Akt-mediated Cardiomyocyte Survival Pathways Are Compromised by Gαq-induced Phosphoinositide 4,5-Bisphosphate Depletion*

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Expression of the wild type α subunit of Gq (GqWT) in cardiomyocytes induces hypertrophy, whereas a constitutively active Gqα subunit (GqQ209L) induces apoptosis. Akt phosphorylation increases with GqWT expression but is markedly attenuated in cardiomyocytes expressing GqQ209L or in those expressing GqWT and treated with agonist. A membrane-targeted Akt rescues GqQ209L-expressing cardiomyocytes from apoptotic cell death. In contrast, leukemia inhibitory factor fails to activate Akt or promote cell survival in these cells. Association of Akt and PDK-1 with the membrane is also diminished in GqQ209L-expressing cardiomyocytes. Phosphatidylinositol 3,4,5-bisphosphate (PIP3), the primary regulator of Akt, increases significantly in GqWT-expressing cells but not in cardiomyocytes expressing GqQ209L. Levels of phosphatidylinositol 4,5-bisphosphate (PIP2), the immediate precursor of PIP3, are also markedly lower in GqQ209L-expressing compared to control cells. Expression of a GqQ209L mutant that has diminished capacity to activate phospholipase C does not decrease PIP2 or Akt or induce apoptosis. In transgenic mice with cardiac Gqα overexpression, heart failure and increased cardiomyocyte apoptosis develop during the peripartal period. Akt phosphorylation and PIP2 levels decrease concomitantly. Our findings suggest that an Akt-mediated cell survival pathway is compromised by the diminished availability of PIP2 elicited by pathological levels of Gqα activity.

Gqα signaling plays a key role in the hypertrophic growth of cardiomyocytes. Hormones that act through receptors coupled to the heterotrimeric protein Gq (e.g. norepinephrine, PGF2α).}

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§The abbreviations used are: PGF2α, prostaglandin F2α; PLC, phospholipase C; NRVMs, neonatal rat ventricular myocytes; PI3K, phosphoinositide 3-kinase; PDK-1, phosphoinositide-dependent kinase-1; Akt, protein kinase B; CT-1, cardiotrophin-1; LIF, leukemia inhibitory factor; ITS, insulin/transferrin/selenium; myr-Akt, myristoylated Akt; PP, protein phosphatase; HPLC, high pressure liquid chromatography.

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and leukemia inhibitory factor (LIF) (19–21)). Akt has been reported to protect cardiomyocytes from apoptosis induced by serum deprivation or hypoxia (14, 15). Furthermore, in vivo gene transfer of activated Akt protects against cell death and the development of cardiomyopathies caused by ischemia-reperfusion and doxorubicin treatment (22–24). The critical role of Akt in cardioprotection underscores the need to examine the regulation of PI3K, PIP3 formation, and PDK-1 activity and determine how these control Akt activation in cardiomyocytes.

We report here that increasing Gα signaling to pathological levels results in a loss of the protective PI3K/Akt signaling pathway. This occurs at a time point that precedes the appearance of apoptotic markers such as cytochrome c release and nuclear fragmentation. This contrasts directly with the enhanced Akt phosphorylation seen in cardiomyocytes that undergo hypertrophy in response to expression of GαWT. Our investigation into the pathways leading to the down-regulation of Akt activation in cardiomyocytes expressing GαQ209L revealed an unexpected decrease in cellular levels of PIP2, the substrate for both PLC and PI3K, which appeared to be related to the Gα-mediated increase in PLC activity. Importantly, in vivo studies also revealed decreased Akt phosphorylation and PIP3 levels in a Gα-expressing model of heart failure. We suggest that PIP3 generation and Akt activation are influenced by the availability of PIP2 and that this serves as a nodal point in determining the balance between hypertrophic and apoptotic pathways.

**EXPERIMENTAL PROCEDURES**

Cardiomyocyte Culture and Adenoviral Infection—NRVMs were prepared and infected with adenoviruses as described previously (8). Briefly, cardiomyocytes were plated at a density of 1.5 × 10^6 cells per 6-cm dish or 3 × 10^5 per well of a 6-well plate in serum-containing media overnight. The cells were then washed, and the medium was replaced with serum-free medium supplemented with insulin/transferrin/selenium (ITS). Cells were infected with adenoviruses at 200–500 viral particles/cell for 16–18 h or as indicated. Cells were subsequently washed and maintained in serum-free medium with supplements. The p110WT and p110caax expressing adenoviruses were

![Fig. 1. Expression of GαQ209L decreases Akt phosphorylation.](image)

NRVMs were infected with control adenovirus, GαWT, or GαQ209L overnight. The GαWT+PGF2α dishes were treated with 1 μM PGF2α coincident with adenoviral infection. 8 h after washing out the viruses, cell lysates were prepared and analyzed by SDS-PAGE. A, representative Western blots using the phospho-specific Ser-473 antibody (upper panel) and the Akt antibody for total protein levels (bottom panel) are shown. Similar results were obtained with the Thr-308 antibody. B, average ± S.E. of normalized values obtained from densitometric analysis from 4 to 6 experiments using the antibody directed against the phosphorylated Ser-473 site. *, p < 0.05 compared with control, GαWT+PGF2α or GαQ209L, **, p < 0.01 compared with control or GαWT.

![Fig. 2. Activated Akt prevents induction of apoptotic end points by GαQ209L.](image)

NRVMs were infected overnight with control adenovirus, GαQ209L, myr-Akt, or GαQ209L and myrAkt viruses together. A, cells were harvested 36 h after removal of viruses and processed for DNA laddering, as described previously (12). DNA fragments were separated on 2% agarose gels, stained with ethidium bromide, and visualized under UV light. Results shown are representative of three independent experiments. B and C, cells grown on laminin-coated chamber slides were fixed with 3.7% formaldehyde 48 h after infection, permeabilized, and stained with rhodamine-conjugated phalloidin and Hoechst 33342, as described under “Experimental Procedures.” Phalloidin staining (B) was visualized using an Axiovert fluorescent microscope, and images were captured on a digital SPOT camera. For quantification of nuclear fragmentation (C), over 300 nuclei were examined per condition, and each condition was tested in quadruplicate. Values are means ± S.E. *, p < 0.001 compared with GαQ209L.

![Graphs](image)
kindly provided by Dr. Jerry Olefsky (University of California, San Diego) (25). The virus encoding myristoylated Akt was obtained from Dr. Kenneth Walsh (Tufts University) (26). The Gq, WT and Gq, Q209L adenoviruses have been characterized previously, and the Gq, Q209L adenovirus was subcloned into adenovirus from a plasmid obtained from Dr. John Exton (Vanderbilt University), by using our method published previously (8).

**Apoptosis Measurements—Oligonucleosomal DNA fragmentation** was analyzed by DNA laddering as described previously (12). Nuclear condensation was visualized using fluorescence microscopy after staining with Hoechst 33342 as described previously (12).

**Western Blot Analysis**—Cells were harvested in lysis buffer, and Bradford analysis was performed. Equal micrograms of protein (10–30 μg) were loaded onto SDS-PAGE, run, transferred to an Immobilon membrane, and the resulting blot probed using the following antibodies. The Ser-473 and Thr-308 phosphospecific antibodies for Akt, as well as the Pan-Akt antibody, were purchased from Cell Signaling Technologies. Anti-SHIP-2 was purchased from Santa Cruz Biotechnology, Inc. Anti-PTEN and anti-p85 were purchased from Upstate Biotechnology, Inc. Anti-phospho-PDK-1 antibody (Cell Signaling) was a kind gift from Dr. Stephanie Watts (Michigan State University). The phospho-PDK-1 antibody (Cell Signaling) was a kind gift from Dr. Alexandra Newton’s laboratory (University of California, San Diego).

**Measurement of [3H]PIP₃**—NRVMs plated at 4 × 10⁶ cells on 10-cm plates were labeled with 30 μCi/mL myo-[3H]inositol for 36 h, and then infected with control, Gq, WT, or Gq, Q209L adenoviruses overnight, and harvested 24 h later. Cells were extracted with 5% trichloroacetic acid, 5 mM EDTA, 5 mM phytic acid; the lysates were sonicated for 10 s, and resulting Ins(1,4,5)P₃ was assayed by competitive binding assay (BioTrak IP3 Assay protocol, Amersham Biosciences), as described by the supplier.

**Cell Fractionation Studies**—Fractionation was performed as described (29). Briefly, NRVMs were harvested in detergent-free lysis buffer (20 mM HEPES, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 40 μg/mL leupeptin, 1 mM sodium vanadate (NaVO₃)), frozen at −80 °C for 5 min, and then sonicated for 2 min in a bath sonicator. The resulting cytoplasmic fraction was cleared by ultracentrifugation at 100,000 × g for 30 min. After removing the supernatant (cytosolic fraction), the pellet was re-suspended in lysis buffer (50 mM Tris, 5 mM MgCl₂, 1 mM EGTA, 0.5% Nonidet P-40, 2.5% glycerol), sonicated for 2 min, and centrifuged again at 100,000 × g for 30 min. The resulting supernatant was analyzed as the membrane fraction, and the pellet as the insoluble fraction.

**Statistical Analysis**—All results are reported as mean ± S.E. Comparisons of two groups only were accomplished using unpaired Student’s t-test. Experiments with more than two groups were compared by one-way analysis of variance followed by the Tukey post-hoc test for comparison between groups.
RESULTS

Phospho-Akt Levels Are Diminished in GqQ209L-expressing NRVMs—Expression of a constitutively active mutant of Gqq (GqQ209L) induces apoptosis in NRVMs, whereas expression of the GqWT protein induces a sustained hypertrophy with no evidence of cell death (8). We hypothesized that this might reflect differential activation of protective pathways such as those initiated by phosphorylation of Akt. NRVMs were infected with viruses expressing GqWT, GqQ209L, or control virus backbone for 16 h followed by washing out of the viruses. Cells were harvested immediately after removal of the virus or after a further 8 or 24 h. All of these time points precede the development of apoptosis (36–48 h after overnight infection) (12) and exhibited similar results. As shown in Fig. 1, at 8 h after virus removal, GqWT expression caused a 3-fold increase in Akt phosphorylation (Fig 1, A and B) relative to control. Treating GqWT-expressing myocytes with PGF2α further enhance Ginger activity abolished this increase in Akt phosphorylation. Enhancing Gq activity even further, via expression of the constitutively active GqQ209L, resulted in a marked (45%) decrease in Akt phosphorylation, relative to control. Total Akt levels did not differ in GqWT or GqQ209L-expressing NRVMs versus control (Fig. 1A).

Akt Can Prevent GqQ209L-induced Apoptosis—The observation that levels of phospho-Akt were markedly decreased in GqQ209L-expressing cells suggested that the loss of this survival pathway could be a critical factor in the apoptotic response to GqQ209L. If this is so, then direct activation of Akt should prevent GqQ209L-induced apoptosis. To examine this possibility, NRVMs were coinfected with GqQ209L along with adenovirus expressing a constitutively activated Akt construct in which the c-Src myristoylation sequence was fused to the N terminus of Akt (myr-Akt). This membrane-targeted form of Akt has been reported to prevent serum deprivation-induced apoptosis in cultured cardiomyocytes and to reduce apoptosis caused by ischemia-reperfusion in vivo (15) or hypoxia in vitro (14).

Several independent indices of apoptosis were examined 36–48 h after infection to establish whether myr-Akt could protect the myocytes from GqQ209L-induced apoptosis. The characteristic laddering pattern resulting from oligonucleosomal DNA fragmentation is prominent in GqQ209L-expressing cells undergoing apoptosis (8), and this was reduced in cells in which myr-Akt was expressed along with GqQ209L (Fig. 2A). In addition, whereas disorganization of myofilaments is evident in cells expressing GqQ209L, the expression of myr-Akt along with GqQ209L preserved myofilament organization (Fig. 2B). Finally, Hoechst staining was used to quantitate the number of cells with fragmented nuclei following infection with GqQ209L, myrAkt, or both viruses together. Expression of myr-Akt reduced the number of fragmented nuclei in GqQ209L-infected cells by ~60% (Fig. 2C). The effect of enhancing Akt activity supports the notion that decreases in Akt activity play a central role in the development of GqQ209L-induced apoptosis.

LIF Does Not Prevent Apoptosis or Activate Akt in GqQ209L-expressing NRVMs—Cytokines that act via the gp130 receptor, including LIF and cardiostatin 1 (CT-1), induce PI3K-dependent Akt phosphorylation (21, 30) and can prevent apoptosis induced by serum deprivation in NRVMs (31). When NRVMs were placed in serum-free medium lacking supplementation with ITS, apoptosis ensued. LIF was able to prevent the development of apoptosis as assessed by DNA laddering (Fig. 3A, 5th and 6th lanes). In contrast, LIF failed to protect against GqQ209L-induced DNA laddering (Fig. 3A, 2nd to 4th lanes).

Because LIF may not be sufficiently stable to signal for the periods needed for protection from cell death, we added additional LIF to the GqQ209L-expressing cells, throughout the course of GqQ209L expression (data not shown). Even under these conditions, LIF failed to rescue the GqQ209L phenotype. In addition, whereas acute treatment with LIF markedly increased Akt phosphorylation in control NRVMs, it failed to do so in GqQ209L-expressing myocytes (Fig. 3B). The lack of Akt activation by LIF would appear to be responsible for the inability of this cytokine to prevent GqQ209L-expressing NRVMs from undergoing apoptosis.

Phosphatase Inhibition Does Not Restore Akt Phosphorylation in GqQ209L-expressing NRVMs—We considered the possibility that GqQ209L expression might diminish Akt phosphorylation by activating phosphatases, such as PP2A, that can dephosphorylate Akt (32). Calyculin, a phosphatase inhibitor with specificity for PP1 and PP2A, was used to test this possibility. In both control-infected and GqQ209L-expressing NRVMs pretreated for 30 min with calyculin, basal Akt phosphorylation was enhanced, demonstrating some endogenous control by phosphatases PP1 and PP2A. However, LIF was unable to increase Akt phosphorylation in GqQ209L-expressing cells even when these phosphatases were inhibited (Fig. 3C). This argues against enhanced dephosphorylation of Akt as the
mechanism by which GqQ209L expression limits Akt activation and cell survival.

**GqQ209L-expressing Cells Do Not Exhibit an Increase in PIP3**—Because it was not possible to stimulate Akt phosphorylation using the cytokine LIF, and a calyculin-sensitive phosphatase did not appear to be involved, we turned our focus upstream of Akt phosphorylation. Activation of Akt is often used as a surrogate for changes in PIP3 levels because Akt phosphorylation by PDK-1 is PIP3-dependent. We therefore asked whether there was diminished PIP3 production in GqQ209L-expressing cells. To our knowledge, there are no published reports of direct PIP3 measurements in cardiomyocytes, and PIP3 levels at rest are thought to be extremely low. To establish the feasibility of measuring [3H]PIP3 in NRVMs, we labeled cells (4 x 10^6 per plate) with 30 μCi/ml myo-[3H]inositol for 36 h and used an adenosivial vector to express a membrane-targeted, and thus constitutively active, PI3K catalytic subunit (p110caax). The wild type p110 subunit of PI3K (p110WT), which does not target to membranes, served as a control. [3H]Inositol phospholipids were extracted and quantified using HPLC as described under “Experimental Procedures.” Chromatographic analysis of extracts from cells expressing p110caax revealed a peak at the expected elution time of PIP3. The corresponding peak in p110WT-expressing cells was substantially smaller, confirming the identity of the peak as PIP3 (Fig. 4). We then examined [3H]PIP3 levels in cells infected with control, GqWT, and GqQ209L-expressing adenoviruses. HPLC analysis revealed significant levels of [3H]PIP3 in GqWT-expressing cells (Fig. 5). In contrast, PIP3 was barely detectable in cells expressing GqQ209L.

**PIP3-dependent Membrane Translocation Is Not Stimulated in GqQ209L-expressing Cardiomyocytes**—Akt phosphorylation, and thus activation, occurs when PDK-1 and Akt are colocalized at the membrane following PIP3 generation (33). The localization of these enzymes was therefore examined by fractionation and immunoblotting of cells to determine whether alterations in PIP3 formation led to diminished membrane associations. The fractionation method was verified by Western blot analysis using an antibody against the sodium/calcium exchanger, an ion transporter found on the plasma membrane (Fig. 6C). The pattern of Akt phosphorylation observed in membrane fractions was similar to that seen in whole cell lysates, with an increase in phospho-Akt observed in cells expressing GqWT, and less phosphorylated Akt at the membrane in cells expressing GqQ209L (Fig. 6A). The pattern of PDK-1 membrane localization was consistent with that of Akt, its substrate (Fig. 6A). When control-infected myocytes were stimulated with LIF for 90 s prior to fractionation, recruitment of both Akt and PDK-1 to the membrane fraction was observed (Fig. 6B). In contrast, but consistent with the inability of LIF to stimulate Akt phosphorylation in GqQ209L-expressing NRVMs, LIF treatment did not cause an increase in membrane-associated Akt or PDK-1 in cells expressing GqQ209L (Fig. 6B). These data imply that limited PIP3 formation in GqQ209L-expressing cells precludes recruitment of Akt and PDK-1 to the membrane for activation.

**PIP3 Regulatory Enzymes Are Not Altered with GqQ209L Expression**—We explored the possibility that the differential effects of GqWT and GqQ209L expression on PIP3 formation and Akt phosphorylation resulted from altered expression of PIP3K or PIP3 phosphatases. No differences in the p85, p110α, or p110β PIP3 subunits or in PTEN or SHIP2 expression were evident in control, GqWT, and GqQ209L-expressing cells based on immunoblotting experiments (Fig. 7). The only difference observed was an increase in PI3Kγ expression in GqQ209L-expressing cells, which would not explain the diminished PIP3...
levels. These findings do not support the concept that changes in expression of PI3Ks or PIP3 phosphatases are the basis for the altered PIP2 formation and Akt activation in GqWT-versus GqQ209L-expressing cells.

**PIP2 Is Depleted in Cells Expressing Constitutively Active Goq**—The substrate for PI3K is PIP2. The hydrolysis of PIP2 is directly stimulated by binding of the activated α subunit of Gq to one of the β isoforms of PLC (34, 35). We have shown previously that GqWT expression in cardiomyocytes activates PLC and that expression of the constitutively active GqQ209L elicits far greater inositol phosphate formation (8). This led us to consider the possibility that membrane PIP2 levels might be altered by sustained PLC activation in GqQ209L-expressing NRVMs, and that this might be limiting for PIP3 formation. Cardiomyocyte PIP2 levels were measured, at time points that preceded the incidence of apoptosis, in cells labeled with myo-[3H]inositol for 36 h and then infected with wild-type or GqQ209L adenovirus. After overnight infection with GqQ209L there was a marked decrease in [3H]PIP2 observed at all time points tested (Fig. 8, A and B). This same assay also revealed marked decreases in both [3H]PI and [3H]PIP with GqQ209L expression (data not shown), suggesting that the entire phospholipid pool was diminished in an attempt to replenish the diminished PIP2. Decreased PIP2 was also observed when this was measured by mass assay (Fig. 8C).

To explore further the relationship between Gq-mediated PLC activation, PIP2 hydrolysis, and Akt activation, we compared the effects of GqQ209L with those of a mutant GqQ209LDNE which retains constitutive activity due to diminished GTPase activity but has a reduced ability to activate PLC (36, 37). The extent of PLC activation by the adenovirally expressed mutant GqQ209LDNE was less than a third of that observed with GqQ209L (data not shown). Strikingly, PIP2 was not significantly decreased (Fig. 9A) by GqQ209LDNE expression, and Akt activation paralleled that seen with GqWT expression (Fig. 9B). Phenotypically, the mutant GqQ209LDNE causes cardiomyocyte hypertrophy without observable apoptosis, as seen with GqWT expression (data not shown). On the other hand, when Gq and PLC activation are enhanced by the addition of PGF2α to NRVMs expressing GqWT, cellular PIP2 levels are significantly reduced to ~43% of those in control
cardiomyocytes (data not shown), there is a concomitant decrease in Akt phosphorylation (Fig. 1), and these cells ultimately undergo apoptosis (12).

Akt Phosphorylation and PIP2 Levels Are Decreased in Gq Transgenic Mice—Mice with cardiac-specific GqWT overexpression develop significant hypertrophy associated with modest decreases in ventricular function (38). However, in the peripartum period (after giving birth) these mice develop a cardiomyopathy associated with significant amounts of cardiomyocyte apoptosis (8, 39). The changes seen in the peripartum period have been suggested to result from enhanced Gq activity and are similar to those induced by GqQ209L expression in cardiomyocytes. To determine whether our in vitro observations had a parallel in vivo, we examined Akt phosphorylation in ventricles from hearts isolated from mice 5 days postpartum.

Representative Western blots (Fig. 10A), and pooled data from densitometric analysis from 7 animals per group (Fig. 10B), revealed a significant decrease in Akt phosphorylation in ventricles from the transgenic mice. PIP2 mass was then assessed. Consistent with our data in isolated cardiomyocytes, a significant decrease in PIP2 mass was observed in Gq transgenic mouse hearts versus nontransgenic littermate control mouse hearts at 5 days postpartum (Fig. 10C).

**DISCUSSION**

The current study reveals dramatic differences in the level of signaling through the protective PI3K/Akt signaling pathway as a result of increasing Gq activity. In contrast to the ability of GqWT expression to increase PIP2 levels and Akt phosphorylation, GqQ209L expression results in decreased Akt phosphorylation and a reduced ability to generate PIP2. The ability of GqQ209L to depress PIP3 levels and Akt phosphorylation is associated with marked decreases in PIP2 and is paralleled by similar changes in Akt and PIP2 in an in vivo model in which hypertrophy transitions to heart failure.

Although the physiological significance of constitutive Gq activation could be questioned, we have also demonstrated that increasing Gq activity by stimulating myocytes expressing GqWT with PGF2α enhances PLC activity and results in cardiomyocyte apoptosis (12), whereas either stimulus alone induces hypertrophy. We show here that PIP2 and Akt phosphorylation also decrease under the same conditions. These data suggest that the increased level of PLC activation achieved under physiological conditions in which Gq expression is enhanced, and/or availability of Gqα-coupled receptor agonists is increased, may be sufficient to lead to diminished PIP2 and Akt phosphorylation. In this regard, and relevant to cardiac pathophysiology, it is noteworthy that up-regulation of the Gqα protein has been reported in the border zones of the myocardium after infarct (40), a paradigm in which there is also enhanced release of Gqα-coupled receptor agonists (41). In addition, increased expression of PLCβ3 has been reported to occur in a model of chronic heart failure (42). Furthermore, in two models of heart failure (the cardiomyopathic hamster and post-myocardial infarct), there is evidence for decreased PIP2 mass and decreased expression of phosphatidylinositol 4-phosphate 5-ki-
nase, the enzyme required to replete stores of PIP$_2$ (42, 43). We show here that in another model of heart failure, the peripartum cardiomyopathy seen in transgenic mice expressing Go$_q$, both PIP$_2$ levels and Akt phosphorylation are decreased compared with nontransgenic littermate controls. Therefore, significant reductions in PIP$_2$ are detectable in multiple models of cardiomyopathy, and our data are the first to suggest that pathophysiological levels of Go$_q$ signaling can lead to PIP$_2$ reduction and concomitant loss of the Akt survival pathway.

The relationship between decreased PIP$_2$ and loss of Akt phosphorylation has not been directly proven here. However, it is important to note that in vitro reductions in total cellular PIP$_2$ were evident at early time points following Go$_q$ expression, prior to the time when we observed appearance of cellular apoptotic markers (e.g., Hoechst staining of fragmented nuclear oligosomes, DNA fragmentation, and cytochrome c release) in our previous studies (12). Thus reduced lipid levels do not appear to be secondary to loss of membrane integrity associated with apoptosis. These data imply that reduced PIP$_2$ is an early event and a potentially causal factor in the apoptotic response following expression of activated Go$_q$. Furthermore, G$_q$Q209L and G$_q$WT plus PGF$_2$,$\alpha$ and in an in vivo model of Go$_q$-associated peripartum cardiomyopathy.

The finding that adrenoviral expression of myr-Akt was able to protect against G$_q$Q209L-induced apoptosis is consistent with the now well documented role of Akt in cardiomyocyte survival (15, 23). Interestingly, whereas expression of membrane-targeted Akt was able to prevent G$_q$Q209L-induced apoptosis, addition of LIF was ineffective. LIF, like CT-1, works for hormonal stimulation of PI3K-mediated PIP$_3$ formation and concomitantly loss of the Akt survival pathway.
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