP Histone (designated as control on graph) or GFP-PIP 5-K (designated as PIP 5-K on graph). Cells were then [^{32}P]-orthophosphate labelled, after which phospholipids were extracted and analysed by TLC. Plotted are the resulting PtdIns(4,5)P_2 levels. Error bars display standard deviation of triplicate samples. This graph is typical of three independent experiments. (E) PIP 5-Kα attenuates Gαq*-induced apoptosis. HeLa cells were transfected with the constructs shown. For these experiments a myc-tagged PIP 5-K construct was used. Cells were left for 36 hrs, collected, fixed and stained with Hoechst 33258. Cells were then examined for apoptosis (as defined by nuclear fragmentation). Only transfected cells that were GFP positive were scored. Typically, a total of 500 cells were counted for each sample. Mean values of duplicate samples are plotted. Shown is a graph representative of three independent experiments.

markedly depleted. In line with previous data from cardiomyocytes, expression of Gαq* caused apoptosis in HeLa cells in a caspase-dependent manner (Howes, 2003) (Figure 1C and Figure 1E). A causal link between Gαq*-mediated PtdIns(4,5)P_2 depletion and apoptosis has never been made and to test this point, we sought to inhibit cell death by rescuing PtdIns(4,5)P_2 levels. It has been previously reported by our group and others that the expression of PIP 5-K isoforms elevates PtdIns(4,5)P_2 levels by 1.5 – 2 fold in the majority of adherent cell lines (this figure is based upon 70 % of a cell population ectopically expressing PIP 5-K) and indeed this is also the case in HeLa cells (Figure 1D). Therefore we attempted to rescue

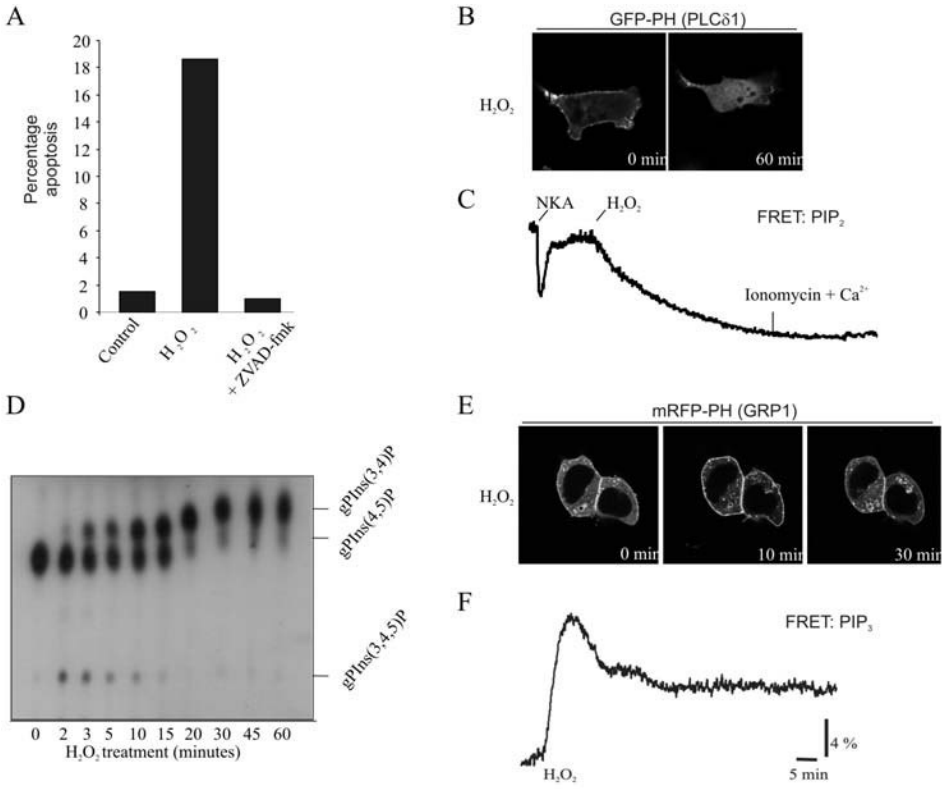

Figure 2, H_2O_2 causes apoptosis in a caspase-dependent manner. (A) HeLa cells were transfected with a GFP Histone. Cells were stimulated with 600 μM H_2O_2 in the presence or absence of ZVAD-fmk as indicated for 24 hr and fixed. The nuclei of cells were then stained with Hoechst 33258 and examined using fluorescence microscopy to identify transfected cells that displayed fragmented apoptotic nuclei. Plotted graphically are the mean values of duplicate samples. The number of apoptotic cells displaying fragmented nuclei is plotted as a percentage of the total number of transfected cells. Typically, a total of 500 cells were counted for each sample. The data shown are representative of three different experiments. (B) H_2O_2 induces PtdIns(4,5)P_2 depletion in HeLa cells. Cells were grown overnight on glass coverslips and transfected with GFP-PH^{PLC}. Cells were stimulated with 600 μM H_2O_2 and confocal images taken every minute for 60 min. Shown are representative images of cells prior to stimulation (0 min) with H_2O_2 and 60 min after stimulation with H_2O_2. (C) PtdIns(4,5)P_2 depletion by H_2O_2 can be visualised using FRET. Cells were transfected with YFP and CFP chimeras of the PH domain from PLCδ1. FRET changes were followed on a wide-field microscope by calculating the ratio of the CFP and YFP fluorescence (Van der Wal, 2001). Prior to stimulation the responsiveness of each cell was assessed by NKA addition. Cells were then treated with 600 μM H_2O_2 and the fluorescence ratio was monitored. 45 minutes post stimulation ionomycin and Ca^{2+} were added. The points of NKA, H_2O_2, ionomycin and Ca^{2+} addition are indicated. Shown is a representative FRET trace of many independent experiments. (D) H_2O_2 induces changes in phosphoinositide metabolism. Cells were [^{32}P]-orthophosphate labelled for two hours. They were stimulated with H_2O_2 for the time indicated and the phospholipids extracted from the cells. Lipid samples were then deacylated and the resulting glycerophosphoinositides separated on a PEI-cellulose plate. The positions of gPIns(4,5)P_2, gPIns(3,4)P_2 and gPIns(3,4,5)P_3 are indicated. (E) H_2O_2 induces transient synthesis of PtdIns(3,4,5)P_3. HeLa cells expressing mRFP-PH^{Grp1} were treated with 600 μM H_2O_2 and confocal images taken every minute for 60 min. Shown are representative images of cells prior to stimulation with H_2O_2, 10 min and 30 min after stimulation with H_2O_2. (F) Transient PtdIns(3,4,5)P_3 production by H_2O_2 can be visualised using a FRET based assay. Cells expressing both GFP-PH^{Grp1} and mRFP-PH^{Grp1} were treated with 600 μM H_2O_2 and FRET changes were followed on a wide-field microscope by calculating the ratio of the GFP and mRFP fluorescence.

$PtdIns(4,5)P_2$ levels in HeLa cells by co-expressing murine PIP 5-Kα. In support of the idea that $PtdIns(4,5)P_2$ depletion is a critical step during Gαq*-induced apoptosis, expression of PIP 5-Kα potently blocked cell death (Figure 1E).

Apoptotic stress stimuli cause sustained $PtdIns(4,5)P_2$ depletion.

Having demonstrated that $PtdIns(4,5)P_2$ depletion is essential for Gαq*-mediated apoptosis, the study was broadened to test whether other apoptotic stimuli also negatively regulate $PtdIns(4,5)P_2$ levels.

Therefore, we began by assessing H_2O_2-induced apoptosis in HeLa cells. It is important to note that H_2O_2 influences cell behaviour in a concentration-dependent manner as higher concentrations cause necrosis while lower concentrations cause cell cycle arrest and apoptosis (Finkel, 2003). It was therefore critical to establish a concentration of H_2O_2 that induced apoptosis rather than necrotic cell death. In our cell system, 600 μM H_2O_2 reproducibly caused apoptosis, which was potently blocked by the caspase inhibitor, ZVAD-fmk (Figure 2A).

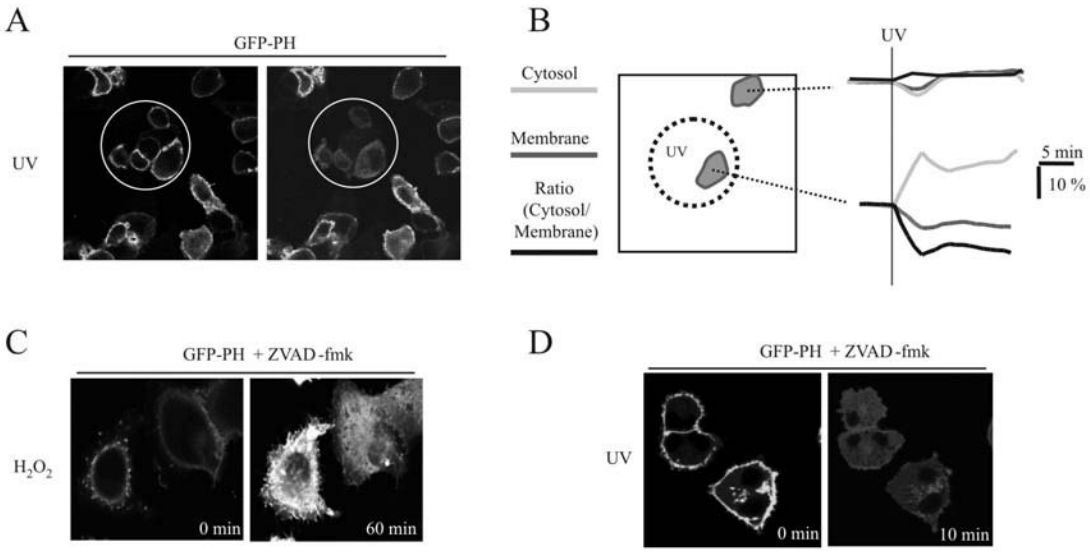

Figure 3, UV-irradiation induces $PtdIns(4,5)P_2$ depletion. (A) HeLa cells were grown overnight on glass coverslips. They were then transfected with $GFP\text{-}PH^{PLC}$. Using confocal imaging, living cells were recorded for 2-3 min and then stimulated with UV-irradiation for 30 seconds. By closing the field diaphragm only the centre cells (within the white circle) were exposed to UV-irradiation. Cells were then imaged for 10 min with images being recorded every 30 seconds. Shown are representative images of cells prior to stimulation and 10 mins after UV-irradiation. (B) The cartoon highlights two analysed cells for which the intensities of the fluorescence of the membrane (red line), and of the cytosol (blue line), and the membrane/cytosol ratio (black line) were plotted in time. The graphs for each cell is indicated via a dashed line. Note the drop in the membrane/cytosol ratio of the cell within the circle (indicated by the dotted line) after UV-irradiation and that the membrane/cytosol ratio of the cell outside the circle remains constant. Scale bar signal shows percent deviation from baseline ratio value. Time is also indicated, as is the point of UV-irradiation. (C) H_2O_2 induces $PtdIns(4,5)P_2$ depletion in a caspase-independent manner. HeLa cells were grown overnight on glass coverslips. They were then transfected with a $GFP\text{-}PH^{PLC}$ construct. 16 hours post-transfection, cells were incubated with 50 μM (final concentration) ZVAD-fmk for one hour. Cells were then treated with 600 μM H_2O_2 and the cells were imaged for 30 minutes with images being taken every minute. (D) Same as in (C) however cells were stimulated with UV-irradiation for 30 seconds. Cells were then imaged for 10 mins with images being recorded every 30 seconds. Shown are representative images prior to treatment and after treatment with H_2O_2 or UV-irradiation (as indicated).

Cells were stimulated with 600 μM H_2O_2 and the localisation of GFP-PH^{PLC} was monitored using live confocal imaging techniques. H_2O_2 caused translocation of GFP-PH^{PLC} from the plasma membrane indicative of sustained PtdIns(4,5)P_2 depletion (Figure 2B). Even after prolonged stimulation with H_2O_2, PtdIns(4,5)P_2 levels at the plasma membrane did not recover, as GFP-PH^{PLC} remained diffusely spread throughout the cell. To monitor PtdIns(4,5)P_2 depletion with increased temporal resolution, we used a FRET based assay (Van der Wal, 2001). During each experiment, cells were stimulated with neurokinin A to test the responsiveness of individual cells to agonist-induced PtdIns(4,5)P_2 changes. NKA mediated stimulation of PLC activity caused a transient decrease in PtdIns(4,5)P_2 which recovered to near basal levels within 2 minutes. Conversely, H_2O_2 treatment caused a sustained decrease in the FRET ratio, indicating prolonged and sustained PtdIns(4,5)P_2 depletion. Complete activation of PLC by the addition of ionomycin and extracellular Ca^{2+} did not elicit further decreases in the FRET ratio indicating that plasma membrane PtdIns(4,5)P_2 had already been depleted (Figure 2C).

To support our confocal studies, we sought to demonstrate PtdIns(4,5)P_2 depletion by an independent method. Therefore we labelled cells with [^{32}P]-orthophosphate and monitored phosphoinositide levels during a time course of H_2O_2 treatment. H_2O_2 markedly decreased PtdIns(4,5)P_2 labelling compared to untreated controls (Figure 2D). The labelling data was in good agreement with the FRET and confocal data, indicating that apoptotic concentrations of H_2O_2 negatively regulate PtdIns(4,5)P_2 levels. Moreover, this was a rapid process as PtdIns(4,5)P_2 labelling was significantly depleted within twenty minutes of H_2O_2 treatment. As previously observed by our group and others, both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 labelling were increased after H_2O_2 treatment (Van der Kaay, 1999; Halstead, 2001) (Figure 2D). The increase in PtdIns(3,4,5)P_3 labelling however was clearly transient. Intriguingly, the increase in PtdIns(3,4)P_2 labelling was sustained after H_2O_2 treatment suggesting that synthesis of this lipid occurs in the absence of observable PtdIns(3,4, 5)P_3 levels. The transient production of PtdIns(3,4,5)P_3 inferred by our labelling studies was confirmed with live cell imaging in which PtdIns(3,4,5)P_3 levels were monitored after H_2O_2 treatment in real-time using a monomeric RFP fusion of the PH domain of Grp1 (mRFP-PH^{Grp1}) as an *in vivo* PtdIns(3,4,5)P_3 probe (Gray, 1999) . This probe, which is predominantly cytosolic was transiently recruited to the plasma membrane after H_2O_2 treatment (Figure 2E). The transient synthesis of PtdIns(3,4,5)P_3 production after treatment with H_2O_2 was further confirmed by a FRET based assay in which mRFP-PH^{Grp1} and GFP-PH^{Grp1} were used as a FRET pair to follow PtdIns(3,4,5)P_3 production with improved temporal resolution (Figure 2F).

To examine whether depletion of PtdIns(4,5)P_2 occurred with other apoptotic stress stimuli, HeLa cells expressing GFP-PH^{PLC} were stimulated with UV-irradiation and monitored using confocal microscopy. UV–irradiation also triggered translocation of GFP-PH^{PLC} from the plasma membrane to the cytosol (Figure 3A and Figure 3B). The time-scale of the translocation of GFP-PH^{PLC} after UV treatment was also rapid occurring within 10 min. Together, these data demonstrate that two apoptotic stress stimuli trigger sustained decreases in

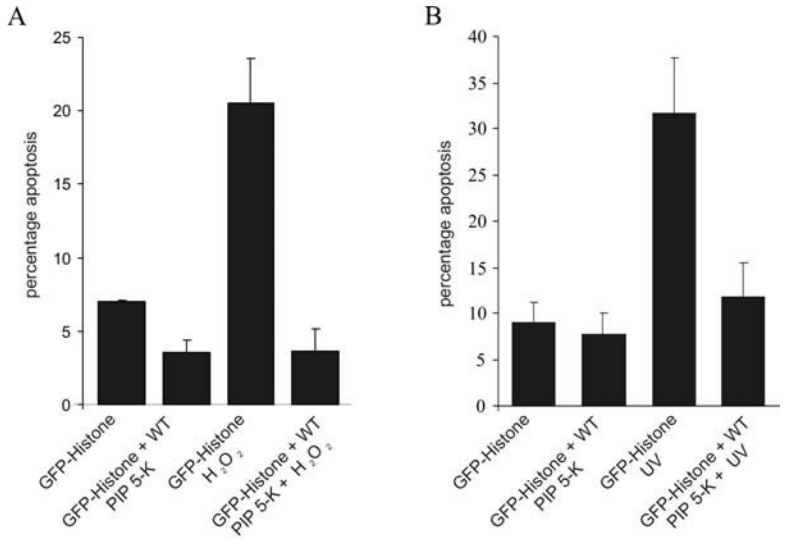

Figure 4, PIP 5-K protects against H_2O_2 induced apoptosis. (A) HeLa cells were transfected with various constructs (as indicated). Cells were stimulated with 600 μM H_2O_2 for 24 hr and fixed. The nuclei of cells were then stained with Hoechst 33258 and examined using fluorescence microscopy to identify transfected cells that displayed fragmented apoptotic nuclei. Plotted graphically are the mean values of triplicate samples. The number of apoptotic cells displaying fragmented nuclei is plotted as a percentage of the total number of transfected cells. Typically, a total of 500 cells were counted for each sample. The data shown are representative of at least three independent experiments. Error bars display standard deviation of triplicate samples. (B) PIP 5-K protects against UV-irradiation induced apoptosis., Cells were irradiated with UV (18 mJ/cm^2) and incubated for a further 24 hours prior to fixation and staining with Hoechst 33258 . The data shown are representative of 3 independent experiments. Error bars display standard deviation of triplicate samples.

PtdIns(4,5)P_2 levels.

PtdIns(4,5)P_2 depletion is essential for stress induced apoptosis.

The *in vivo* synthesis of PtdIns(4,5)P_2 is dependent upon the PIP 5-K family of lipid kinases and it is possible that the observed decreases in PtdIns(4,5)P_2 resulted from inactivation of PIP 5-K activity. As apoptosis by H_2O_2 and UV-irradiation are both caspase dependent and one isoform of human PIP 5-K is cleaved and inactivated by caspases, we considered the possibility that PtdIns(4,5)P_2 depletion may occur downstream of caspase activation. While pre-incubation of HeLa cells with the general caspase inhibitor (ZVAD-fmk) attenuated H_2O_2 induced apoptosis (Figure 2A), ZVAD-fmk, failed to block depletion of PtdIns(4,5)P_2 induced by either H_2O_2 (Figure 3C) or UV-irradiation (Figure 3D). These data demonstrate that apoptotic stress stimuli deplete PtdIns(4,5)P_2 levels in a caspase-independent manner.

Our data indicated that PtdIns(4,5)P_2 depletion was an essential step during Gαq*-dependent apoptosis. To determine if H_2O_2-dependent apoptosis was also reliant upon PtdIns(4,5)P_2 depletion, we sought to block cell death by expressing PIP 5-Kα. Importantly, we found that ectopic expression of PIP 5-Kα completely blocked apoptosis by H_2O_2 in HeLa cells (Fig. 4a). This block in H_2O_2-induced apoptosis was dependent upon the lipid products of PIP 5-Kα, as a 'kinase dead' version of PIP 5-K

(that retains 1% residual activity compared to the wild-type enzyme) offered negligible protection against H_2O_2-induced apoptosis (data not shown). Furthermore, the rescue of cells from apoptosis was not limited to H_2O_2 treatment, as PIP 5-Kα also rescued HeLa cells from UV-irradiation-induced apoptosis (Fig. 4b). As PIP 5-Kα synthesises PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 *in vivo* (Halstead, 2001), we examined if the protective effect of PIP 5-K was linked to the activation of PKB. Cells were transfected with PIP 5-Kα or a constitutively activated PI 3-kinase (CAAX PI 3-kinase) in the absence or presence of PKB and PKB activity was assessed. While the CAAX PI 3-kinase potently activated PKB, no such stimulation was observed with PIP 5-Kα (Figure 5A). This result was confirmed by examining endogenous PKB phosphorylation at Serine^{473} and Threonine^{308}. Insulin, EGF and CAAX PI 3-kinase were potent activators of PKB however PIP 5-Kα failed to promote phosphorylation of either amino acid (Figure 5B). As H_2O_2 triggered PtdIns(4,5)P_2 depletion, it was possible that the levels of PtdIns(4,5)P_2 required for PtdIns(3,4,5)P_3 synthesis were maintained by PIP 5-Kα expression. H_2O_2 transiently activated PKB, as Ser^{473} phosphorylation was lost after 60 minutes (Figure 5C). Importantly, ectopic expression of PIP 5-Kα failed to prolong PKB Ser^{473} phosphorylation upon long-term H_2O_2 treatment (Figure 5D). Furthermore, it is unlikely that PIP 5-Kα-mediated PtdIns(3,4,5)P_3 synthesis attenuated H_2O_2-induced apoptosis, as the PI 3-kinase inhibitor LY294002 (Vlahos, 1994), which blocks the ability of PIP 5-Kα to generate PtdIns(3,4,5)P_3, had no effect upon the PIP 5-K-dependent rescue of cells after H_2O_2 treatment (data not shown). Together, these data indicated that PIP 5-K-dependent rescue from apoptosis was dependent upon PtdIns(4,5)P_2 and not PtdIns(3,4,5)P_3; and that PtdIns(4,5)P_2 depletion is an essential step during H_2O_2 and UV-irradiation-induced apoptosis.

Apoptotic stress stimuli attenuate PIP 5-K activity during apoptosis

As there was a close temporal correlation between the labelling decreases in both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 after H_2O_2 treatment (production of both these lipids is dependent upon PIP 5-K activity), we considered the possibility that PIP 5-K activity was inhibited after prolonged oxidative stress. To test this, we assayed endogenous PIP 5-K activity from HeLa cells after *in vivo* treatment with 600 μM H_2O_2. Endogenous PIP 5-K activity was inhibited after 20 minutes incubation with H_2O_2 (Figure 6A). The inhibition was transient as PIP 5-K activity recovered with time, demonstrating that PIP 5-K inhibition by apoptotic concentrations of H_2O_2 was reversible. As H_2O_2 inactivated PIP 5-K in a transient manner, this mechanism was unlikely to fully explain the sustained depletion of PtdIns(4,5)P_2 levels we observed by confocal and labelling studies. Therefore, we examined other potential mechanisms for the down-regulation of PIP 5-K activity. It is known that PIP 5-K activity is abundant in membrane fractions and PIP 5-K localises to membranous structures and is enriched at the plasma membrane (Figure 6B). As the PIP 5-K substrate PtdIns4P is membrane localised and a number of reports have linked *in vivo* PIP 5-K activity to membrane localisation (Kunz, 2000; Kunz, 2002), we tested whether treatment with apoptotic concentrations of H_2O_2 altered PIP 5-K localisation. Indeed, within minutes H_2O_2 treatment, a GFP fusion of PIP5-Kα (GFP-PIP 5-Kα) translocated from the plasma membrane to the cytoplasm in a manner reminiscent of the H_2O_2- induced trans-

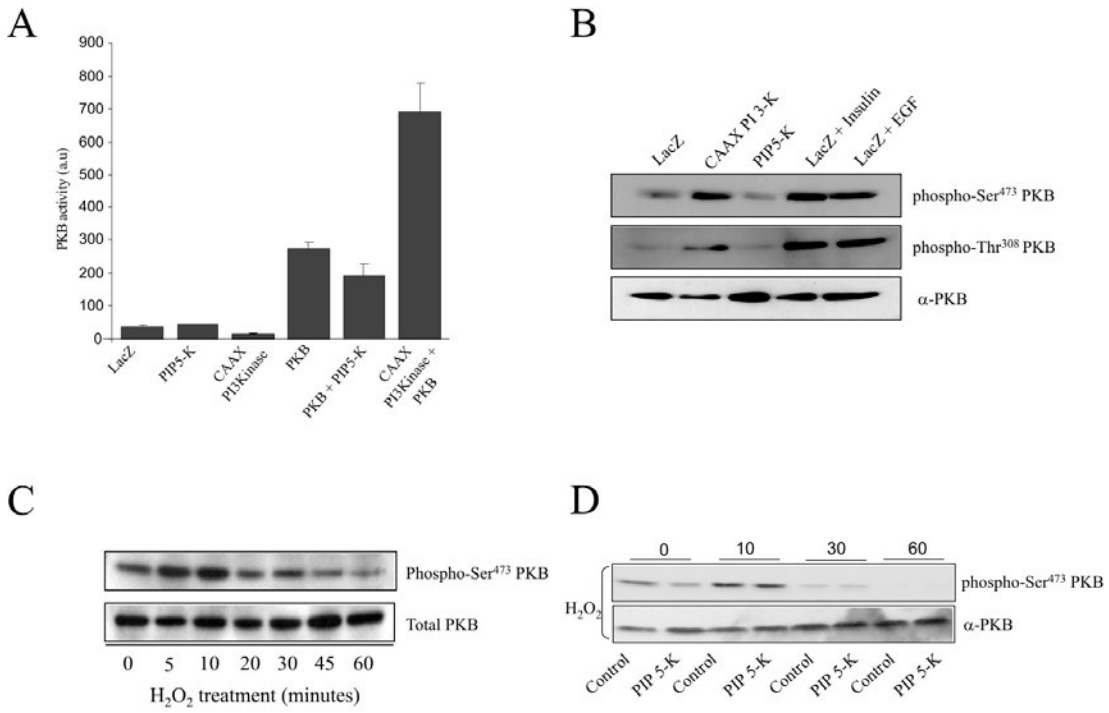

Figure 5, the PIP 5-K rescue from apoptosis is not mediated through PtdIns(3,4,5)P$_3$ production. (A) PIP 5-K expression has no effect upon PKB activity whereas CAAX PI 3-kinase potently activates PKB *in vivo*. Activity of ectopically expressed PKB was assayed after co-transfection with the various constructs shown. (B) PIP 5-K expression has no effect upon endogenous PKB activity. Activity of endogenous PKB was examined using phospho-specific antibodies raised against the phosphorylated forms of the PKB amino acids Threonine^{308} and Serine^{473}. Phosphorylation of these residues is indicative of PKB activation. (C) Transient phosphorylation of PKB in cells after treatment with 600 μM H_2O_2. Endogenous PKB activation was examined using an antibody that specifically recognises PKB upon phosphorylation of residue Serine^{473}. (D) PIP 5-K does not prolong activation of endogenous PKB after treatment with 600 μM H_2O_2.

location of GFP-PH^{PLC} (Figure 6B). The translocation of GFP-PIP 5-Kα was also observed after UV-irradiation (Figure 6C). In both cases, we were unable to detect enrichment of GFP PIP 5-Kα at the plasma membrane several hours after treatment. It has been shown previously that PIP 5-K mutants which localise to the cytosol do not efficiently elevate PtdIns(4,5)P_2 levels *in vivo* (Kunz, 2000; Kunz, 2002). The translocation of the enzyme from the plasma membrane could therefore constitute a primary mechanism by which PtdIns(4,5)P_2 synthesis at the membrane could be attenuated. As GFP PIP 5-Kα translocation after H_2O_2 treatment correlated with a decrease in PtdIns(4,5)P_2, we tested whether sustained PtdIns(4,5)P_2 hydrolysis would alter GFP PIP 5-K localisation. Cells were co-transfected with GFP PIP 5-Kα and a mutated version of the neurokinin A (NKA) receptor, which is not de-sensitised after stimulation, leading to prolonged PLC activation and sustained PtdIns(4,5)P_2 hydrolysis (Alblas, 1995). To observe changes in both GFP-PIP 5-K localisation

and PtdIns(4,5)P_2 levels, we used a monomeric red fluorescent protein fusion of the PH domain of PLCδ1 (mRFP-PH^{PLC}). After NKA stimulation, mRFP-PH^{PLC} translocated from the plasma membrane to the cytosol within 20 seconds (Figure 6D). GFP PIP 5-Kα however remained localised at the plasma membrane indicating that depletion of PtdIns(4,5)P_2 alone, or activation of downstream targets of the PLC pathway, was insufficient to cause translocation of the lipid kinase. If the translocation of PIP 5-K from the plasma membrane was important for the observed loss of PtdIns(4,5)P_2 levels it would be expected that the translocation of the lipid kinase would precede the translocation of the *in vivo* PtdIns(4,5)P_2 probe. In support of this idea, we observed translocation of GFP PIP 5-Kα in HeLa cells prior to

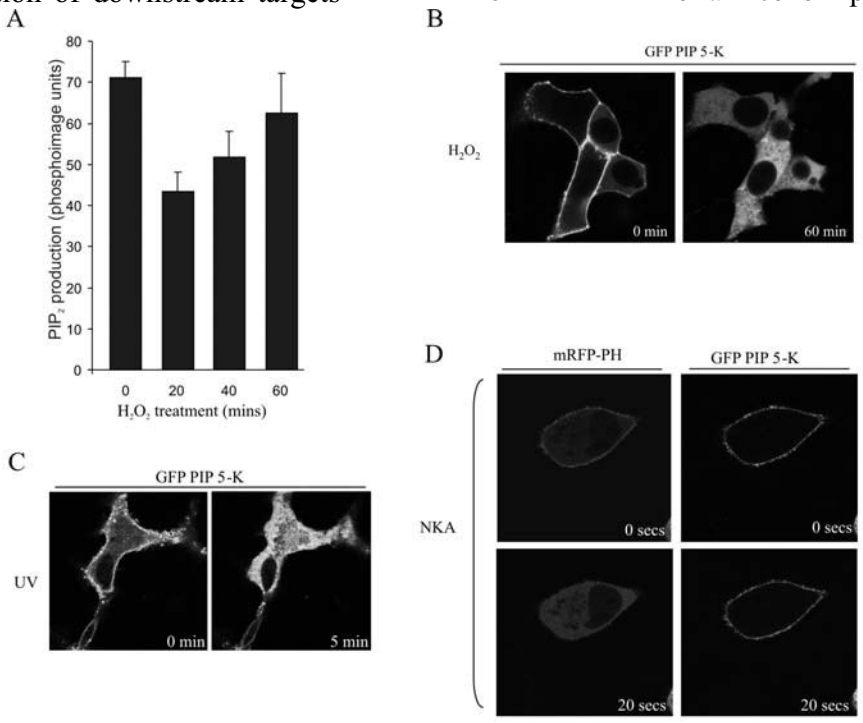

Figure 6, Endogenous PIP 5-K is catalytically inactivated *in-vivo* after treatment with H_2O_2. (A) HeLa cells were stimulated for the time indicated. Cells were lysed and endogenous PIP 5-kinase was immunoprecipitated using a polyclonal antibody. Immunoprecipitates were tested for associated PIP 5-kinase activity using a mixed micelle containing PtdIns(4)P as a substrate (see materials and methods). Samples were extracted and the lipids resolved on using TLC. Plotted graphically are the mean values of triplicate samples. Error bars represent the standard deviation of the mean of triplicate samples. (B) H_2O_2 induces delocalisation of PIP 5-K from the plasma membrane. Shown are confocal images of the mid-section of cells expressing GFP PIP 5-Kα. Cells were grown on glass cover slips over night prior to transfection. 16 hours post-transfection, cells were stimulated with H_2O_2. Shown is a representative image of cells prior to stimulation and 60 min post stimulation with H_2O_2. (C) UV-irradiation causes delocalisation of PIP 5-K from the plasma membrane. Identical to Figure 6B however 16 hours post-transfection, cells were stimulated with UV-irradiation and images taken every 20 seconds. Shown is a representative image of cells prior to stimulation and 5 min after treatment with UV. (D) PtdIns(4,5)P_2 depletion does not cause delocalisation of PIP 5-K from the plasma membrane. Shown are the confocal images of the mid-section of HeLa cells transfected with GFP PIP 5-K, mRFP-PH and truncated NKA receptor. Cells were grown on glass cover slips over night prior to transfection. 16 hours post transfection cells were stimulated with NKA and images taken prior to stimulation and for 30 minutes after stimulation. Shown is an image of a representative cell prior to stimulation and 20 seconds after NKA addition. It should be noted that throughout the entire time course of the experiment, GFP PIP 5-K remained localised at the plasma membrane.

translocation of mRFP-PH^{PLC} in cells treated with H_2O_2 (data not shown). These data suggested that distinct signalling events are initiated after H_2O_2 treatment that attenuate PIP 5-K activity at the plasma membrane leading to transient inactivation of the enzyme and displacement of the enzyme away from the plasma membrane. Both these events would serve to deplete PtdIns(4,5)P_2 levels after H_2O_2 treatment, which we have demonstrated are essential for stress-induced apoptosis.

Discussion

We show that apoptotic stress stimuli trigger irreversible PtdIns(4,5)P_2 depletion. PtdIns(4,5)P_2 depletion occurs almost immediately after H_2O_2 or UV-irradiation treatment and is independent of caspase activation. Therefore we propose that H_2O_2 or UV-irradiation mediated PtdIns(4,5)P_2 depletion constitutes an early apoptotic signalling event. A previous report (Mejillano, 2001) has shown that activated caspase-3, cleaves and inactivates human PIP 5-Kα (homologue of the murine PIP 5-Kβ). Primary structure analysis of the other PIP 5-K isoforms (murine PIP 5-Kα,γ or H), failed to reveal homologous caspase cleavage sites indicating that these enzymes are unlikely to be directly regulated by caspase cleavage. However, our data and the data from Mejillano *et al*. indicate that apoptotic stimuli down-regulate PIP 5-K activity in both a caspase-dependent and -independent manner. It is possible that caspase-independent mechanisms are required during the early stages of apoptosis and that caspase-dependent mechanisms are employed later on during apoptosis (post-caspase activation) for the inactivation of the murine β isoform (homologue of human PIP 5-Kα). Furthermore, as isoforms of PIP 5-K appear to have non-redundant functions, caspase-dependent and -independent mechanisms may serve to differentially regulate specific pools of PtdIns(4,5)P_2 (nuclear versus cytoplasmic for example).

Expression of PIP 5-Kα attenuates both H_2O_2 and UV-irradiation induced apoptosis and moderately inhibits TNFα/cyclohexaminde-induced apoptosis (JRH, unpublished data), the latter being in good agreement with a previous report (Mejillano, 2001). However, we were unable to observe decreases in PtdIns(4,5)P_2 levels after TNFα/cyclohexamide treatment suggesting that certain apoptotic stimuli (stress stimuli) are more dependent on PtdIns(4,5)P_2 depletion than others (death receptor agonists such as TNFα). A role for PIP 5-K in death receptor mediated cell death has been previously suggested as RNAi mediated suppression of PIP 5-Kγ sensitises HeLa cells to apoptosis induced by TRAIL (Aza-Blanc, 2003). The anti-apoptotic effect of PIP 5-K is dependent upon kinase activity as a kinase dead version of PIP 5-K offered negligible protection against H_2O_2 and UV-mediated cell death. As LY294002 can block PIP 5-K-induced PtdIns(3,4,5)P_3 formation but not its ability to attenuate apoptosis, it is unlikely that PtdIns(3,4,5)P_3 generation is the mechanism for the inhibition of stress induced-apoptosis. In agreement with this data, expression of PIP 5-K failed to augment or affect PKB activation although it completely rescued cells from H_2O_2-induced apoptosis. We suggest that PtdIns(4,5)P_2 depletion enables an essential event for apoptosis, such as the inhibition of a survival strategy or the activation of a pro-apoptotic signal. With respect to this issue, previous *in vitro* data suggested that PtdIns(4,5)P_2 can inhibit

caspase 8 and 9 activity'. However, so far we were unable to demonstrate that overexpression of PIP 5-Kα, which attenuated H_2O_2-induced apoptosis, also blocked caspase activation (unpublished data). Modulation of the cytoskeleton using agents such as cytochalasins potently induces apoptosis (Yamazaki 2000; Rubtsova 1998). As PtdIns(4,5)P_2 is a key regulator of cytoskeletal dynamics, an enticing possibility is that PtdIns(4,5)P_2 depletion induces apoptosis through the modulation of the actin cytoskeleton.

In this paper, we present evidence to support the idea that inactivation of PIP 5-K causes sustained PtdIns(4,5)P_2 depletion during H_2O_2-induced apoptosis. Negative regulation of murine PIP 5-Kα (homologue of human PIP 5-Kβ) occurs through both the inhibition of PIP 5-K catalytic activity and through its relocalisation away from its substrate at the plasma membrane. In our model, inactivation of PIP 5-K by H_2O_2 prevents re-synthesis of PtdIns(4,5)P_2 thereby depleting the cell of this lipid. Interestingly, both H_2O_2 and UV-irradiation can also activate PLC and PtdIns(4,5)P_2 hydrolysis (Scheiven, 1993). Thus, the coordinated activation of PLC and inactivation of PIP 5-K may be the underlying mechanism for stress-induced PtdIns(4,5)P_2 depletion. In *S.cerevisiae*, mutation of the phospholipase C gene, *plc-1* sensitises yeast to UV-irradiation (Andoh, 1998). Furthermore, up-regulation of PKC signalling in mammalian cells can attenuate UV-irradiation induced apoptosis (Matsumura, 2003). Both these data indicate that PLC activity and the resulting activation of PKC may contribute to apoptotic resistance. Thus, maintenance of PtdIns(4,5)P_2, by over-expression of PIP 5-K, may attenuate apoptosis through maintenance of the PLC signalling pathway.

PtdIns(4,5)P_2 is a key intracellular phosphoinositide required for the regulation of many different signalling pathways including the PLC and PtdIns(3,4,5)P_3/PKB pathway. Furthermore PtdIns(4,5)P_2 is essential for vesicle generation and actin dynamics. Dysfunction of these processes is unlikely to be compatible with life. PIP 5-K appears to be a central player in response to oxidative damage. For that reason it is tempting to speculate that PIP 5-K acts as an essential intracellular stress sensor, governing the switch between survival and apoptosis. For example at the point where accumulation of oxidative damage abrogates PIP 5-K activity, the decision is made to embark into a pathway of programmed cell death. The ensuing apoptotic signalling cascade may be triggered by the concomitant decreases in PtdIns(4,5)P_2 levels. It follows therefore that maintenance of PIP 5-K activity and hence PtdIns(4,5)P_2 levels will enable cells to escape apoptosis in response to cellular stress. Clinically, this raises the issue of PIP 5-K in diseases that are characterised by high levels of oxidative stress, such as certain human cancers (Szatrowski, 1991). It is tempting to speculate that these tumour cells may maintain higher than normal levels of PtdIns(4,5)P_2 or alternatively that they have developed a PIP 5-K that is insensitive to H_2O_2. Intriguingly, evidence for aberrant PIP 5-K activity already exists in tumours (Singhal, 1994A; 1994B). In conclusion, PtdIns(4,5)P_2 has an essential role in cellular survival and maintaining levels of this lipid is sufficient to circumvent programmed cell death. Accordingly, certain apoptotic stimuli signal the depletion of PtdIns(4,5)P_2 in the early stages of apoptosis by inactivating PIP 5-K.

Acknowledgements

We would like to thank Professor J. Borst, Professor W. Moolenaar, Dr W. van Blitterswijk, Dr S.W. Tait and Dr D. Weinkove for critical reading of the

manuscript and the members of the phosphoinostide lab, NKI, Amsterdam for helpful discussion. We would also like to thank Dr T. Balla for the kind gift of the monomeric RFP-PH^{PLC} construct. J.R.H. is supported by the KWF. J.v.R. is supported by the NWO and K.J. and N.D are supported by the NKI (AvL).

Materials and Methods

Materials, cell culture and Plasmids
Synthetic PtdIns(4)P dipalmitoyl esters were purchased from Echelon (Salt Lake City, UT). Phosphatidylserine, phosphatidic acid, Z-VAD-fmk and hydrogen peroxide were all purchased from Sigma (St. Louis, MO). Topro-Red was purchased from Molecular Probes. ZVAD-fmk was purchased from Sigma. GFP, YFP and CFP-$PH^{PLC\delta1}$ constructs are described elsewhere[24]. GFP-PH^{Grp1} was was a kind gift from Professor P. Downes and was subsequently cloned into a mRFP construct. GFP-Histone H2B and activated Gαq* was provided by Prof. W Moolenaar.. GFP-PIP 5-Kα and myc-pcDNA PIP 5-Kα were PCR cloned as previously described in our laboratory from a murine brain cDNA library (Divecha, 2000). The CAAX PI 3-kinase construct was a gift from Dr Len Stephens. HeLa cells were routinely cultured in 10 % fetal bovine serum (Gibco) in dulbecco's modified Eagle's medium (DMEM) and transfected using Fugene transfection reagent (Boehringer-Mannheim).

Apoptosis Assays
During our study, two methods were employed to examine cellular apoptosis. The first method was based upon the nuclear morphology of cells visualised using fluorescence microscopy after staining with *bis*benzimide (Hoescht 33258, Sigma). Cells were collected 24 hours post-apoptotic stimulation including those that had detached from the cell culture plate. Cells that were still attached were washed twice with PBS (washes were saved) and removed by trypsinization. All fractions including PBS wash steps were then pooled and washed a further two times with PBS. The cellular pellet was then fixed in 50 µl of a 3.7 % (v/v) formaldehyde/PBS solution. After 10 min at room temperature, the fixative was removed and the cells were resuspended in 15 µl of PBS containing 16 µg/ml *bis*benzimide. A 10 µl aliquot was placed on a glass slide, and approximately 500 cells per slide were scored in duplicate or triplicate for the incidence of apoptotic nuclear changes under a Zeiss Axiovert 135 fluoresence microscope. To be classified as apoptotic we scored the incidence of fragmented nuclei only and did not include the incidence of condensed or partially condensed nuclei as apoptotic. This generated highly reproducible data sets. The second method was also based on nuclear morphology and was used for imaging experiments. Briefly, cells were transfected with GFP Histone2B. After apoptotic stimulation cells were fixed and treated with the nuclear stain Topro-Red and imaged using confocal microscopy (Leica).

PKB assay
Activation of PKB was determined using a phospho-specific antibody that recognises $Serine^{473}$ of PKB or $Threonine^{308}$. The PKB activity assays were performed as described previously (Welch, 1998).

FRET and confocal imaging
For confocal imaging, a Leica DM-IRBE inverted microscope fitted with a TCS-SP scanhead (Leica, Mannheim, Germany) was used. Excitation of enhanced GFP was with the 488-nm argon ion laserline, and emission was collected at 500-565 nm. For translocation studies, series of confocal images were taken at 2-10-s intervals and stored on disc. PtdIns(4,5)P_2 FRET studies were performed as described previously (ref) and PtdIns(3,4,5)P_3 FRET studies adapted from these methods. Visualisation and analysis was performed off-line using LCS and Qwin software (Leica). Qwin software was used to automize the assignment of regions of interest .

[^{32}P]-orthophosphate labelling and PIP 5-K *in vitro* assays
Transfected or non-transfected cells were orthophopshate labelled as previously described[13]. After extraction, phospholipids were treated with monomethylamine and the deacylated products analysed using PEI-cellulose plates (Whatman) as described previously (Halstead, 2001). For the in vitro PIP 5-K assays, cells were transfected as described and left for 16 h, after which they were lysed [1 ml lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1% NP-40)], scraped, and nuclei and cellular debris removed by centrifugation (14 000 r.p.m., 4°C Eppendorf centrifuge). Immunoprecipitation of the endogenous PIP 5-K from Hek293T cells was carried out using a polyclonal antibody raised against a peptide present all three isoforms of PIP 5-K. The immunoprecipitates were collected using protein G–Sepharose, and washed three times with IP wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA 0.1% Tween-20), then twice with 1x PIP 5-K buffer. For the PIP 5-K assays, lipid vesicles were

prepared using 1 nmol of PtdIns4P together with 1 nmol PtdSer and 3 nmol of PtdOH. Reactions were carried out at 30°C for 20 min in 1 x PIPkinase buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 70 mM KCl) containing cold ATP (20 µM) and 1 µCi of [^{32}P]ATP in a final volume of 100 µl. Reactions were quenched with 0.5 ml of chloroform :methanol [1:1 (v:v)] and the phases were split by the addition of 125 µl of 2.4 M HCl. The lower phases were removed to a new tube, dried and separated by TLC either using an alkaline solvent [chloroform:methanol:ammonia (28%):water 45:35:2:8]. Incorporation into PtdIns(4,5)P_2 was quantitated using a phosphoimager.

References

Alblas, J., van Etten, I., Khanum, A. and Moolenaar, W.H. (1995). C-terminal truncation of the neurokinin-2 receptor causes enhanced and sustained agonist-induced signaling. Role of receptor phosphorylation in signal attenuation. J. Biol. Chem. *270*, 8944-8951.

Adams, J.W. *et al*. (1998). Enhanced G-alpha q signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. Proc.Natl. Acad. Sci. *18*, 10140-10145.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., et al. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. *15*, 6541-6551.

Althoefer, H., Eversole-Cire, P and Simon, M.I. (1997). Constitutively active Galphaq and Galpha13 trigger apoptosis through different pathways. J. Biol. Chem. *272*, 24380-24386.

Andersen J.K. (2004). Oxidative stress in neurode-generation: cause or consequence? Nat. Med., S18-25.

Andoh, T., Kato, T. Jr., Matsui, Y. and Toh-e, A. (1998). Phosphoinositide-specific phospholipase C forms a complex with 14-3-3 proteins and is involved in expression of UV resistance in fission yeast. Mol. Gen. Genet. *258*, 139-147.

Aza-Blanc P., Cooper C.L., Wagner K., Batalov S., Deveraux Q.L., Cooke M.P. (2003) Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. Mol. Cell *12*, 627-637.

Azuma, T., Koths, K., Flanagan, L and Kwiatkowski, D. (2000). Gelsolin in complex with phosphatidylinositol 4,5-bisphosphate inhibits caspase-3 and -9 to retard apoptotic progression. J. Biol. Chem. *275*, 3761-3716.

Chang, J.D., Field, S.J., Rameh, L.E., Carpenter, C.L. and Cantley, L.C. (2003). Identification and characterization of a phosphoinositide phosphate kinase homolog. J. Biol. Chem. *279*, 11672-11679.

Divecha, N., Roefs, M., Halstead, J.R., D'Andrea, S., Fernandez-Borga, M., Oomen, L., Saqib, K.M., Wakelam, M.J. and D'Santos, C. (2000). Interaction of the type Ialpha PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4, 5-bisphosphate in the regulation of PLD2 activity. EMBO J. *19*, 5440-5449.

Finkel T. Oxidant signals and oxidative stress. (2003). Curr. Opin. Cell Biol. *15*, 247-254.

Gray A., Van Der Kaay J and Downes C.P. (1999). The pleckstrin homology domains of protein kinase B and GRP1 (general receptor for phos-phoinositides-1) are sen-sitive and selective probes for the cellular detection of phosphatidylinositol3,4-bis-phosphate and/

or phosphatidylinositol 3,4,5 trisphosphate in vivo. Biochem J. *344*, 929-936.

Halstead, J.R., Roefs, M., Ellson, C.D., D'Andrea, S., Chen, C., D'Santos, C.S and Divecha, N. (2001). A novel pathway of cellular phosphatidy-linositol(3,4,5)-tris-phosphate synthesis is regulated by oxidative stress. Curr. Biol. *11*, 386-395

Hilgemann, D.W. (2004). Oily barbarians breach ion channel gates. Science *304*, 265-270.

Howes, A.L., Arthur, J.F., Zhang, T., Miyamoto, S., Adams, J.W., Woodcock, E.A. and Heller Brown J. (2003). Akt-mediated cardiomyocyte survival pathways are compromised by Galpha q-induced PIP2 depletion. J Biol. Chem. *278*, 40343-40351.

Ishihara, H., Shibasaki, Y., Kizuki, N., Wada, T., Yazaki, Y., Asano, T. and Oka, Y. (1998). Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. J. Biol. Chem. *273*, 8741-8748.

Itoh, T and Takenawa, T. (2004). Regulation of endocytosis by phosphatidylinositol 4,5-bisphosphate and ENTH proteins. Curr. Top. Microbiol. Immunol. *282*, 31-47.

Jiang D, Jha N., Boonplueang R and Andersen J.K. (2001). Caspase 3 inhibition attenuates hydrogen peroxide-induced DNA fragmentation but not cell death in neuronal PC12 cells. J. Neurochem. *76*, 1745-55.

Kisseleva M.V., Cao L and Majerus PW. (2002). Phosphoinositide-specific inositol polyphosphate 5-phosphatase IV inhibits Akt/protein kinase B phosphorylation and leads to apoptotic cell death. J. Biol. Chem. *277*, 6266-62672.

Kunz J., Wilson M.P., Kisseleva M., Hurley J.H., Majerus P.W and Anderson R.A. (2000). The activation loop of phos-phatidylinositol phosphate kinases determines signaling specificity. Mol. Cell *5*, 1-11.

Kunz J., Fuelling A., Kolbe L and Anderson RA. (2002). Stereo-specific substrate recognition by phosphatidylinositol phosphate kinases is swapped by changing a single amino acid residue. J. Biol. Chem. *277*, 5611-5619.

Loijens, J.C. and Anderson, R.A. (1996). Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family. J. Biol. Chem. *271*, 32937-32943.

Matsumura, M., Tanaka, N., Kuroki, T., Ichihashi ,M. and Ohba, M. (2003).The eta isoform of protein kinase C inhibits UV-induced activation of caspase-3 in normal human keratinocytes. Biochem. Biophys. Res. Commun. *303*, 350-356.

Mejillano, M., Yamamoto, M., Rozelle, A.L., Sun, H.Q., Wang, X. and Yin, H.L. (2001). Regulation of apoptosis by phosphatidylinositol 4,5-bisphosphate inhibition of caspases, and caspase inactivation of phosphatidylinositol phosphate 5-kinases. J. Biol. Chem. *276*, 1865-1872.

Rubtsova, S.N., Kondratov, R.V., Kopnin, P.B., Chumakov, P.M., Kopnin, B.P. & Vasiliev, J.M. (1998).Disruption of actin microfilaments by cytochalasin D leads to activation of p53. FEBS Lett. *430*, 353-357.

Scheid, M.P. and Woodgett, J.R. (2003). Unravelling the activation mechanisms of protein kinase B/Akt. FEBS. Lett. *546*,108-112.

Schieven, G.L., Kirihara, J.M., Gilliland, L.K., Uckun, F.M and Ledbetter, J.A. (1993). Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signaling in lymphocytes. Mol. Biol. Cell. *4*, 523-530.

Singhal, R.L., Yeh, Y.A., Look, K.Y., Sledge, G.W. Jr. & Weber, G. (1994) Coordinated increase in activities of the signal transduction enzymes PI kinase and PIP kinase in human cancer cells. Life Sci. *55*, 1487-1492.

Singhal, R.L., Prajda, N., Yeh, Y.A. & Weber, G. (1994). 1-Phosphatidylinositol 4-phosphate 5-kinase: a proliferation- and malignancy-linked signal transduction enzyme. Cancer Res. *54*, 5574-5578.

Stauffer, T.P., Ahn, S., and Meyer, T. (1998). Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. Curr. Biol. *8*, 343-346.

Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F. and Hawkins P.T. (1997). Dual role of phosphatidyinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science. *277*, 567-570.

Szatrowski, T. P. and Nathan, C. F. (1991). Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. *51*, 794-798.

Van der Kaay J., Beck M, Gray A. and Downes C.P. (1999). Distinct phosphatidylinositol 3-kinase lipid products accumulate upon oxidative and osmotic stress and lead to different cellular responses. J. Biol. Chem. *274*, 35963-35968.

van der Wal, J., Habets, R., Varnai, P., Balla, T. and Jalink, K. (2001). Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. J. Biol. Chem. *276*, 15337-15344.

Varnai P and Balla T. (1998). Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo- [3H] inositol -labeled phosphoinositide pools. J. Cell Biol. *143*, 501-510.

Vlahos, C.J., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. *269*, 5241-5248.

Welch H., Eguinoa A., Stephens L.R, and Hawkins P.T. (1998). Protein kinase B and rac are activated in parallel within a phosphatidylinositide 3OH-kinase-controlled signaling pathway. J. Biol. Chem. *273*, 11248-11256.

Yamazaki, Y., Tsuruga, M., Zhou, D., Fujita, Y., Shang, X., Dang, Y., Kawasaki, K. and Oka, S. (2000). Cytoskeletal disruption accelerates caspase-3 activation and alters the intracellular membrane reorganization in DNA damage-induced apoptosis. Exp. Cell Res. *259*, 64-78.

Yin, H.L. and Janmey, P.A. (2003). Phosphoinositide regulation of the actin cytoskeleton. Annu. Rev. Physiol. *65*, 761-789.

Zhao ZQ. (2004). Oxidative stress-elicited myocardial apoptosis during reperfusion. Curr. Opin. Pharmacol. *2*, 159-65.