Attenuation of Protein Kinase C and cAMP-dependent Protein Kinase Signal Transduction in the Neurogranin Knockout Mouse*

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Neurogranin (Ng) is a brain-specific, postsynaptically located protein kinase C (PKC) substrate, highly expressed in the cortex, hippocampus, striatum, and amygdala. This protein is a Ca\(^{2+}\)-sensitive calmodulin (CaM)-binding protein whose CaM-binding affinity is modulated by phosphorylation and oxidation. To investigate the role of Ng in neural function, a strain of Ng knockout mouse (KO) was generated. Previously we reported (Pak, J. H., Huang, F. L., Li, J., Balschun, D., Reymann, K. G., Chiang, C., Westphal, H., and Huang, K.-P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11232–11237) that these KO mice displayed no obvious neuroanatomical abnormality, but exhibited deficits in learning and memory and activation of Ca\(^{2+}/\)CaM-dependent protein kinase II. In this report, we analyzed several downstream phosphorylation targets in phorbol 12-myristate 13-acetate- and forskolin-treated hippocampal slices from wild type (WT) and KO mice. Phorbol 12-myristate 13-acetate caused phosphorylation of Ng in WT mice and promoted the translocation of PKC from the cytosolic to the particulate fractions of both the WT and KO mice, albeit to a lesser extent in the latter. Phosphorylation of downstream targets, including mitogen-activated protein kinases, 90-kDa ribosomal S6 kinase, and the cAMP response element binding protein (CREB) was significantly attenuated in KO mice. Stimulation of hippocampal slices with forskolin also caused greater stimulation of protein kinase A (PKA) in the WT as compared with those of the KO mice. Again, phosphorylation of the downstream targets of PKA was attenuated in the KO mice. These results suggest that Ng plays a pivotal role in regulating both PKC- and PKA-mediated signaling pathways, and that the deficits in learning and memory of spatial tasks detected in the KO mice may be the result of defects in the signaling pathways leading to the phosphorylation of CREB.

Neurogranin (Ng)\(^{1}\) is a brain-specific, Ca\(^{2+}\)-sensitive calmodulin (CaM)-binding phosphoprotein, and is highly expressed in the neuronal cell bodies and dendrites within the hippocampus, neocortex, amygdala, and striatum (1–5). Ng is a specific substrate of protein kinase C (PKC), and it can also be modified by nitric oxide and other oxidants to form intramolecular disulfide (6–9). Both the phosphorylation and oxidation of Ng attenuate its binding affinity for CaM (7, 9–11). To investigate the role of Ng in neural function, a strain of Ng knockout mouse (KO) was generated (12). These mutant mice displayed no obvious neuroanatomical abnormality; however, they exhibited deficits in learning the spatial tasks when tested with Morris water maze. In addition, Ca\(^{2+}/\)CaM-dependent protein kinase II (CaMKII) in the hippocampal slices of these KO mice is less readily autophosphorylated as compared with those of the wild type (WT) mice upon treatments that enhance Ng phosphorylation and oxidation.

Induction of hippocampal long term potentiation (LTP, an experimental model of learning and memory) is well recognized to be initiated by the stimulation of N-methyl-D-aspartate receptor, and perhaps also metabotropic glutamate receptors, and is dependent on the influx of Ca\(^{2+}\) through N-methyl-D-aspartate receptors as well as voltage-dependent calcium channel. Subsequently, the expression and the maintenance of LTP and the eventual consolidation and storage of information into the long term memory are known to depend on the stimulation of several Ca\(^{2+}\)/CaM-sensitive enzymes (including CaMKII), PKC, protein kinase A (PKA), and other kinases in the phosphorylation cascade, and eventual de novo protein synthesis (13–17). Judging from its CaM-binding property, Ng would have assumed a fairly upstream regulatory role in the biochemical mechanisms involved in the memory formation.

Mitogen-activated protein kinase (MAP kinase), also known as extracellular signal-regulated kinase, is a family of serine/threonine protein kinases having widespread distributions. MAP kinase cascade was originally discovered as a critical regulator of cell division and differentiation (18–20). Interestingly, MAP kinase signaling cascades were discovered also to play a pivotal role in synaptic plasticity and learning and memory (16, 21–27). Moreover, many studies indicated that signal transductions lead to activation of either PKC or PKA can elicit hippocampal MAP kinase activation, and MAP kinase is an important regulator of cAMP response element-binding protein (CREB) phosphorylation in the hippocampus (15, 22, 24, 28). Some of these studies (15, 22, 29) also show that 90-kDa ribosomal S6 kinase (RSK2) is a likely candidate coupling MAP kinase to CREB phosphorylation. RSK2, which is directly activated by MAP kinase via phosphorylation, in turn phosphorylates CREB at Ser-133 and thereby regulates its activity as a transcriptional activator. In light of its unique CaM-binding ability in the absence of Ca\(^{2+}\), Ng generally can be considered as a CaM depot. Upon activation of glutamatergic neurons, the rising intracellular Ca\(^{2+}\) would be expected to displace Ng from the CaM/Ng complex, and the resulting Ca\(^{2+}\)/CaM became available for the various Ca\(^{2+}\)/CaM-dependent enzymes, these

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1 The abbreviations used are: Ng, neurogranin; PKC, protein kinase C; PKA, protein kinase A; CaM, calmodulin; KO, knockout; WT, wild type; PMMA, phorbol 12-myristate 13-acetate; ACSF, artificial cerebrospinal fluid; MAP, mitogen-activated protein; RSK2, 90-kDa ribosomal S6 kinase; CREB, cAMP response element-binding protein; CaMKII, Ca\(^{2+}/\)CaM-dependent protein kinase II; LTP, long term potentiation; TTBS, Tris-buffered saline with Tween 20; S, cytosolic; P, particulate membrane.
in turn regulate the downstream targets including those in the aforementioned MAP kinase cascade. In the absence of Ng, as in the KO mice, lack of the CaM depot will lead to the perturbation of most, if not all, of these signaling steps.

In the present study, we analyzed several downstream phosphorylation components in responding to phorbol 12-myristate 13-acetate (PMA) and forskolin in the hippocampal slices of both WT and KO mice. The results show that both PKC and PKA are activated to greater degrees by PMA and forskolin, respectively, in the WT than those of KO mice. Consequently, the activation of downstream signaling components, including MAP kinases, p90RSK, and CREB, are greatly attenuated in KO as compared with the WT mice. These results, together with our previous observation of the defect of CaMKII autophosphorylation in the KO mice, strongly support the notion that Ng plays an important role in neuronal signal transductions. As a result of genetic knockout of Ng, many defects including those reported presently render the mutant mice poorer learners as compared with WT mice. These Ng KO mice will be a useful model to delineate the signal transduction pathways in the hippocampus-dependent memory acquisition, storage, and retrieval.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the indicated sources: PMA, LC Laboratories; forskolin, Alexis Biochemicals; Ng peptide (residues 28–43) and Leu-Arg-Ang-Aza-Ser-Leu-Gly (Kemptide), Bachem Inc.; PKA inhibitor peptide (residues 5–22), SynPop Corp.; [γ-32P]ATP, PerkinElmer Life Sciences; AG 1-X8 resin, Avanti Polar Lipids; and bovine serum albumin and cAMP, Sigma.

Preparation and Treatment of Mouse Hippocampal Slices—Hippocampi from adult mouse brain (3–4 months old) were removed immediately after decapitation and placed into ice-cold artificial cerebrospinal fluid (ACSF, in mM: NaCl 125, KCl 2.5, CaCl2 2.0, NaHCO3 26, NaH2PO4 1.25, MgCl2 1.0, glucose 25) bubbling with 95% O2,5% CO2 at room temperature for at least 3 h before use. Transverse hippocampal slices (400 μm thick) were prepared on a vibratome, stored in ACSF in the dark at 4°C, and used for experiments within the next 6 h. Immediately after decapitation and placed into ice-cold ACSF, the brains were quickly removed and frozen on dry ice. The hippocampal slices were obtained by using a Vibratome. The slices of 400 μm thickness were mounted in a chamber containing oxygenated ACSF (95% O2,5% CO2). For experiments involving time course, the slices were perfused with ACSF at a rate of 0.5 ml/min for 20 min. Then the slices were incubated in the desired conditions for the required period, followed by another 20 min wash with ACSF and stored at −80°C until use.

Preparation of Cytosolic and Particulate Fractions and PKC Assay—Cytosolic and particulate fractions were isolated by the standard methods. The hippocampal slices were homogenized at 4°C for 20 s at 20,000 × g for 5 min at 4°C. The supernatant was used for protein determination and PKA assay. PKA activity was assayed by measuring the incorporation of 32P into a synthetic peptide substrate, Kemptide, with or without 10 μM cAMP, and 1 μg of protein of tissue extract. Reactions were stopped, and the incorporation into peptide was determined as described (32). PKC activity was assayed by measuring the PKC activity incorporated into peptide substrate/μg of protein/5 min. PKC activities were expressed as picomoles of 32P incorporated into peptide substrate/μg of protein/5 min. Activities measured with cAMP were considered as total PKA activity, which remained constant throughout the incubation period whereas activities measured without cAMP increased following forskolin treatment as a result of elevated level of cAMP. Thus, increase in cAMP activities calculated as percentage of total activity (+cAMP) was used as an index of PKA activation. Kinase assay in the presence of synthetic PKA inhibitor peptide (residues 5–22), abolished nearly completely the cAMP-activated activity and the increase in the cAMP activity resulting from forskolin treatment.

Throughout the study, protein concentrations were determined by the Bradford method (31) with bovine serum albumin as standard.

Immunoblotting—For MAP kinase, RSK2, and CREB blots, 30 μl of protein from each sample was loaded per lane for SDS-PAGE (10% gel). For PKC and Ng, 20 μg of protein was used for electrophoresis in SDS-PAGE (8 and 10–20% gradient gels, respectively). After electrophoresis and transfer of proteins onto nitrocellulose membrane at 4°C, the membrane was washed for 10 min with TTBS (20 mM Tris–Cl, pH 7.5, containing 0.15 μM NaCl, and 0.05% Tween 20), blocked with 5% nonfat dried milk in TTBS for 40 min, washed three times with TTBS, and then incubated with primary and secondary antibodies in TTBS, consecutively, for 3–4 and 1 h, respectively.

For phospho-MAP kinase, an anti-dual phospho-MAP kinase monoclonal antibody (Cell Signaling Technology; selectively detects doubly phosphorylated Thr-202 and Tyr-204 of p44 and p42 MAP kinases) was used at 1:1000 dilution. In this and all other Western blot protocols described below, the blots were washed extensively in TTBS after incubations with primary and secondary antibodies (typically three washes, each for 10 min). For monoclonal antibodies, horseradish peroxidase-conjugated IgG and goat anti-rabbit IgG antibodies were used at 1:5000 dilution. For phospho-MAP kinase, an anti-dual phospho-MAP kinase monoclonal antibody (Cell Signaling Technology; selectively detects doubly phosphorylated Thr-202 and Tyr-204 of p44 and p42 MAP kinases) was used at 1:1000 dilution. In this and all other Western blot protocols described below, the blots were washed extensively in TTBS after incubations with primary and secondary antibodies (typically three washes, each for 10 min). For monoclonal antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG was used. Both were used at 1:5000 dilution. Signals were revealed via enhanced chemiluminescence reagent (ECL, PerkinElmer Life Sciences).

For phospho-RSK2, we used two different primary antibodies specific for two separate phosphorylation sites, phospho-p90RSK Ser-380 (Upstate Biotechnology, Inc.) and Thr-359/362Ser-364 (Cell Signaling Technology). For phospho-CREB, the primary antibody (diluted 1:1000, Cell Signaling Technology) detects CREB only when activated by phosphorylation at Ser-133. For total MAP kinase and total CREB immunoreactivities, the membranes previously blotted with phosphorylation-dependent antibodies were stripped using buffer containing 62.5 mM Tris–Cl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS for 30–60 min at 55°C with constant shaking. The blots were washed several times in TTBS and treated in the same way as for the phosphospecific antibody. Both total MAP kinase antibodies (New England Biolabs), recognize both p44 and p42 MAP kinases, and total CREB antibodies are phosphorylation state-independent and were used at 1:1000 dilution.

For PKC, an anti-conventional PKC antibodies previously prepared in our laboratory (32) was used at 1:3000 dilution. Use of antibodies 3615 and 270 for the detection of phosphorylated total Ng were described previously (6, 8, 12).

Data Analysis—The data were expressed as mean ± S.E. of at least three independent experiments when using KO mice, while the responses elicited by the various conditions were minimal, and of five to six independent experiments using WT mice. Quantitative analysis for immunoblot was done by scanning the x-ray film and determined using the Fodotyne Gel-Pro Analyzer program. Statistical analysis was conducted by one-way analysis of variance followed by paired comparisons using Student’s t test.
RESULTS

Deletion of Ng Causes Different Kinetics of PMA-mediated PKC Activation—Under control conditions, specific PKC activities determined in the cytosolic (S) and particulate membrane fractions (P) of WT and KO mice hippocampal slices were, respectively, 188 ± 27.4 (WT, S), 173 ± 9.3 (KO, S), 155 ± 11.8 (WT, P), and 145 ± 10.0 (KO, P) pmol/µg of protein/5 min. Exposure of these hippocampal slices to PMA, a PKC activator, caused an apparent translocation of PKC activity, and the reduction of cytosolic PKC in the WT was significantly greater than that of the KO (80 ± 4.7% (WT, S, n = 7) versus 91 ± 8.2% (KO, S, n = 4), p < 0.05). After 5 min of incubation, PKC activity in the cytosolic fraction of WT mice slightly recovered, but then decreased continuously until the end of incubation at 60 min. In contrast, in the KO mice, there was a slow decrease of PKC activity in cytosolic fraction. At 60 min, the remaining level of cytosolic PKC in the WT was much less than that of the KO mice (53 ± 6.4% (WT, S, n = 7) versus 71 ± 14.0% (KO, S, n = 4), p < 0.05). On the other hand, in the particulate membrane fractions, at 2 min, the translocated PKC activity is significantly higher in the WT mice than that in the KO mice (percentage of control values, 126 ± 6.0% (WT, P, n = 7) versus 111 ± 12.1% (KO, P, n = 4), p < 0.05). For the WT, after an initial increase in the activity of the particulate fraction at 2 min, there was a slight dip at 10 min, and then the level immediately increased after 10 min until the end of incubation. In contrast, there was only a small and slow increase of PKC activity in the particulate fractions of KO mice. The level of particulate PKC in the KO at 60 min was significantly less than that of the WT mice (118 ± 16.3% (KO, P, n = 4) versus 140 ± 11.2% (WT, P, n = 7), p < 0.05). The data suggested that the degree of translocation of PKC in the KO is less than that of the WT mice. The immunoblot analysis with a conventional PKC antibody (Fig. 1A) supported this conclusion.

PMA Induces Ng Phosphorylation in Hippocampal Slices of WT Mice—Ng has been shown to be a specific PKC substrate. As PMA treatment promotes the translocation and activation of PKC in hippocampal slices (Fig. 1), it is of interest to test the status of Ng phosphorylation during PMA treatment. Fig. 2A (top panel) shows that 4 µM PMA, the concentration used in the previous PKC translocation experiment, induced a time-dependent phosphorylation of Ng determined by immunoblot analysis with antibodies specific for phosphorylated Ng. It reached a maximal level in 10 min and declined slightly afterward until the end of incubation at 60 min (Fig. 2B). Degrees of Ng phosphorylation at 2, 5, 10, 30, and 60 min were, respectively, 135 ± 5.5, 148 ± 11.3, 157 ± 11.4, 129 ± 9.9, and 127 ± 13.5% of basal level (p < 0.001). Total Ng levels were unchanged by the above treatments (Fig. 2A, bottom panel), as shown in the immunoblot analyses with antibodies independent of phosphorylation status of Ng. These results demonstrate that the phosphorylation of Ng does occur in the neurons when PKC is activated either directly by PMA (present study) or...
indirectly by carbachol.2 Needless to say, such reaction does not take place in the KO mice, as Ng is completely devoid in these mice.

Ng KO Mice Exhibit Lesser Degree of Activation of MAP Kinase Cascades—It was reported that PKC activation in hippocampal slices resulted in an activation of mostly p42 MAP kinase, an effect that has been observed in a wide variety of cell types (16, 33, 34). In the present studies we found that PMA elicited activation of both p44 and p42 MAP kinases. Fig. 3A (left panels) showed that, in hippocampal slices of WT mice, 4 μM PMA produced a rapid increase in immunoreactivities of phospho-MAP kinases within 2 min of stimulation; the phosphorylation and activation remained high and only declined to near basal level after 60 min of incubation. In contrast, the same concentration of PMA did not induce much increase in MAP kinase phosphorylation in Ng KO mice (Fig. 3A, right panels). After 2, 5, 10, and 30 min of incubations, activations of p42 MAP kinase in WT mice were averaged 1.4–1.9-fold greater than those of the KO mice (percentages of control values were, for WT, 171 ± 16.9%, 2 min; 190 ± 24.7%, 5 min; 204 ± 22.2%, 10 min; and 162 ± 29.8%, 30 min; n = 6; and for KO, 100.3 ± 0.9%, 2 min; 107 ± 2.7%, 5 min; 110 ± 1.2%, 10 min; and 114 ± 1.8%, 30 min; n = 3, p < 0.05) (Fig. 3B). It is worth of noting that the phospho-p44 MAP kinase, although its immunoreactivity is relatively less intense than that of phospho-p42 MAP kinase, also shows a time-dependent increase in the WT mice parallel to that of the phospho-p42 MAP kinase. Again, such an increase is hardly discernible in the KO mice.

These observations suggest that PKC activation in hippocampal slices of WT mice leads to activations of both p44 and p42 MAP kinases, and at no time point in KO mice was stimulation of phospho-MAP kinases meaningfully obtained. Total MAP kinase levels were unchanged by the above treatments in both WT and KO mice (Fig. 3A, bottom panels).

We next analyzed the downstream target of MAP kinases in the hippocampus. We focused on RSK2, because RSK2 lies immediately downstream of MAP kinase in the phorbol ester- and growth factor-mediated signaling pathways; additionally, RSK2 has been shown to be activated by MAP kinase in vitro and in vivo via phosphorylation. So far, several phosphorylation sites of RSK2, including Ser-381 (referred to as Ser-380 for the Upstate Biotechnology antibodies) and Thr-360/Ser-364, have been identified to be important for its activation (35, 36). Fig. 4 shows that there were time-dependent increases of phosphorylation of p90RSK at Ser-381 as well as at Thr-360/Ser-364 in the WT mice during PMA treatment as analyzed with phosphorylation site-specific anti-p90RSK antibodies. However, similar exposure of hippocampal slices of Ng KO mice to PMA caused no appreciable increase of phospho-p90RSK immunoreactivities.

Transcription factor CREB is a downstream target of MAP kinase in the hippocampus (15, 22, 24). MAP kinase has been demonstrated previously to couple to CREB phosphorylation via the intervening RSK kinases in PC12 cell and neurons (15, 22, 36). RSK2 activates CREB by the phosphorylation of CREB at Ser-133. As shown in Fig. 5, the application of PMA to

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2 J. Wu and F. L. Huang, unpublished data.
hippocampal slices of WT mice resulted in a robust increase in CREB phosphorylation (2 min, 177 ± 19.4%; 5 min, 194 ± 20.0%; 10 min, 188 ± 18.5%, percentage of control, n = 6). In contrast, Ng KO mice exhibited a reduced degree of phosphorylation of CREB, a step that is known to be critical in the formation of long term memory.

**Forskolin Produces Different Kinetics of PKA Activation between WT and KO Mice—**Under control conditions, cAMP-independent activities in the hippocampal extracts of both WT and KO mice were, respectively, 11.3 ± 1.5 (n = 7) and 10.7 ± 2.2 (n = 5) pmol/μg of protein/5 min, which corresponded to 22.6 ± 1.53% (n = 7) and 21.4 ± 2.2% (n = 5), respectively, of total (+cAMP) activities. Forskolin (50 μM) produced minimal activation of MAP kinase in KO mice (Fig. 7A). Den- sitometric analysis indicated that at 10 min the activation of p42 MAP kinase in KO mice was only 1.25-fold, and there was no further activation through 60 min of incubation. Again, it is worth noting (Fig. 7A) that the p44 MAP kinase underwent a degree of activation comparable with those of p42 MAP kinase at least in the WT mice. It is clear that the total MAPK levels were unchanged in both WT and KO mice by the above treatments (Fig. 7A, lower panels).

As shown in Fig. 8, exposure of hippocampal slices of WT mice to 50 μM forskolin also caused a time-dependent increase of phospho-p90RSK at Ser-381 (Ser-380 for Upstate Biotechnology antibodies) and at Thr-360/Ser-364. At no time point in KO mice did such stimulation of phospho-p90RSK occur at a significant level.

Many reports (15, 22, 26, 28) indicated that the PKA activity is important for CREB phosphorylation. As shown in Fig. 9, forskolin caused a significant increase in CREB phosphorylation in the hippocampal slices of WT mice. After 10 min of incubation, the CREB phosphorylations in the WT and KO mice were 190 ± 3.5% versus 104 ± 3.5% of control (p < 0.01). Although, in WT mice, CREB phosphorylation was transient and did not remain high through 60 min of incubation, we found that, in KO mice, dephosphorylation of CREB was already taking place between 30 and 60 min of incubation. Again, the total CREB level remained unchanged throughout the whole period of incubation in both WT and KO mice (Fig. 9A, bottom panels).

**DISCUSSION**

In the adult mouse brain, Ng is postnatally expressed at high levels in the neocortex, hippocampus, and amygdala, brain...
areas known to be important for learning and memory in vertebrates (39–41). Although the physiological functions of Ng have not been unequivocally defined, its biochemical properties and postsynaptic localization have implicated it in several neuronal signal transduction pathways. Recent work has shown that Ng is phosphorylated in rat hippocampal slices after the induction of LTP and that intracellular injection of antibodies against Ng prevents the maintenance phase of LTP (40, 41). As a result of Ng phosphorylation and/or oxidation, CaM is released from Ng to form Ca\(^{2+}\)/CaM, which could then activate CaM-dependent enzymes, including CaM-dependent kinases, adenylyl cyclases, and NO synthase, etc., that are involved in the regulation of synaptic plasticity. Indeed, our previous experiments showed that the Ng KO mice exhibited severe performance deficits in the Morris water maze. In addition, Ng KO mice displayed reduced potentiation in hippocampal CA1 region of WT mice leads to secondary activation of MAP kinase and the downstream components of RSK2, and CREB phosphorylations. However, the phosphorylations and activations of MAP kinase, RSK2, and CREB were significantly attenuated in the KO as compared with those of WT mice. It is worth mentioning that, in all our analyses, in contrast to the other reports (15, 16), p44 MAP kinase was found to be as readily phosphorylated and activated as p42 MAP kinase, and the time course of p44 MAP kinase activation paralleled that of p42 MAP kinase (Figs. 3 and 7).

Ng is phosphorylated and oxidized Ng in turn may lead to an availability of Ca\(^{2+}\)/CaM for the activation of Ca\(^{2+}\) and PKC activation. Our data show that Ng was phosphorylated when hippocampal slices were treated with PMA (present data) as well as phorbol 12,13-dibutyrate (data not shown), and it could also be oxidized by sodium nitroprusside and other NO donors (7, 8, 10). The phosphorylated and oxidized Ng in turn may lead to an availability of Ca\(^{2+}\)/CaM for the activation of Ca\(^{2+}\)/CaM-dependent enzymes at specific sites. It has also been noted (19, 20) that PKC can activate Raf-1 directly or indirectly via Ras, then lead
to MAP kinase cascades activations. Our data indicated that the observed decrease in PKC translocation must have contributed in part to a reduced activation of MAP kinase cascades in the KO mice. In the mean time, the lack of Ng phosphorylation and/or oxidation, as in the KO mice, would have led to an aberrant regulation of neuronal Ca\(^{2+}\) and CaM levels, which affect the activation of Ca\(^{2+}\)/CaM-dependent enzymes. In another word, in the absence of Ng, the fine turning of making Ca\(^{2+}\)/CaM available at the right time and at the right site is obstructed. Previously, it has been shown that LTP is associated with an increased phosphorylation of PKC substrates including Ng, and these phosphorylations can be blocked by PKC inhibitors (39–41). The attenuated PKC signaling mechanism demonstrated in this study may explain our previously observed deficits in synaptic plasticity in these Ng KO mice.

The present data showed that stimulation of the hippocampal slices with forskolin resulted in a greater activation of PKA in the WT mice as compared with those of the KO mice. The phosphorylations of the downstream targets of PKA were significantly attenuated in the KO mice, which may be largely the result of the reduced PKA activation in these mice. It was reported that Ca\(^{2+}\)/CaM-dependent form of adenylyl cyclases, namely AC1 and AC8, are enriched in the hippocampus and are likely responsible for forskolin action (45–47). Our present experiments with hippocampal slices have clearly demonstrated this notion that, in the presence of forskolin, stimulation of such adenylyl cyclases is likely operating to activate PKA, which is critical for the late phase LTP and long term memory (47). It is well recognized that phosphorylation of Rap-1 at Ser-179 by PKA activates B-Raf and then leads to the activation of mitogen-activated protein kinase kinase and its substrate MAP kinases (37, 38). Our results suggest that Ng may also plays a role in controlling PKA activation via Ca\(^{2+}\)/CaM-dependent adenylyl cyclases. All in all, the data obtained with the Ng KO mice support the hypothesis that Ng also regulates brain adenylyl cyclases by way of its interactions with CaM.

CREB is a nuclear protein that modulates the transcription of genes with cAMP-responsive elements in their promoters. Increase in the concentration of either Ca\(^{2+}\)/CaM or cAMP is found to trigger the phosphorylation and activation of CREB. Transcription factor CREB has also been shown to be a downstream target of the MAP kinase cascade in the hippocampus. Genetic and pharmacological studies in mice and rats demonstrate that phosphorylation of CREB is required for the establishment of a variety of complex forms of memory, including spatial and emotional learning; thus, CREB may be a universal modulator of processes involved in memory formation (14, 46–48). Our data indicate that the deficits in the learning and memory of spatial tasks seen in the Ng KO mice may be in part a result of the defects in the signaling pathways leading to the attenuated phosphorylation of CREB.

In summary, the current study adds to our growing understanding on the various deficits in the multifarious signaling pathways in these Ng KO mice. These results suggest that Ng functions as an upstream modulator and plays a key role in both the PKC- and PKA-mediated signaling pathways via its modulation of free Ca\(^{2+}\) and CaM. Both the PKC and PKA pathways, acting via the regulation of MAP kinases and CREB phosphorylation, control gene expression required in LTP and other lasting forms of hippocampal synaptic plasticity. Thus, the attenuation of CREB phosphorylation in Ng KO mice may be one of the reasons that result in deficits in hippocampal synaptic plasticity and hippocampus-dependent spatial learning.

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