Arachidonic acid and its metabolites have been previously implicated in the regulation of endothelial cell proliferation. Arachidonic acid may be liberated from cellular phospholipids by the action of group VIA calcium-independent phospholipase A2 (iPLA2-VIA). Consequently, we tested the hypothesis that iPLA2-VIA activity is linked to the regulation of endothelial cell proliferation. Inhibition of iPLA2 activity by bromoel lactone (BEL) was sufficient to entirely block endothelial cell growth. BEL dose-dependently inhibited endothelial cell DNA synthesis in a manner that was reversed upon the exogenous addition of arachidonic acid. DNA synthesis was inhibited by the S-isomer and not by the R-isomer of BEL, demonstrating that endothelial cell proliferation is mediated specifically by iPLA2-VIA. iPLA2-VIA activity was critical to the progression of endothelial cells through S phase and is required for the expression of the cyclin A/cdk2 complex. Thus, inhibition of iPLA2-VIA blocks S phase progression and results in exit from the cell cycle. Inhibition of iPLA2-VIA-mediated endothelial cell proliferation is sufficient to block angiogenic tubule formation in co-culture assays. Consequently, iPLA2-VIA is a novel regulator of endothelial cell S phase progression, cell cycle residence, and angiogenesis.

The endothelium lines the luminal surface of all blood vessels and is of critical importance to a variety of vascular processes, including the regulation of blood clotting and blood pressure (1). In addition, the endothelium plays a central role in angiogenesis, the process of new blood vessel formation from pre-existing vessels (2, 3). The growth of tumors is vitally dependent on the development of a neovascular supply by angiogenesis (2, 4) with antiangiogenic therapy being seen as one of the most promising strategies to restrict tumor growth and prevent metastasis (5). Upon the induction of angiogenesis, endothelial cells proliferate, migrate, and differentiate to form new vessels (2, 6). Thus, a promising and viable antiangiogenic route is inhibition of endothelial cell proliferation (5).
iPLA$_2$ and Endothelial Cell Proliferation

The iPLA$_2$ group of enzymes consists of two members in mammalian cells, iPLA$_2$-VIA and iPLA$_2$-VIB, of which iPLA$_2$-VIA is the best characterized. Here we report that iPLA$_2$-VIA but not iPLA$_2$-VIB is critical to endothelial cell proliferation, angiogenesis, and S phase progression. These findings shed light on the functional role of iPLA$_2$-VIA activity in proliferating endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—Affinity-purified goat polyclonal anti-iPLA$_2$ (T14) and rabbit polyclonal anti-Ki67 (H300) antibodies were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-PCNA (610664), anti-cyclin A (611268), and anti-cdk2 (610145) antibodies were purchased from BD Transduction Laboratories. Rabbit polyclonal anti-GRASP55 antibodies were provided by S. Ponnambalam (University of Leeds, UK). Horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce. Bromoelolactone (BEL) was purchased from Cayman Chemical. All other reagents were obtained from Sigma or Invitrogen unless otherwise stated.

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as described previously (7, 23). Human dermal microvascular endothelial cells (HDMECs) were purchased from Promocell. Cells were cultured in endothelial cell basal medium supplemented with the endothelial cell growth factor kit 2 (PromoCell) containing human recombinant epidermal growth factor (5 ng/ml), hydrocortisone (0.2 μg/ml), vascular endothelial growth factor (0.5 ng/ml), human recombinant basic-fibroblast growth factor (10 ng/ml), recombinant basic-fibroblast growth factor-1 (IGF-1, 20 ng/ml), ascorbic acid (1 μg/ml), heparin (22.5 μg/ml), amphotericin B (50 μg/ml), gentamicin (50 μg/ml), and 2% (v/v) fetal calf serum. All endothelial cell cultureware was coated with 0.1% (w/v) pig skin gelatin. Endothelial cells were not used in excess of three passages and expressed the characteristic endothelial markers von Willebrand factor and platelet-endothelial cell adhesion molecule-1. All cells were grown at 37 °C in a humid incubator containing 5% CO$_2$.

Cell Proliferation ELISA—HUVEC and HDMEC proliferation rates in the presence or absence of varying concentrations of BEL were compared using a 5-bromo-2’-deoxyuridine (BrdUrd) incorporation-based ELISA (Roche Diagnostics). For inhibitor studies, cells were seeded at 1 × 10$^4$ cells/well (0.55 × 10$^5$ cells/cm$^2$) in 96-well plates, and cells were grown for 24 h and then processed according to manufacturer’s instructions. For inhibitor studies, the BrdUrd incorporation period was fixed at 16 h. For BEL recovery studies, BrdUrd was incorporated for a period of 1 h at various time points after BEL washout.

iPLA$_2$ Activity Assay—The activity of endogenous iPLA$_2$ was determined using a modified cPLA$_2$ assay (Cayman Chemicals), as described previously (24, 25).

SDS-PAGE and Immunoblotting—Samples (20 μg of protein) were resolved for 60 min at 30 mA/gel on 10% SDS-PAGE mini-gels using a discontinuous buffer system (26). For immunoblotting, protein was transferred onto nitrocellulose mem-

branes for 3 h at 300 mA (27). Membranes were blocked in 5% (w/v) nonfat milk in phosphate-buffered saline for 30 min and then incubated overnight with primary antibody (1:500) at room temperature. For antigenic adsorption, antibodies were preincubated with blocking peptide (1:5 ratio of μg of antibody to μg of peptide) for at least 3 h prior to incubation with membranes overnight. After incubation with horseradish peroxidase-conjugated anti-goat IgG (1:3000) for 1 h, immunoreactive bands were visualized using a West Pico enhanced chemiluminescence (ECL) detection kit (Pierce). A Fujifilm Intelligent Dark Box II image reader using Fuji Las-1000 Pro software was used to capture images. Band intensities were determined densitometrically using Aida (Advanced Image Data Analyzer) 2.11 software.

Reverse Transcriptase-PCR—Total mRNA was isolated from proliferating HUVECs using TRizol reagent. 2 μg of total mRNA was then reverse-transcribed using Superscript II RNase H reverse transcriptase and then subjected to 36 cycles of PCR (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) with iPLA$_2$-VIA specific primers, as described previously (28). Primers used for PCR were: sense, 5’-AAGCTTTCCAGGC-CTCCC-3’; antisense, 5’-GAGGTCTCTCACCCTTGG-3’. PCR products were analyzed on 1% agarose gels.

Flow Cytometry—Subconfluent HUVECs were cultured for 16 h in the presence or absence of 5 μM BEL. Cells were then harvested, fixed in 70% ice-cold ethanol, and incubated with propidium iodide (50 μg/ml) and RNase A (20 μg/ml) for 3 h. Propidium iodide-stained DNA content was then assessed using a FACSCalibur flow cytometer (BD Biosciences), and the percentage of cells in each phase of the cell cycle was analyzed using ModFit software (Verity Software House).

RNA Interference—HUVECs were transfected with no siRNA (control), 50 nm non-targeting control siRNA (mock-transfected; D-001210-01; Dharmacon), or 50 nm annealed iPLA$_2$-VIA siRNA (siRNA; 139141; Ambion) for 4 h using the Lipofectamine2000 transfection reagent (Invitrogen).

Angiogenesis Assay—Co-cultures of HUVECs seeded on a bed of human fibroblasts (TCS Cellworks) were cultured for 7 days in the presence or absence of 5 μM BEL. Tubules were then fixed and stained for PECA-1. Stained tubules were then imaged by phase contrast microscopy, and tubule length was quantified using NIH Image J software.

RESULTS

BEL Inhibits Endothelial Cell Growth—BEL is a catalytic site-directed inhibitor of iPLA$_2$ activity (29, 30). To assess the effects of BEL treatment on endothelial cell growth, HUVECs were seeded at a density of 3 × 10$^5$ cells/cm$^2$ and were allowed to settle for 24 h. HUVECs were then maintained in normal growth medium for a period of 7 days in the presence or absence of 5 μM BEL (Fig. 1A). At 24-h intervals, cells were harvested for determination of cell numbers. As expected, when grown in the absence of BEL, cell numbers increased as a function of time. After 7 days in culture, cell numbers had increased 18.5-fold to reach 55.4 ± 4.5 × 10$^5$ cells/cm$^2$. However, when HUVECs were cultured in the presence of BEL, growth was entirely blocked. Upon incubation with BEL, no significant increase in cell density was observed at any time.
point relative to the previous time point. Thus, in the presence of BEL, HUVEC density remained constant throughout the growth period. Phenotypic differences have been identified between endothelial cells derived from large vessels, such as HUVECs, and endothelial cells derived from the microvasculature (6, 31); however, similar results were seen upon the incubation of HDMECs with 5 \( \mu \)M BEL (Fig. 1B). Inhibition of HUVEC and HDMEC growth was not a consequence of enhanced cell death as incubation with 5 \( \mu \)M BEL for 7 days had no effect on cell viability, as assessed by trypan blue exclusion (data not shown). Furthermore, incubation with 5 \( \mu \)M BEL had no effect on HUVEC or HDMEC morphology (Fig. 1C). Therefore, BEL-mediated reduction of growth was not due to cellular toxicity. HUVECs grown for 5 days in the presence of 5 \( \mu \)M BEL recovered from 4.6 ± 0.6 \( \times \) 10³ to 96 ± 3.6 \( \times \) 10³ cells/cm² upon inhibitor washout after recovery for 8 days (Fig. 1D). Thus, the inhibitory effect of BEL on endothelial cell growth was also fully reversible.

<iPLA_2> Activity Mediates Endothelial Cell DNA Synthesis—</i>

The effects of BEL on HUVEC DNA synthesis was assessed by determining BrdUrd incorporation into cellular DNA in the presence of varying concentrations of BEL (Fig. 2A). HUVECs, seeded at a density of 0.55 \( \times \) 10³ cells/cm², were cultured for 24 h and then allowed to incorporate BrdUrd for 16 h in the presence or absence of inhibitor. BEL acted in a dose-dependent manner, inhibiting HUVEC proliferation by 18.9 ± 6, 73.1 ± 2.3, and 91.5 ± 0.7% at concentrations of 1, 5, and 10 \( \mu \)M, respectively. Similarly, upon incubation with 1, 5 and 10 \( \mu \)M BEL, HDMEC proliferation was inhibited by 25.3 ± 3.7, 53.5 ± 3.7, and 93.4 ± 1.2% (Fig. 2B). Although BEL selectively targets iPLA₂ over other PLA₂s (29, 30), it may also inhibit other cellular enzymes. Despite this, the exogenous addition of 100 nM AA...
significantly attenuated the antiproliferative effect of 5 μM BEL (Fig. 2C), suggesting that iPLA₂-mediated AA release is involved in the control of endothelial cell DNA synthesis. However, the exogenous addition of other downstream products of iPLA₂ activity (lysophosphatidyl choline and diacylglycerol) was not able to recover the inhibitory effects of BEL.

Recovery of iPLA₂ Activity Is Correlated with the Induction of HUVEC Proliferation—HUVECs seeded at a density of 0.55 × 10⁵ cells/cm² were cultured in the presence of 5 μM BEL for 5 days prior to inhibitor washout and recovery. HUVEC DNA synthesis was then assessed at varying time points after inhibitor washout by determining BrdUrd incorporation into cellular DNA (Fig. 3A). No significant increase in HUVEC DNA synthesis was observed up to 8 h after recovery. However, after 16 h of recovery, HUVEC BrdUrd incorporation was elevated 5.9-fold relative to 0 h after recovery.

To establish whether the recovery of iPLA₂ activity is involved in the recovery of HUVEC proliferation, iPLA₂ activity was assessed at varying times after BEL washout (Fig. 3B). Similarly, no significant increase in iPLA₂ activity was observed up to 8 h after inhibitor washout. Furthermore, in correlation with BrdUrd incorporation, there was a significant increase in iPLA₂ activity after 16 h of recovery. These findings suggested that iPLA₂ activity is indeed vital to the proliferation of endothelial cells.

**iPLA₂-VIA but Not iPLA₂-VIB Mediates Endothelial Cell DNA Synthesis**—Due to its ability to inhibit both iPLA₂-VIA and iPLA₂-VIB, it is difficult to distinguish the effects of BEL on each family member. However, separation of BEL into its two enantiomers reveals that the S-isofom of BEL is significantly more potent toward iPLA₂-VIA than iPLA₂-VIB (32). In contrast, the R-isofom is considerably more effective at inhibiting iPLA₂-VIB than iPLA₂-VIA (32). Thus, the enantiomers of BEL can be used to attribute the effects of BEL to either iPLA₂-VIA or iPLA₂-VIB. The effects of 10 μM S-BEL and 10 μM R-BEL on HUVEC proliferation were assessed by determining BrdUrd incorporation over 16 h (Fig. 4A). In the presence of S-BEL, HUVEC proliferation was inhibited by 72.9 ± 3.3%, whereas R-BEL had no significant effect on cellular proliferation. Consequently, the antiproliferative effects of BEL are due to its inhibition of iPLA₂-VIA and not iPLA₂-VIB. The expression of
iPLA₂-VIA in endothelial cells was confirmed upon detection of iPLA₂-VIA protein and mRNA in HUVECs by Western blotting and RT-PCR, respectively (Fig. 4, B and C). Western blotting using a well characterized affinity-purified antibody directed toward the C-terminal region of iPLA₂-VIA (33) detected an immunoreactive band of 80 kDa corresponding to iPLA₂-VIA. In addition, iPLA₂-VIA immunoreactivity could be eliminated upon preabsorption of antibody with antigenic peptide (Fig. 4B, T-14). Furthermore, as predicted, a 218-bp RT-PCR product was obtained from HUVEC mRNA using primers specific to sequences in the C terminus of iPLA₂-VIA (Fig. 4C). PCR of total mRNA without a prior RT step did not produce any product, excluding the possibility of the amplification of genomic DNA (data not shown). The identity of this RT-PCR product was confirmed upon restriction endonuclease digestion (HhaI) to produce fragments of 101 and 117 kb as expected (Fig. 7.2 and 46). Thus, we decided to further investigate the role of iPLA₂-VIA in the control of the endothelial cell cycle.

**iPLA₂-VIA Mediates Cell Cycle Residence**—The role of iPLA₂-VIA in cell cycle progression was assessed by investigating the cell cycle distribution of HUVECs by flow cytometry. HUVECs were cultured for 16 h in the presence or absence of 5 μM BEL and then stained with propidium iodide prior to flow cytometry. In the presence of BEL, the number of cells residing in the S phase of the cell cycle was reduced by 71.3% (Fig. 5A). As a consequence, significantly more cells resided in the G₀–G₁ phases of the cell cycle, suggesting that iPLA₂-VIA plays a key role in G₁ to S phase progression. This was further reinforced upon analysis of Ki67 expression levels in response to iPLA₂-VIA inhibition. Ki67 is a nuclear protein expressed in the G₁, S, G₂, and mitotic phases of the cell cycle but not in the G₀ phase of quiescence (34, 35). Incubation with BEL significantly reduced Ki67 expression in HUVECs (Fig. 5B), indicating that fewer cells resided in the cell cycle. Thus, iPLA₂-VIA activity mediates endothelial cell cycle residence.

**iPLA₂-VIA Mediates S Phase Progression**—Transition from G₁ to S phase is dependent on the assembly and activation of the DNA replication complex and initiation of DNA synthesis. The replication clamp proliferating cell nuclear antigen (PCNA) is a vital component of the replication complex. PCNA expression is low throughout the cell cycle until early S phase and initiation of replication clamp formation (36, 37). Consequently, S phase entry and replication clamp formation can be monitored by assessing the cellular levels of PCNA. Upon incubation for 16 h with 5 μM BEL, the cellular levels of PCNA were unaffected (Fig. 6, A and B), suggesting that in the presence of BEL, endothelial cells still enter S phase; however, cells are unable to progress through S phase as DNA synthesis is inhibited upon iPLA₂-VIA inhibition. The activation of preassembled replication complexes and initiation of DNA synthesis is mediated by the cyclin A/cdk2 complex in early S phase. Mammalian cells cannot synthesize DNA or progress through S phase in the absence of cyclin A/cdk2 activity (38, 39). Inhibition of iPLA₂-VIA significantly reduced the expression of endothelial cell cyclin A/cdk2 as assessed by Western blotting (Fig. 6A). Analysis of immunoreactive band intensities revealed that incubation with 5 μM BEL reduced cyclin A and cdk2 expression levels to 34.3 ± 7.2 and 46 ± 4.8% of controls, respectively (Fig. 6B).

Reduced cyclin A/cdk2 expression but unaffected PCNA expression are consistent with a block in early S phase and account for the block in G₁-S phase progression and inhibition of DNA synthesis seen previously. Thus, iPLA₂-VIA activity may play a critical role in endothelial cell cycle regulation by mediating S phase progression and the initiation of DNA synthesis.

**Inhibition of iPLA₂-VIA Prior to S Phase Entry Blocks HUVEC Cell Cycle Progression**—The previous data suggested that iPLA₂-VIA mediates endothelial cell S phase progression and the expression of cyclin A/cdk2. However, these effects may be an indirect effect due to a lack of progression from G₀. Thus, the effects of iPLA₂-VIA inhibition were assessed at different points in the endothelial cell cycle (Fig. 7). HUVECs were grown to confluence to synchronize cells in G₀ (40). HUVECs were then harvested and seeded at a lower density (1 in 4) to induce proliferation. At varying time points after seeding, HUVECs were lysed, and the levels of cyclin A expression were analyzed by Western blotting (Fig. 7A). As...
ern blotting. The addition of BEL up to 12 h after seeding (before S phase entry) almost completely blocked the induction of cyclin A expression relative to control cells. However, the addition of BEL to cells after 15, 18, and 21 h of growth (after S phase entry) had no effect on the induction of cyclin A expression. Thus, iPLA$_2$-VIA activity in early S phase is critical to endothelial cell S phase progression.

**siRNA-mediated Knockdown of Endogenous iPLA$_2$-VIA**—The previous data are consistent with a vital role for iPLA$_2$-VIA in the regulation of endothelial cell cycle progression. Despite this, the use of BEL to inhibit iPLA$_2$-VIA activity is also known to inhibit a number of other enzyme activities (41). Thus, the effects of BEL on cell proliferation could possibly be due to inhibition of these and not inhibition of iPLA$_2$-VIA. To exclude this possibility, iPLA$_2$-VIA-targeted siRNA was used to corroborate the previous pharmacological studies. Consistent with the inhibitor studies, knockdown of endogenous iPLA$_2$-VIA significantly reduced the levels of cyclin A and cdk2 expression to 3 and 22% of non-targeting siRNA controls (mock), respectively (Fig. 8, A and B). Consequently, the siRNA-mediated knockdown of endogenous iPLA$_2$-VIA was sufficient to almost completely inhibit HUVEC proliferation (98% inhibition relative to mock; Fig. 8C). Thus, iPLA$_2$-VIA plays a vital role in the control of endothelial cell S phase progression and proliferation.

**iPLA$_2$-VIA Activity Is Critical to Angiogenesis**—Upon the induction of angiogenesis, endothelial cells proliferate, migrate, and differentiate to form new blood vessels. Consequently, the induction of endothelial cell proliferation is central to the angiogenic response. As iPLA$_2$-VIA mediates endothelial cell proliferation, we hypothesized that its activity may be essential to the angiogenic process. Endothelial cell angiogenesis can be assessed using co-culture assays. In these assays, HUVECs are seeded on a bed of human dermal fibroblasts and cultured for 7 days. Under these conditions, endothelial cells form tubules with patent lumens, reminiscent of mature capillaries (6). Incubation with BEL reduced tubule length to 59.1 ± 2.9 and 30 ± 3.3% of controls at 5 and 10 μM, respectively (Fig. 9, A and B). Thus, iPLA$_2$-VIA-mediated endothelial cell proliferation plays a central role in the formation of new blood capillaries by angiogenesis in vitro assays.

**DISCUSSION**

In this study, we define a novel role for iPLA$_2$-VIA in the regulation of endothelial cell S phase progression and angiogenesis. iPLA$_2$-VIA has previously been implicated in the regulation of the proliferation of a variety of cell types (33, 41–47). Despite this, the mechanism of the regulation of cellular proliferation by iPLA$_2$-VIA remains undefined. Studies with Jurkat T-cells and CHO-K1 cells suggest that in these cell types, iPLA$_2$-VIA activity mediates cell cycle progression and proliferation by regulating glycerophospholipid metabolism throughout the cell cycle. Contrary to this model, a recent study documents that stable suppression of iPLA$_2$-VIA in
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INS-1 β-cells has no effect on cellular lipid content or composition but still results in inhibition of cellular proliferation (41). In addition, overexpression of iPLA₂-VIA in INS-1 β-cell results in elevated proliferation (44), consistent with a role for iPLA₂-VIA in the mitogenic signaling of INS-1 β-cells but not in phospholipid remodeling. Similarly, a role for iPLA₂-VIA in the mitogenic signaling of thrombin-stimulated vascular smooth muscle cells was recently proposed (42). Our results complement these latest findings by documenting that endothelial cell proliferation and S phase progression are dependent on iPLA₂-VIA-mediated AA release. Thus, it is an emerging concept that iPLA₂-VIA plays a central role in the mitogenic signaling of a variety of cell types.

Although previous studies have suggested an involvement of iPLA₂-VIA in proliferation, our study is the first to define the role of iPLA₂-VIA in the regulation of cellular proliferation. Here we show that iPLA₂-VIA mediates the progression of endothelial cells through S phase. Thus, inhibition of iPLA₂-VIA blocks the initiation of DNA synthesis and results in exit from the cell cycle. This is consistent with observations in Jurkat T-cells showing that iPLA₂-VIA activity is a requirement for exit from the G₀ phase of quiescence (43). Furthermore, in studies that have analyzed the activity of iPLA₂-VIA activity throughout the cell cycle, it appears that its activity increases at the beginning of S phase to reach a peak at G₂/M (33, 43). An up-regulation of iPLA₂-VIA activity in early S phase may be responsible for promoting S phase progression and the subsequent induction of cyclin A/cdk2 activity required for the initiation of DNA synthesis. An early event in the induction of angiogenesis is the entry of quiescent endothelial cells back into the cell cycle. Consequently, the regulation of endothelial cell proliferation and cell cycle progression is a key component of the angiogenic response (2). We find that inhibition of iPLA₂-VIA-mediated endothelial cell proliferation is sufficient to block angiogenic tubule formation in co-culture assays. Thus, inhibition of iPLA₂-VIA activity may represent a viable antiangiogenic route for the treatment of cancer. The potential of iPLA₂-VIA as a therapeutic target without adverse side effects is reinforced by the fact that the only reported phenotypic defect in iPLA₂-VIA knock-out mice is the production of spermatozoa with impaired motility (48).

We have previously implicated cPLA₂α-mediated AA release in the regulation of endothelial cell proliferation (7). Consequently, it appears that there are at least two distinct AA mobi-
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lization mechanisms, which are under the regulation of iPLA₂-VIA and cPLA₂α, in endothelial cells. Furthermore, inhibition of cPLA₂α blocks S phase progression and results in the down-regulation of cyclin A/cdk2 expression, suggesting that cPLA₂α and iPLA₂-VIA play cooperative roles in regulating endothelial cell proliferation. Similarly, a potential cooperative role for both cPLA₂α and iPLA₂-VIA was previously suggested for the control of A549 cell proliferation (47). Thus, it is possible that basal iPLA₂-VIA activity or stimulated cPLA₂α activity alone are insufficient to promote S phase progression and that DNA synthesis can only proceed upon the combined activation of iPLA₂-VIA and cPLA₂α. Alternatively, it may be that both iPLA₂-VIA and cPLA₂α mediate S phase progression by differing downstream mechanisms. To fully understand the roles of iPLA₂-VIA and cPLA₂α in cellular proliferation, the next challenges will include elucidation of the specific downstream metabolites of AA involved in both iPLA₂-VIA-mediated and cPLA₂α-mediated cell proliferation.

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REFERENCES

1. Vane, J. R., Anggard, E. E., and Botting, R. M. (1990) N. Engl. J. Med. 323, 27–36
2. Carmeliet, P. (2000) Nat. Med. 6, 389–395
3. Carmeliet, P. (2005) Nature 438, 932–936
4. Folkman, J., and Shing, Y. (1992) J. Biol. Chem. 267, 10931–10934
5. Ferrara, N., and Kerbal, R. S. (2000) J. Biol. Chem. 275, 28802–28807
6. Staton, C. A., Stribbling, S. M., Tazzyman, S., Hughes, R., Brown, N. J., and Munaron, L. (2003) J. Cell. Physiol. 197, 370–378
7. Sa, G., Murugesan, G., Jaye, M., Ishvashchenko, Y., and Fox, P. L. (1995) J. Biol. Chem. 270, 2360–2366
8. Antoniotti, S., Fiorio Pla, A., Pregnolato, S., Mottola, A., Lovisolo, D., and Munaron, L. (2003) J. Cell. Physiol. 197, 370–378
9. Fafeur, V., Jiang, Z. P., and Bohlen, P. (1991) J. Cell. Physiol. 149, 277–283
10. Santer, B., and McDonald, D. (1992) J. Biol. Chem. 267, 1174–1178
11. Rickard, A., Portell, C., Kell, P. J., Vinson, S. M., and McHowat, J. (2005) Am. J. Physiol. Cell Physiol. 288, F714–F721
12. McHowat, J., Kell, P. J., O’Neill, H. B., and Creer, M. H. (2001) Biochemistry 40, 14921–14931
13. Evans, J. H., Spencer, D. M., Zweifach, A., and Leslie, C. C. (2001) J. Biol. Chem. 276, 30150–30160
14. Hirabayashi, T., Murayama, T., and Shimizu, T. (2004) Biol. Pharm. Bull. 27, 1168–1173
15. Nowatzke, W., Ramanadham, S., Ma, M., Hsu, F. F., Bohrer, A., and Turk, J. (1998) Endocrinology 139, 4073–4085
16. Howell, G. J., Herbert, S. P., Mitchell, A., Ed., Luan, C., Mohamed, M., Hunter, A. R., Simpson, N., Turner, A., Zachary, I., Walker, J. H., and Ponnambalam, S. (2004) Mol. Membr. Biol. 21, 413–421
17. Smani, T., Zakharov, S. I., Csutora, P., Leno, E., Trepakova, E. S., and Bolotina, V. M. (2004) Nat. Cell Biol. 6, 113–120
18. Smani, T., Zakharov, S. I., Leno, E., Csutora, P., Treepakova, E., and Bolotina, V. M. (2003) J. Biol. Chem. 278, 11909–11915
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
21. Larsson, P. K., Claesson, H. E., and Kennedy, B. (1998) J. Biol. Chem. 273, 207–214
22. Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) J. Biol. Chem. 270, 445–450
23. Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P., and Gross, R. W. (1991) J. Biol. Chem. 266, 7227–7232
24. Jackson, C. J., and Nguyen, M. (1997) Int. J. Biochem. Cell Biol. 29, 1167–1177
25. Jenkins, C. M., Han, X., Mancuso, D. J., and Gross, R. W. (2002) J. Biol. Chem. 277, 32807–32814
26. Manguikian, A. D., and Barbour, S. E. (2004) J. Biol. Chem. 279, 52881–52892
27. Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U., and Stein, H. (1984) J. Immunol. 133, 1710–1715
28. Schluter, C., Duchrow, M., Wohlenberg, C., Becker, M. H., Key, G., Flad, H. D., and Gerdes, J. (1993) J. Cell Biol. 123, 513–522
29. Takahashi, T., and Caviness, V. S. (1993) J. Neurocytol. 22, 1096–1102
30. Paunesku, T., Mittal, S., Pott, M., Oryhon, J., Korolev, S. V., Joachimiak, A., and Woloschak, G. E. (2001) Int. J. Radiat. Biol. 77, 1007–1021
31. Coverley, D., Laman, H., and Laskey, R. A. (2002) Nat. Cell Biol. 4, 523–528
32. Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N. J. (1991) Cell 67, 1169–1179
33. Okada, T., Lopez-Lago, M., and Giancotti, F. G. (2003) J. Cell Biol. 171, 361–371
34. Bai, S., Bohrer, A., Ramanadham, S., Jin, W., Zhang, S., and Turk, J. (2006) J. Biol. Chem. 281, 187–198
35. Yellaturu, C. R., and Rao, G. N. (2003) J. Biol. Chem. 278, 43831–43837
36. Roshak, A. K., Capper, E. A., Stevenson, C., Eichman, C., and Marshall, L. A. (2000) J. Biol. Chem. 275, 35692–35698
37. Ma, Z., Ramanadham, S., Wohltmann, M., Bohrer, A., Hsu, F. F., and Turk, J. (2001) Lipoic Acid 36, 689–700
38. Sanchez, T., and Moreno, J. J. (2001) J. Cell. Physiol. 193, 293–298
39. Sanchez, T., and Moreno, J. J. (2001) Biochem. Pharmacol. 61, 811–816
40. Choudhury, Q. G., McKay, D. T., Flower, R. J., and Croxall, J. D. (2000) Br. J. Pharmacol. 131, 255–265
41. Bao, S., Miller, J. R., Ma, Z., Wohltmann, M., Eng, G., Ramanadham, S., Molley, K., and Turk, J. (2004) J. Biol. Chem. 279, 38194–38200

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