Membrane Depolarization Induces the Undulating Phosphorylation/Dephosphorylation of Glycogen Synthase Kinase 3β, and This Dephosphorylation Involves Protein Phosphatases 2A and 2B in SH-SY5Y Human Neuroblastoma Cells*

Received for publication, December 13, 2004, and in revised form, March 25, 2005 Published, JBC Papers in Press, March 30, 2005, DOI 10.1074/jbc.M413987200

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Changes in plasma membrane electrical potential evoke signals that regulate the expressions of various genes in the nervous system. However, the role of glycogen synthase kinase 3β (GSK-3β) in this process has not been elucidated. Thus, this study was performed to examine whether membrane depolarization can regulate the phosphorylation of GSK-3β and to identify the molecular mechanisms involved in this regulation. The depolarization by treating with 100 mM KCl for 5 min resulted in the undulating phosphorylation of GSK-3β at Ser-9 in SH-SY5Y human neuroblastoma cells, in H19–7/IGF-IR rat embryonic hippocampal cells, and in PC12 rat pheochromocytoma cells, but not in A172 human glioblastoma cells. Cellular β-catenin contents showed a temporal pattern similar to that of the Ser-9 phosphorylation of GSK-3β. Treatment with wortmannin or calphostin C or the expression of dominant negative Akt inhibited phosphorylation of GSK-3β at Ser-9 following the KCl-induced depolarization of SH-SY5Y cells. Moreover, pretreatment with okadaic acid or cyclosporin A blocked the dephosphorylation of GSK-3β at Ser-9 at 0, 15, and 30 min after KCl-induced depolarization, and the activity of protein phosphatases (PP) 2A and 2B increased at these times. Treatment with nifedipine or calcium-free medium inhibited GSK-3β dephosphorylation following membrane depolarization, and the amounts of co-immunoprecipitated GSK-3β and PP2A changed in parallel with GSK-3β dephosphorylation. Our study demonstrated that KCl-induced depolarization caused undulating GSK-3β phosphorylation/dephosphorylation, which was regulated for the most part by phosphatidylinositol 3-kinase and Akt (phosphorylation) and PP2A and PP2B (dephosphorylation), respectively.

* This work was supported by Grant R01-2002-000-00144-0 from the basic research program of the Korea Science and Engineering Foundation, by Grant M1-0108-00-0082 from the Korean Ministry of Science and Technology, by Grant 03-2002-020 from the Seoul National University Hospital Research Fund, and by the 2004 BK21 Project for Medicine, Dentistry, and Pharmacy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Glycogen synthase kinase 3 (GSK-3)1 was originally identified as an enzyme that phosphorylates glycogen synthase, the rate-limiting enzyme in glycogen biosynthesis. Subsequent studies have shown that GSK-3 phosphorylates a broad range of substrates, including several transcription factors such as c-Myc, c-Jun, and β-catenin (1). Thus, GSK-3 has been implicated in many fundamental biological processes, which include cell fate determination, metabolism, transcriptional control, and oncogenesis (2). GSK-3 also plays crucial roles in many major signaling processes that are involved in key brain functions and has been associated with several dysfunctions of the central nervous system. For example, the dysregulated activity of β isoform of GSK-3 (GSK-3β) has been implicated in certain psychiatric diseases like bipolar mood disorder and neurodegenerative diseases such as Alzheimer disease. Moreover, several mood-stabilizing drugs such as lithium and valproate are known to inhibit GSK-3β activity, which supports the idea that GSK-3β activity modulation may have a therapeutic benefit in those with behavioral conditions in addition to its known benefits in the treatment of Alzheimer disease (3).

GSK-3β is subject to multiple regulatory mechanisms so that it can phosphorylate diverse substrates that are involved in a myriad of cellular responses. The phosphorylation of GSK-3β is the most widely studied regulatory mechanism, but protein complex formation and intracellular localization also have important regulatory influences on GSK-3β activity (3). The activity of GSK-3β can be reduced by the phosphorylation of Ser-9 (1), and several kinases have been found to be capable of mediating this modification, e.g. p70 S6 kinase, p90 Rsk (also called MAPKAP kinase-1), Akt (also called protein kinase B), certain isoforms of protein kinase C, and cyclic AMP-dependent protein kinase (protein kinase A). Moreover, much research has been directed toward identifying the receptor-coupled signaling systems used by each of these kinases to control GSK-3β activity (3). However, in stark contrast to the many kinases known to be involved in the phosphorylation of GSK-3β at Ser-9, protein phosphatase 2A (PP2A) is the only known protein phosphatase that can directly dephosphorylate GSK-3β (4).

Changes in the electrical potential of the plasma membrane are accompanied by synaptic transmission and axonal conduc-

1 The abbreviations used are: GSK-3, glycogen synthase kinase-3; GSK-3β, glycogen synthase kinase 3β; APC, adenomatous polyposis coli gene product; EGFR, epidermal growth factor receptor; PD, post-depolarization; PISK, phosphatidylinositol 3-kinase; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B (also called calcineurin); DMEM, Dulbecco’s modified Eagle’s medium.
Regulation of GSK-3β Phosphorylation by Depolarization

KCL-induced Depolarization Induced the Undulating Phosphorylation of GSK-3β at Ser-9 in Neuroblastoma Cells and Rat Hippocampal Cells—We examined first whether KCl-induced depolarization could regulate GSK-3β phosphorylation in SH-SY5Y cells. The depolarization of SH-SY5Y cells caused by treating them with 100 mM KCl for 5 min resulted in the undulating phosphorylation of GSK-3β at Ser-9. Untreated control cells showed strong basal phosphorylation at Ser-9, which was drastically decreased at 0 min (i.e. after 5 min of depolarization). Ser-9 phosphorylation increased to reach a peak 1.5-fold that of the control at 13 min after the termination of depolarization (post-depolarization (PD)), which was followed by a dephosphorylation at 15 min PD. However, another phosphorylation/dephosphorylation cycle was observed between 15 and 50 min PD with maximal phosphorylation at 21 min PD (Fig. 1, A and B). The phosphorylation of GSK-3β at Ser-9 at 15 min was significantly less than that at 13 and 18 min PD, respectively (p < 0.05, Mann-Whitney U test). In contrast, the phosphorylation of Tyr-216 and the total amount of GSK-3β did not show any significant change post-depolarization. This result indicates that KCl-induced depolarization can induce the undulating phosphorylation of GSK-3β at Ser-9, suggesting that membrane depolarization might regulate the activity of GSK-3β. Two bands of GSK-3β were observed in the Western blots of SH-SY5Y cells, which corresponded to the alternative spliced forms of the enzyme (8).

To examine whether the undulating regulation of GSK-3β phosphorylation by membrane depolarization is specific to neuroblastoma. This study was designed to determine whether membrane depolarization can regulate GSK-3β phosphorylation and, if so, to elucidate the regulatory mechanism responsible. We found that KCl-induced depolarization induced the undulating phosphorylation of GSK-3β and that the protein phosphatases PP2A and PP2B, as well as phosphatidylinositol 3-kinase (PI3K) and Akt, are involved in the regulation of GSK-3β phosphorylation in SH-SY5Y human neuroblastoma cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—SH-SY5Y human neuroblastoma cells and H19–7/1GF-IR rat embryonal hippocampal cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and PC12 rat pheochromocytoma cells and A1272 human glioblastoma cells were from the Korean Cell Line Bank. SH-SY5Y cells, PC12 cells, and A1273 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (JBL, Korea) and 100 units/ml penicillin/streptomycin, in a CO₂ incubator at 37 °C. H19–7/1GF-IR cells were grown in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 200 μg/ml G418. Cells were washed and incubated in fresh DMEM overnight before depolarization. Membrane depolarization was induced by treating the cells with 100 mM KCl in complete medium for 5 min PD, as was followed by immediate wash with fresh medium, and then fresh medium (containing drugs as indicated) was added. KCl and EDTA were purchased from Sigma, cyclosporin A, and bovine serum albumin, 100 μM phosphoprotein substrate (amino acid sequence: RRA[pT]VA), and 5–10 μg of sample protein. The enzyme reaction was started by adding the sample and allowed to progress for 30 min at 37 °C. The reaction was terminated by adding molybdate dye solution, and color was developed by incubating the mixture for 30 min at room temperature. The standard curve was prepared using inorganic phosphate at concentrations ranging from 10 to 200 μM. Absorbance at 660 nm of the solution of phosphate released was calculated using the standard curve.

RESULTS

KCl-induced Depolarization Induced the Undulating Phosphorylation of GSK-3β at Ser-9 in Neuroblastoma Cells and Rat Hippocampal Cells—We examined first whether KCl-induced depolarization could regulate GSK-3β phosphorylation in SH-SY5Y cells. The depolarization of SH-SY5Y cells caused by treating them with 100 mM KCl for 5 min resulted in the undulating phosphorylation of GSK-3β at Ser-9. Untreated control cells showed strong basal phosphorylation at Ser-9, which was drastically decreased at 0 min (i.e. after 5 min of depolarization). Ser-9 phosphorylation increased to reach a peak 1.5-fold that of the control at 13 min after the termination of depolarization (post-depolarization (PD)), which was followed by a dephosphorylation at 15 min PD. However, another phosphorylation/dephosphorylation cycle was observed between 15 and 50 min PD with maximal phosphorylation at 21 min PD (Fig. 1, A and B). The phosphorylation of GSK-3β at Ser-9 at 15 min was significantly less than that at 13 and 18 min PD, respectively (p < 0.05, Mann-Whitney U test). In contrast, the phosphorylation of Tyr-216 and the total amount of GSK-3β did not show any significant change post-depolarization. This result indicates that KCl-induced depolarization can induce the undulating phosphorylation of GSK-3β at Ser-9, suggesting that membrane depolarization might regulate the activity of GSK-3β. Two bands of GSK-3β were observed in the Western blots of SH-SY5Y cells, which corresponded to the alternative spliced forms of the enzyme (8).

To examine whether the undulating regulation of GSK-3β phosphorylation by membrane depolarization is specific to neu-
rons, the effect of KCl-induced depolarization on the phosphorylation of GSK-3β at Ser-9 was also analyzed in H19–7/IGF-IR rat embryonic hippocampal cells, PC12 rat pheochromocytoma cells, and in A172 human glioblastoma cells. Depolarization by KCl resulted in the undulating phosphorylation of GSK-3β at Ser-9 in H19–7/IGF-IR cells and in PC12 cells, without changing GSK-3β expression, and the time course of this undulating phosphorylation was similar to that observed in SH-SY5Y cells (Fig. 1C). In contrast, the same depolarization did not change the phosphorylation status of GSK-3β at Ser-9 in A172 glioblastoma cells. This result suggests that KCl-induced depolarization can result in the undulating phosphorylation of GSK3β at Ser-9 only in excitable neuronal cells.

To examine whether the undulating phosphorylation of GSK3β at Ser-9 can elicit corresponding cellular responses, temporal changes in β-catenin content after depolarization were compared with Ser-9 phosphorylation. This was done because β-catenin is a transcription cofactor of T cell factor/lymphoid enhancer factor in the Wnt pathway and is degraded via GSK-3β-mediated phosphorylation and proteasomal degradation (9). β-Catenin contents were found to show an undulating temporal pattern similar to that of Ser-9 phosphorylation of GSK-3β. More specifically, β-catenin levels decreased at 0, 13, and 30 min PD when Ser-9 phosphorylation also decreased except at 13 min, and increased at 5 and 18 min PD when Ser-9 phosphorylation increased (Fig. 1D). These parallel changes in β-catenin content and Ser-9 phosphorylation suggest that the undulating phosphorylation of GSK-3β and the resulting undulating activation of GSK-3β might have caused the observed undulating change in β-catenin content via phosphorylation-mediated degradation.

PI3K and Akt Mediated Most of the Phosphorylation of GSK-3β at Ser-9 after KCl-induced Depolarization in SH-SY5Y Cells—To identify the protein kinase responsible for the phosphorylation at Ser-9 following KCl-induced depolarization, the effects of specific inhibitors of several protein kinases on Ser-9 phosphorylation were determined at 5 min PD in SH-SY5Y neuroblastoma cells. Wortmannin and LY294002, PI3K inhibitors, blocked most of the Ser-9 phosphorylation, and calphostin C, an inhibitor of protein kinase C, also partly inhibited phosphorylation of GSK-3β at Ser-9 (p < 0.05, Mann-Whitney U test) (Fig. 2A). However, the inhibitors of other protein kinases, namely protein kinase A (H89), extracellular signal-regulated kinase (PD98059), Ca2+/calmodulin-dependent protein kinase II (KN62), and S6 kinase (rapamycin) had no significant inhibitory effect on Ser-9 phosphorylation at 5 min PD. This result suggests that the depolarization-induced phosphorylation of GSK-3β might be mediated mainly by PI3K and protein kinase C.

Because PI3K seemed to be the major kinase responsible for the Ser-9 phosphorylation of GSK3β following KCl-induced depolarization in SH-SY5Y cells, SH-SY5Y human neuroblastoma cells were treated with 100 mM KCl (KCl Tx) in DMEM for 5 min and then immediately placed in fresh medium. Cells were harvested at the indicated times and homogenized. The homogenate (50 μg) was separated by 12% SDS-PAGE, and proteins were transferred to nitrocellulose paper. Blots were incubated with antibodies directed against phosphorylated GSK-3β (Ser-9 or Tyr-216), GSK-3β, and β-catenin, and then with a peroxidase-labeled goat anti-rabbit or anti-mouse IgG antibody, respectively. Proteins were visualized by incubating blots with an enhanced chemiluminescence substrate mixture and exposing to x-ray film. The densities of phosphorylated protein bands were determined at 5 min PD in SH-SY5Y neuronal cells. The resulting un-dulating change in β-catenin content via phosphorylation-mediated degradation was similar to that observed in SH-SY5Y cells (Fig. 1C). In contrast, the same depolarization did not change the phosphorylation status of GSK-3β at Ser-9 in A172 glioblastoma cells. This result suggests that KCl-induced depolarization can result in the undulating phosphorylation of GSK3β at Ser-9 only in excitable neuronal cells.
Regulation of GSK-3β Phosphorylation by Depolarization

Because PI3K was identified as the major mediator of Ser-9, we investigated the temporal pattern of Akt activation; Akt is known to be activated by phosphorylation at Thr-308 and Ser-473, a process stimulated by phosphatidylinositol 3-phosphates (products of PI3K reactions), and after phosphorylation it phosphorylates GSK-3β. KCl-induced depolarization led to an undulating phosphorylation of Akt at Ser-473 in SH-SY5Y cells; moreover, the temporal pattern of this phosphorylation was similar to that of the phosphorylation of GSK-3β at Ser-9. This phosphorylation of Akt sharply reduced at 5 min PD, then increased to peak at 11 min PD, and then slightly decreased at 15 min PD. Another phosphorylation/dephosphorylation cycle was observed between 15 and 30 min PD with a phosphorylation peak at 21 min PD (Fig. 2C). The phosphorylation of phosphorylation of Akt at 15 min was significantly decreased from that at 13 and 18 min treatment (p < 0.05, Mann-Whitney U test). Moreover, the total amount of Akt was not changed significantly (Fig. 2C). Similar undulating phosphorylations of Akt were observed in H19–7/IGF-IR embryonic hippocampal cells and in PC-12 cells, but notably not in the A172 glioblastoma cells (data not shown).

To confirm the role of Akt in Ser-9 phosphorylation, a dominant negative mutant of Akt was overexpressed in SH-SY5Y cells, and its effect on Ser-9 phosphorylation was analyzed at 13 min PD, which showed previously a Ser-9 phosphorylation peak. The overexpression of dominant negative Akt reduced the phosphorylations of Akt at Ser-473 and of GSK-3β at Ser-9 almost to the undepolarized control level (p < 0.05, Mann-Whitney U test) (Fig. 2D). Moreover, mock treatment (data not shown) or the expression of vector alone did not change these phosphorylations of GSK-3β. In addition, treatment with wortmannin attenuated the phosphorylations of both Akt and GSK-3β induced by KCl (Fig. 2B). This result indicates that PI3K and Akt represent a major signaling pathway that phosphorylates GSK-3β at Ser-9 following the KCl-induced depolarization of SH-SY5Y cells.

Calcium-dependent Activation of EGFR Was Found to Be Involved in the Phosphorylation of GSK-3β at Ser-9 Following KCl-induced Depolarization in SH-SY5Y Cells—Next, we investigated the effects of several protein kinase inhibitors on GSK-3β phosphorylation at Ser-9 following KCl-induced depolarization. SH-SY cells were pretreated with several protein kinase inhibitors (10 μM H89 for 1 h, 100 μM PD98059 (PD) for 30 min, 100 μM wortmannin (Wort) for 1 h, 15 μM LY294002 (LY) for 30 min, 20 ng/ml rapamycin (Rap) for 1 h, 100 nM calphostin C (Cal C) 1 h, or 10 μM KN62 for 30 min). The pretreated cells were then depolarized by treatment with 100 mM KCl (KCl Tx) for 5 min in the presence of the inhibitors, and GSK-3β phosphorylation was analyzed at 13 min after this depolarization by Western blotting. Phosphorylations are expressed as ratio versus control cells without pretreatment and depolarization. B, effects of wortmannin on the temporal patterns of the phosphorylations of GSK-3β and Akt. SH-SY5Y cells were pretreated with 100 μM wortmannin for 1 h before depolarization. Cells were harvested at the indicated times after depolarization for analysis. Untreated cells (K(-)) and cells pretreated with wortmannin but not depolarized were used as controls. C, temporal pattern of Akt phosphorylation at Ser-473 following KCl-induced depolarization. D, effects of the overexpression of dominant negative Akt on the depolarization-induced phosphorylation of GSK-3β. SH-SY5Y cells were transfected with 10 μg of plasmid encoding vector (pUSEamp) or Myc-tagged dominant negative Akt (K179M Akt, Myc-DN Akt) using Lipofectamine. 24 h after transfection, the phosphorylations of GSK-3β and Akt were analyzed 13 min after depolarization by Western blotting. The blots shown are representative of at least three independent experiments. The histograms represent average and S.E., and the data are expressed as percentages of corresponding densities of control cells that were not treated with KCl. Asterisks indicate significant difference (p < 0.05, Mann-Whitney U test).
tigated the signaling molecules involved in the activation of PI3K that induce the phosphorylation of GSK-3β following KCl-induced depolarization. Many growth factors are known to be involved in the activation of PI3K, and membrane depolarization was reported to trigger the tyrosine phosphorylation of EGFR (10). Thus, we examined the effect of inhibiting EGFR signaling using AG1478, a selective inhibitor of EGFR kinase, on the Ser-9 phosphorylation of GSK-3β following KCI-induced depolarization. The inhibition of EGFR phosphorylation partially blocked the phosphorylations of Akt and GSK-3β in a dose-dependent manner at 13 min PD (Fig. 3A). Pretreatment with AG1478 at 20 μM reduced the phosphorylations of Akt and GSK-3β following KCI-induced depolarization, although the inhibitory effect of AG1478 was not as complete as that of wortmannin (Fig. 3B). Furthermore, KCl-induced depolarization resulted in a temporal pattern of EGFR phosphorylation that was similar to the temporal phosphorylation patterns of Akt1 and GSK-3β (Fig. 3C). The cells treated without KCl showed slight increase in EGFR phosphorylation during the same period without similar undulation. This result suggests that the phosphorylation of EGFR is involved in the phosphorylations of Akt and GSK-3β following KCI-induced depolarization.

To examine whether calcium influx induced by depolarization can cause the phosphorylation of EGFR following KCI-induced depolarization, we studied the effect of nifedipine (a voltage-dependent calcium channel blocker). Nifedipine almost completely inhibited the EGFR phosphorylation induced by depolarization (Fig. 3C), which confirmed that EGFR activation is dependent on calcium influx through calcium channels, and which suggests that the calcium-dependent activation of EGFR might mediate in part the activation of PI3K and the phosphorylations of Akt and GSK-3β following depolarization.

Inhibitors of Protein Phosphatases Inhibited the Dephosphorylation of GSK-3β following KCl-induced Depolarization in SH-SY5Y Cells—The undulating phosphorylation of GSK-3β induced by depolarization seemed to require the action of protein phosphatases as well as protein kinases. Thus, in order to determine whether protein phosphatases are involved in the undulating phosphorylation of GSK-3β, the effects of inhibitors specific for protein phosphatases on the dephosphorylation of GSK-3β were followed. Pretreatment with okadaic acid (an inhibitor of protein phosphatase 1 and 2A) blocked the dephosphorylation of GSK-3β at Ser-9 at 0, 15, and 30 min PD (Fig. 4A). Pretreatment with cyclosporin A, a PP2B inhibitor, also blocked the dephosphorylation of GSK-3β at Ser-9 at 0, 15, and 30 min PD (Fig. 4B). Another PP2B inhibitor, FK-506, also exhibited similar inhibitory effects on the dephosphorylation of GSK-3β (data not shown). However, pretreatment with okadaic acid, cyclosporin A, or FK-506 only partially blocked the dephosphorylation of Akt at 0, 15, and 30 min PD (Fig. 4A and B). These results suggest that PP2A and PP2B are involved in the dephosphorylation of GSK-3β at Ser-9 and that the dephosphorylation of Akt by PP2A and PP2B might be involved in the dephosphorylation of GSK-3β following depolarization.

To support the role of protein phosphatases in the dephosphorylation of GSK-3β, phosphatase activity was analyzed at 0 and 13 min PD in SH-SY5Y cells. The temporal activities of PP2A and PP2B were very similar; both activities were high at 0 min PD and low at 13 min PD, and treatment with nifedipine inhibited the increase in PP2B activity observed at 0 min PD (p < 0.05, Mann-Whitney U test) (Fig. 4C). Moreover, the activities of these protein phosphatases were inversely related to the phosphorylation status of GSK-3β at Ser-9, i.e., high phosphatase activity and low Ser-9 phosphorylation at 0 min PD, and low phosphatase activity and high Ser-9 phosphorylation at 13 min PD. This result suggests that KCl-induced depolarization induces the activations.

![Figure 3](http://www.jbc.org)
treated without KCl showed a slight but continuous decrease in [Ca$^{2+}$], with a transient drop at 3 min PD that might be caused by media replacement. Moreover, this pattern shown by [Ca$^{2+}$], agreed well with PP2B activity and with the dephosphorylation status of GSK-3$\beta$ at Ser-9 following KCl-induced depolarization, and thus supported the role of PP2B in the dephosphorylation of GSK-3$\beta$ at Ser-9. This result suggests that an undulating change in [Ca$^{2+}$] caused the observed undulating activation of PP2B, which then dephosphorylated GSK-3$\beta$ following membrane depolarization.

To confirm the source of elevated [Ca$^{2+}$], following depolarization, we analyzed the effect of calcium-free media, EDTA, and nifedipine on the phosphorylation of GSK-3$\beta$. When SH-SYSY cells were pretreated with calcium-free media, the dephosphorylation of GSK-3$\beta$ at Ser-9 at 0, 15, and 30 min PD was inhibited (Fig. 5C). The pretreatment of these cells with EDTA-containing medium also inhibited the dephosphorylation of GSK-3$\beta$ at Ser-9 (data not shown). Moreover, treatment with nifedipine also exhibited a similar inhibitory effect on GSK-3$\beta$ (Fig. 5C). These results indicate that free calcium ion influx through voltage-gated calcium channels activates PP2B, which in turn is involved in the dephosphorylation of GSK-3$\beta$ following KCl-induced depolarization.

Membrane Depolarization Induced a Change in Complex Formation between GSK-3$\beta$ and PP2A in Parallel with the Dephosphorylation Status of GSK-3$\beta$ in SH-SYSY Cells—GSK-3$\beta$ is known to form a complex with PP2A together with axin, APC, and $\beta$-catenin. Moreover, this complex sequesters GSK-3$\beta$ to act on the components of the complex and plays a central role in the Wnt signaling pathway (13). Because membrane depolarization was found to regulate the activity of PP2A, we examined whether membrane depolarization can also regulate the formation of the complex between GSK-3$\beta$ and PP2A. When GSK-3$\beta$ was immunoprecipitated with a specific antibody, PP2A was found to be co-precipitated. Similarly, GSK-3$\beta$ was co-precipitated with PP2A by antibody against PP2A (Fig. 6A), confirming that GSK-3$\beta$ forms a stable complex with PP2A in vivo. Moreover, more PP2A was co-precipitated with GSK-3$\beta$ in undepolarized SH-SYSY cells and at 0 min PD, and less at 13 min PD (p < 0.05, Mann-Whitney U test). Similarly, more GSK-3$\beta$ was co-precipitated with PP2A in undepolarized cells at 0 min PD, and less at 13 min PD (p < 0.05, Mann-Whitney U test). This result indicates that membrane depolarization may regulate complex formation between GSK-3$\beta$ and PP2A in concert with the dephosphorylation of GSK-3$\beta$.

**DISCUSSION**

The question addressed by this study was whether membrane depolarization can regulate the phosphorylation of GSK-3$\beta$, and if so what molecular mechanisms are involved in this regulation. The main finding of this study is that KCl-induced depolarization causes undulating phosphorylation/dephosphorylation, which is regulated for the most part by PI3K and Akt (phosphorylation) and PP2A and PP2B (dephosphorylation), respectively, in SH-SYSY human neuroblastoma cells.

This study also shows that KCl-induced depolarization causes the undulating phosphorylation of GSK-3$\beta$ at Ser-9 in neuron-derived cells and that it causes a similar undulating change in $\beta$-catenin concentration. KCl-induced depolarization resulted in two phosphorylation/dephosphorylation cycles with phosphorylation peaks at 13 and 21 min after depolarization for 5 min in SH-SYSY human neuroblastoma cells. In general, membrane depolarization precedes synaptic transmission and axonal conduction, and this constitutes a primary element of rapid signaling in the nervous system. Such signals are trans-
Regulation of GSK-3β Phosphorylation by Depolarization

In independent experiments, cells depolarized with KCl in normal toma cells and hippocampal cells, but not in glioblastoma cells. That GSK-3 might mediate signals generated by membrane depolarization due to its enzyme activity. Activated GSK-3β phosphorylates target proteins like β-catenin, which is a transcription factor that regulates the expressions of several genes by binding to T cell factor/lymphoid enhancer factor (19, 20). GSK-3β is considered a primary kinase that is responsible for phosphorylation and down-regulation of β-catenin levels (21, 22). The present study shows that the concentration of β-catenin changes in parallel with the undulating phosphorylation of GSK-3β, indicating that the undulating phosphorylation of GSK-3β results in corresponding undulating β-catenin level changes. However, β-catenin concentration was low at 13 min PD even though

In agreement with this finding, a similar undulating phosphorylation of GSK-3β was observed in rat brain following electroconvulsive shock treatment, where an immediate sharp decrease in the phosphorylation of GSK3β at Ser-9 was observed at 0 min post-shock treatment, which was followed by increased phosphorylation that peaked about 10 min after treatment (18). Thus, our data suggest that this undulating phosphorylation of GSK-3β observed in rat brain might occur in neurons and be the direct result of membrane depolarization caused by the electric shock treatment.

Undulating phosphorylation of GSK-3β following membrane depolarization induces an undulating change in β-catenin concentration, suggesting that the undulating change in GSK-3β phosphorylation results in the undulating regulation of cellular responses including β-catenin-dependent gene expression. Because phosphorylation of GSK-3β at Ser-9 results in the inhibition of its enzyme activity, the undulating phosphorylation of GSK-3β results in an undulating inactivation/activation of the enzyme. Activated GSK-3β phosphorylates target proteins like β-catenin, which is a transcription factor that regulates the expressions of several genes by binding to T cell factor/lymphoid enhancer factor (19, 20).

Depolarization (5). Previous reports that KCl-induced depolarization (14, 15) imply that GSK-3β might be phosphorylated as a result of depolarization. The present study demonstrates that membrane depolarization can regulate the phosphorylation of GSK-3β at Ser-9 in an undulating manner and suggests that GSK-3β might mediate signals generated by membrane depolarization to regulate various cellular responses. However, the difference between the undulating activation and the continuous activation of GSK-3β with respect to the regulation of cellular responses is not known, although it is speculated that such undulating GSK-3β activation can elicit unique responses like calcium oscillation (16, 17). Furthermore, the undulating nature of GSK-3β phosphorylation following depolarization implies that GSK-3β activity varies depending on the time of analysis in experimental systems, and thus the timing of analysis needs to be approached cautiously when examining GSK-3β activities following membrane depolarization.

In the present study, membrane depolarization was found to induce an undulating change in GSK-3β phosphorylation in neuron-derived cells, suggesting that undulating phosphorylation might be involved in neuron-specific functions. This is supported by our finding that such undulating phosphorylation of GSK-3β was observed in neuron-derived cells, e.g. neuroblastoma cells and hippocampal cells, but not in glioblastoma cells.

**Fig. 5.** Dephosphorylation of GSK-3β involves calcium influx through voltage-gated calcium channels following KCl-induced depolarization. A, fluorescence image of intracellular calcium following KCl-induced depolarization. SH-SY5Y cells were preincubated with 5 μM Fluo-4AM for 30 min before depolarization with 100 mM KCl for 5 min ([KCl Tx]). Levels were monitored continuously by measuring fluorescence under a confocal microscope. B, CRTH2 expression was determined using the confocal microscope software. C, effects of calcium-free media and nifedipine on the dephosphorylation of GSK-3β following KCl-induced depolarization. SH-SY5Y cells were pretreated with calcium-free DMEM for 1 h or with 1 μM nifedipine ([Ni]) for 15 min before KCl-induced depolarization. Untreated cells ([K+]) were used as controls. The blots shown are representative of at least three independent experiments.

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Undulating phosphorylation of GSK-3β following membrane depolarization induces an undulating change in β-catenin concentration, suggesting that the undulating change in GSK-3β phosphorylation results in the undulating regulation of cellular responses including β-catenin-dependent gene expression. Because phosphorylation of GSK-3β at on Ser-9 results in the inhibition of its enzyme activity, the undulating phosphorylation of GSK-3β results in an undulating inactivation/activation of the enzyme. Activated GSK-3β phosphorylates target proteins like β-catenin, which is a transcription factor that regulates the expressions of several genes by binding to T cell factor/lymphoid enhancer factor (19, 20), and GSK-3β is considered a primary kinase that is responsible for phosphorylation and down-regulation of β-catenin levels (21, 22). The present study shows that the concentration of β-catenin changes in parallel with the undulating phosphorylation of GSK-3β, indicating that the undulating phosphorylation of GSK-3β results in corresponding undulating β-catenin level changes. However, β-catenin concentration was low at 13 min PD even though

In agreement with this finding, a similar undulating phosphorylation of GSK-3β was observed in rat brain following electroconvulsive shock treatment, where an immediate sharp decrease in the phosphorylation of GSK3β at Ser-9 was observed at 0 min post-shock treatment, which was followed by increased phosphorylation that peaked about 10 min after treatment (18). Thus, our data suggest that this undulating phosphorylation of GSK-3β observed in rat brain might occur in neurons and be the direct result of membrane depolarization caused by the electric shock treatment.
GSK-3β was still highly phosphorylated, which is assumed to result from the degradation of β-catenin phosphorylated by kinases other than GSK-3β. The changes in β-catenin concentration are presumed to cause corresponding changes in the expression of β-catenin-dependent genes. Therefore, the undulating phosphorylation of GSK-3β could regulate various cellular responses via the undulating phosphorylation of β-catenin (2, 3). Furthermore, the undulating regulation of β-catenin by GSK-3β implies that the undulating activation of GSK-3β might phosphorylate other target proteins in the same way and thus elicit a multiplicity of cellular responses.

We found that KCl-induced depolarization results in the phosphorylation of GSK-3β via EGFR, PI3K, and Akt in SH-SY5Y cells. The involvement of PI3K in the major GSK-3β phosphorylation pathway following depolarization is supported by the finding that treatment with wortmannin or LY290924 almost completely inhibited the phosphorylations of Akt and GSK-3β induced by depolarization and by previous reports that depolarization activated PI3K, which protects against apoptosis (15, 23), although PI3K activation by depolarization was also found to depend on the neuronal context (24). The mediation of GSK-3β phosphorylation by Akt following depolarization is supported by our results that Akt phosphorylation changes in parallel with GSK-3β phosphorylation and that the expression of dominant negative Akt blocks GSK-3β phosphorylation following depolarization. Furthermore, Akt that is known to phosphorylate GSK-3β was found to be activated by depolarization, suggesting that this might provide a general mechanism coordinating the effects of growth factors and neural activity on survival (15). The partial inhibition of the phosphorylations of Akt and GSK-3β following membrane depolarization by EGFR kinase inhibitor in the present study, together with a report that membrane depolarization triggers the tyrosine phosphorylation of EGFR (10), indicates that the activation of EGFR is involved in the phosphorylation of GSK-3β following depolarization. However, our observation of a partial inhibition suggests that there may exist other signaling pathways that activate PI3K following membrane depolarization. Furthermore, treatment with the calcium channel blocker, nifedipine, almost completely blocked the activation of EGFR, suggesting that calcium influx through a calcium channel following membrane depolarization induces the EGFR activation. On the other hand, although protein kinase C mediated GSK-3β phosphorylation following membrane depolarization, the inhibitory effect of a protein kinase C inhibitor on GSK-3β phosphorylation was not as strong as that of a PI3K inhibitor, suggesting that PI3K is the major mediator of GSK-3β phosphorylation following membrane depolarization. This result shows that membrane depolarization phosphorylates GSK-3β mainly via the PI3K-Akt pathway, which can be activated by growth factors to integrate signals that regulate cellular responses like survival.

The protein phosphatases, PP2A and PP2B, in addition to kinases such as PI3K and Akt, were found to be involved in the undulating phosphorylation/dephosphorylation of GSK-3β following KCl-induced depolarization in SH-SY5Y cells. The involvement of PP2B in the dephosphorylation of GSK-3β following depolarization is supported by the finding that the inhibition of PP2B with specific inhibitors (cyclosporin A and FK506) blocked the dephosphorylation of GSK-3β following membrane depolarization. It is also supported by the finding that membrane depolarization induced undulating changes in PP2B activity and [Ca^{2+}], which occurred in parallel with the undulating dephosphorylation of GSK-3β. In addition, treatment of cells with calcium-free medium or a calcium channel blocker also blocked dephosphorylation. These results suggest that PP2B can directly dephosphorylate GSK-3β, which requires validation with purified enzymes. PP2B is a member of the serine/threonine protein phosphatase family and is activated in response to Ca^{2+} signals through the direct binding of Ca^{2+} and calmodulin. PP2B is involved in the regulation of important biological processes such as T cell activation, muscle function, memory development, and apoptosis (12, 25). Moreover, PP2B was reported to dephosphorylate the NF-AT transcription factor and to activate its nuclear import, thus enabling the sustained activation of Ca^{2+}-dependent gene expression.
expression, whereas GSK-3β reverses the calcium effect by phosphorylating and promoting the nuclear export of NF-AT (26–28). However, GSK-3β dephosphorylation by PP2B has been implicated indirectly by the findings that apoE4, an apolipoprotein E isoform, induces GSK-3β dephosphorylation in a manner partially dependent on extracellular Ca^{2+} in SH-SY5Y cells (29), and that staurosporine elevates [Ca^{2+}]_i, and dephosphorylates GSK-3β during the formation of extended lamellipodia in human keratinocytes (30). Therefore, our data suggest that PP2B might dephosphorylate and thus activate GSK-3β, a process that has been reported to abrogate the effect of PP2B in specific environments, e.g. in the regulation of NF-AT translocation.

Different relationships between GSK-3β and PP2B are speculated to be the results of different initiation signals and cell types.

PP2A was also found to be involved in the undulating phosphorylation of GSK-3β induced by KCl depolarization in SH-SY5Y neuroblastoma cells. The activity of PP2A also changed in concert with GSK-3β dephosphorylation, and treatment with a PP2A inhibitor blocked the depolarization-induced dephosphorylation of GSK-3β. PP2A has been reported to directly dephosphorylate Ser-9-phosphorylated GSK-3β purified from rabbit skeletal muscle, thus reactivating GSK-3β (31). Therefore, PP2A is believed to be involved in the activation of GSK-3β following depolarization in SH-SY5Y cells. PP2A is composed of a family of protein serine/threonine phosphatases and accounts for the major portion of serine/threonine phosphatase activity in most tissues and cells. Moreover, PP2A can interact with a substantial number of proteins and contribute to the regulation of numerous signaling pathways that regulate the cell cycle, apoptosis, and carcinogenesis (32, 33). Thus, PP2A is believed to mediate various cellular responses evoked by membrane depolarization. We also found that PP2A and PP2B partly dephosphorylate Akt following KCl-induced membrane depolarization, as evidenced by the finding that treatment with a protein phosphatase inhibitor (okadaic acid or cyclosporin A) partially blocks Akt dephosphorylation. Thus, membrane depolarization changes complex formation between GSK-3β and PP2A in concert with GSK-3β dephosphorylation. Therefore, we speculate that the regulation of complex formation between GSK-3β and PP2A might be involved in regulation of cellular responses to membrane depolarization.

In summary, this study shows that KCl-induced depolarization causes undulating GSK-3β phosphorylation/dephosphorylation, which is primarily regulated by PI3K and Akt (phosphorylation) and PP2A and PP2B (dephosphorylation) in SH-SY5Y human neuroblastoma cells. It also shows that membrane depolarization changes the amounts of enzymes in the GSK-3β/PP2A complex. This finding suggests that this undulating change in GSK-3β activity mediates cellular responses to membrane depolarization.

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Membrane Depolarization Induces the Undulating Phosphorylation/Dephosphorylation of Glycogen Synthase Kinase 3 β, and This Dephosphorylation Involves Protein Phosphatases 2A and 2B in SH-SY5Y Human Neuroblastoma Cells

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doi: 10.1074/jbc.M413987200 originally published online March 30, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413987200

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