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Phosphorylation of neurogranin, protein kinase C, and Ca^{2+}/calmodulin dependent protein kinase II in opioid tolerance and dependence

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Abstract

Activation of Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) and protein kinase C (PKC) are hallmarks of opioid tolerance and dependence. It is not known if the actions of these two kinases are synchronized by a common mechanism in opioid tolerance and dependence. Neurogranin (Ng), through mechanisms such as phosphorylation, has been previously proposed to regulate the activities of these protein kinases. We examined the phosphorylation status of neurogranin in mice that were made tolerant to opioids by morphine (100 mg/kg, s.c.). Increase in phosphorylation of neurogranin was found both in brains and spinal cords of morphine-treated mice, as compared to the untreated baseline or saline-treated mice. The effect appeared to correlate with the changes in the activities of PKC and CaMKII, and with the development of opioid tolerance and dependence. We have found that neurogranin activity is regulated in opioid tolerance and dependence. Neurogranin may, therefore, provide a potential mechanism interacting with both CaMKII and PKC in opioid tolerance and dependence.
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Keywords: Pain; Addiction; RC3; CaMKII; PKC; Kinase

Opioids such as morphine are efficacious drugs to treat pain. Tolerance to and dependence on opioids are two major problems associated with the prolonged use of these drugs. The mechanisms underlying opioid tolerance and dependence are not entirely understood [11,12]. A number of studies have suggested that both protein kinase C (PKC) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) are essential for the generation and maintenance of opioid tolerance and dependence (e.g., [2,4,7,15,26,28,30,32]). It is not known whether these two kinases act independently or have a common regulator in opioid tolerance and dependence.

Neurogranin (Ng) has been found to interact with both PKC and calmodulin (CaM) [3,9,18]. Ng, also called RC3 or P17, is expressed in postsynaptic neuronal cell bodies and dendrites of forebrain, hippocampus, amygdala, and striatum [8,20]. Ng regulates the availability of free CaM in cytoplasm, thus affecting the activity of CaM-dependent enzymes such as CaMKII. At low levels of Ca^{2+}, Ng binds to CaM and effectively sequesters CaM at specific regions in cytoplasm. An increase in intracellular Ca^{2+} dissociates the stable CaM–Ng complex, releasing free CaM to activate CaMKII and other enzymes [9]. As a phosphoprotein, Ng is a neuron-specific substrate of PKC [3]. Phosphorylation of Ng by PKC is another mechanism to dissociate Ng–CaM complex, effectively raising the intracellular concentrations of CaM and activity of CaMKII [9]. Recent studies suggest that Ng can also function as an upstream modulator and regulate the activity of PKC [9]. Mice lacking Ng exhibited decreased phorbol ester-activation of PKC, decreased agonist-stimulated autophosphorylation of CaMKII [9,31]. Phosphorylation of Ng may thus serve as a mechanism synchronizing the actions of PKC and CaMKII. In this study, we test if Ng phosphorylation is altered in opioid tolerance and dependence.

All experimental procedures were performed in accordance with the NIH guidelines after approval by the Animal Care Committee at the University of Illinois at Chicago. Male ICR mice (20–25 g, Harlan Industries, Indianapolis, IN) were housed on a 12/12 h light/dark cycle and provided food and

Abbreviations: CaMKII, Ca^{2+}/calmodulin dependent protein kinase II; CaM, calmodulin; CREB, cAMP response element-binding protein; MAP kinase, mitogen-activated protein kinase; Ng, neurogranin; PKA, cAMP dependent protein kinase; PKC, protein kinase C

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water *ad libitum* before experiments. Morphine sulfate was purchased from Abbot Laboratories (North Chicago, IL).

The tail-flick test was used to assess morphine antinociception in mice as previously described [26]. Briefly, distal one-third of the tail was immersed into a water bath maintained at 52 °C. Latencies to a rapid tail flick were recorded before and 30 min after the injection of morphine (10 mg/kg, s.c.). A cut-off of 12 s was applied to prevent tissue damage. Individual groups of eight mice were made acutely dependent on and tolerant to opioids by an injection of morphine (100 mg/kg, s.c.) [2]. Mice were randomly selected for opioid tolerance, dependence and biochemical experiments. Morphine antinociceptive tolerance, defined as significantly reduced antinociception, was determined 0.5, 1 and 4 h later using a test dose of morphine (10 mg/kg, s.c.). To examine the presence of opioid dependence, mice were challenged with naloxone (10 mg/kg, i.p.), and immediately placed inside Plexiglas cylinders. Naloxone-induced withdrawal jumps were counted for 15 min.

After the treatment with morphine (100 mg/kg, s.c.), brain and spinal cord samples were immediately dissected out and homogenized in ice-cold RIPA buffer for Western blotting analyses as described previously [24,29]. Samples (30 μg protein) were separated by 10% polyacrylamide gel electrophoresis and transferred onto PVDF membranes for the analysis of phosphoprotein expression using the following antibodies: anti phospho-neurogranin (1/1000, Upstate Biotechnology, Lake Placid, NY), anti T286-phospho-CaMKII (pan) (1/1000, Promega, Madison, WI), and anti phospho-PKC (γT514) (1/1000, Cell Signaling, Beverly, MA). β-Actin expression was measured in the same blots using a monoclonal antibody (1/10,000, Sigma). Enhanced chemiluminescence (Amersham)-signals were captured by a ChemiDoc imaging system and analyzed using QuantityOne (BioRad, Hercules, CA).

All data are expressed as the mean ± S.E.M. The differences among all groups were first analyzed by ANOVA. When statistical significance was detected, Student's *t*-test was used to determine the post hoc statistical significance between a testing group and its corresponding control group. Statistical significance was established at $p < 0.05$.

In naïve mice, morphine (10 mg, s.c.) produced significant antinociception (Fig. 1A). Following the treatment with morphine (100 mg/kg, s.c.), morphine antinociception decreased gradually over the time course of 0.5–4 h ($p < 0.001$). Significant reduction of antinociception was observed at 1 h ($p < 0.01$), indicative of the on-set of opioid tolerance. At 4 h, antinociceptive tolerance was fully developed ($p < 0.001$). The presence of morphine dependence was revealed by acute administration of naloxone, which had little effect in naïve mice (Fig. 1B). In morphine (100 mg/kg, s.c.) treated mice, naloxone elicited significant withdrawal jumps (163 ± 2%, $p < 0.001$) at 4 h. As we reported previously [2], no significant withdrawal jumps were recorded at 0.5 and 1 h.

In order to correlate the behavioral data with biochemical changes, we next examined the phosphorylation of Ng, which decreases Ng's affinity to CaM. Supraspinal Ng phosphorylation increased within 1 h by 55% ($p < 0.001$) and 4 h by 142% ($p < 0.01$) after the morphine (100 mg/kg) treatment

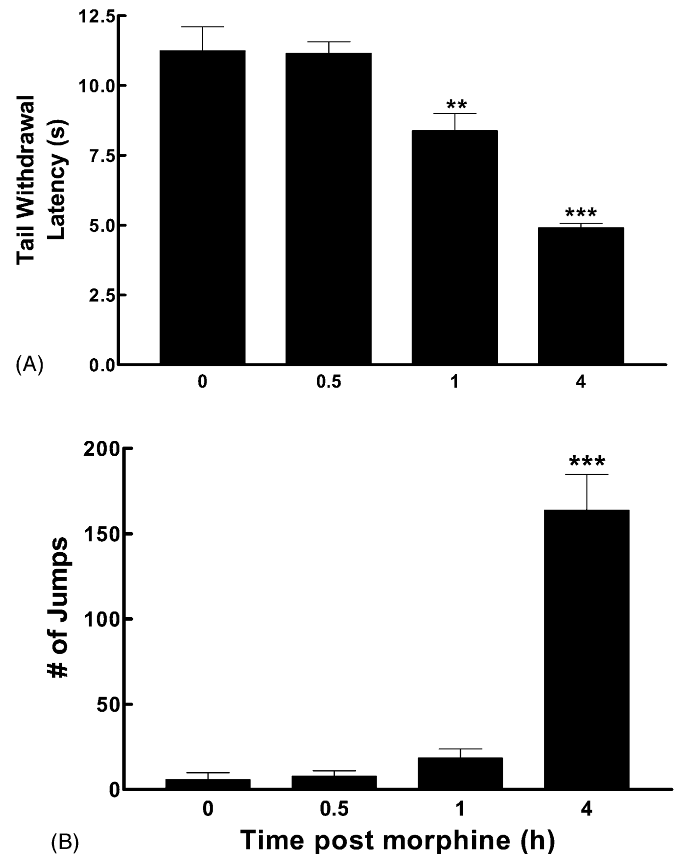

Fig. 1. Development of morphine antinociceptive tolerance (A) and dependence (B) in mice. Separate groups of eight mice were treated with morphine (100 mg/kg, s.c.). Morphine (10 mg/kg, s.c.) antinociception was determined before and 0.5, 1 and 4 h later. Similarly, the presence of opioid dependence was tested at these time points by an administration of naloxone (10 mg/kg, i.p.). Data are expressed as mean ± S.E.M. ($N = 8$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the saline control group.

(Fig. 2). A similar up-regulation of spinal Ng phosphorylation was found. Significant increases of Ng phosphorylation were detected at 0.5 h (by 61%, $p < 0.001$), 1 h (by 106%, $p < 0.001$) and 4 h (by 217%, $p < 0.001$) after the morphine administration (Fig. 3).

Increased phosphorylation of CaMKII has been previously reported [4,24,26,30]. We also examined the phosphorylation of CaMKII in the acute opioid tolerance and dependence model that induced Ng phosphorylation. T286-phosphorylated CaMKII, representing activated CaMKII, was found to increase in brains after the treatment with morphine (100 mg/kg) within 0.5 h (by 13%, $p < 0.05$), 1 h (by 29%, $p < 0.001$) and 4 h (by 86%, $p < 0.01$) (Fig. 2). Similarly, spinal CaMKII activity was increased by 102% ($p < 0.001$) and 221% ($p < 0.001$) at 1 and 4 h, respectively, following the treatment (Fig. 3).

We next compared phosphorylation of PKC in saline and morphine-treated mice using a Western blotting method that specifically detected the phosphorylated (i.e., activated) PKC. We found that both supraspinal and spinal PKC activity was increased by 54% ($p < 0.01$) in mice that were tolerant to morphine (4 h). There was also an increase of spinal PKC activity by 18% ($p < 0.05$) at 1 h post treatment.

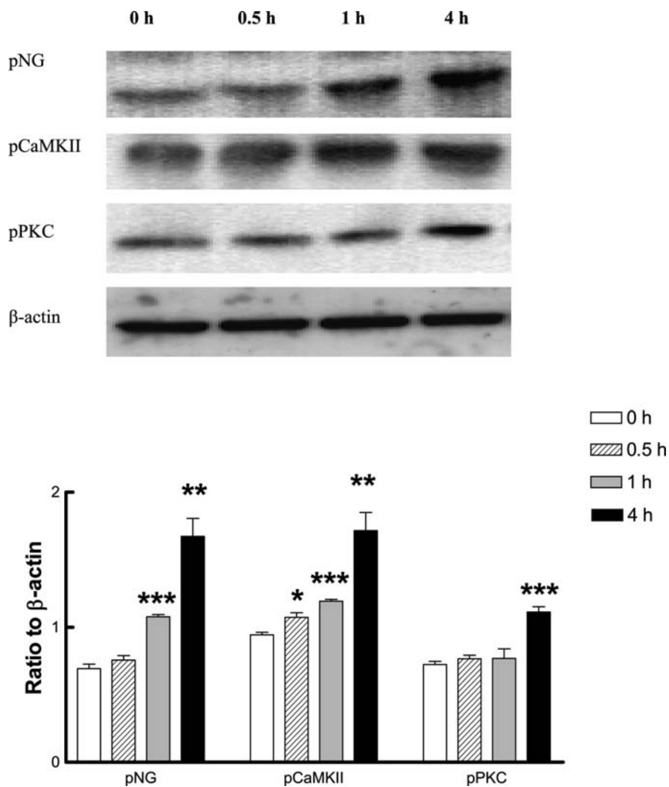

Fig. 2. Effect of morphine on phosphorylation of supraspinal neurogranin, CaMKII, and PKC. Solubilized brain tissue samples were subjected to 10% polyacrylamide gel electrophoresis and transferred onto PVDF membranes, which were then incubated with anti-pNG, pCaMKII, or pPKC antibodies, then HRP-conjugate anti-rabbit secondary antibody. Ratios of the optical densities of pNG, pCaMKII, or pPKC to that of β-actin were calculated for each sample. Histograph data, expressed as mean ± S.E.M., were constructed from the representative figure shown and three other experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to the saline control group.

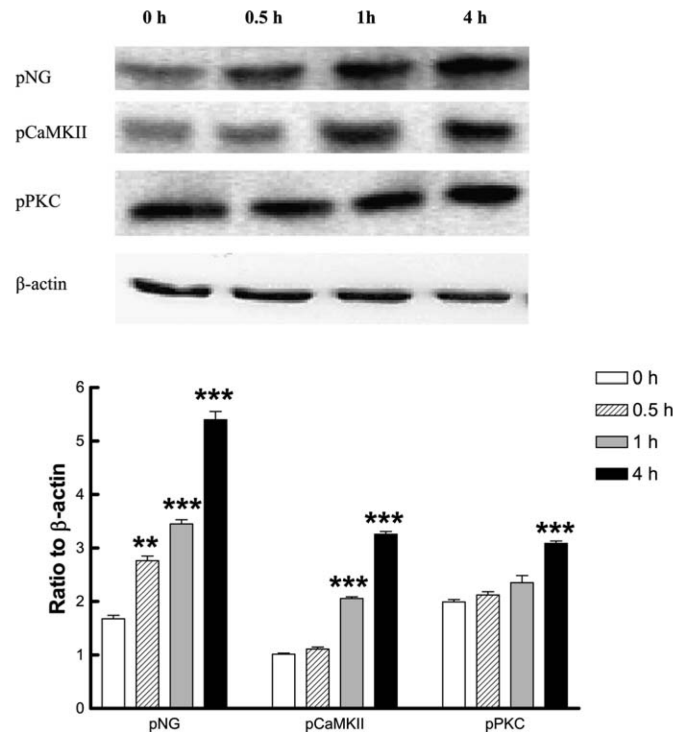

Fig. 3. Effect of morphine on phosphorylation of spinal neurogranin, CaMKII, and PKC. Solubilized spinal tissue samples were subjected to 10% polyacrylamide gel electrophoresis and transferred onto PVDF membranes, which were then incubated with anti-pNG, pCaMKII, or pPKC antibodies, then HRP-conjugate anti-rabbit secondary antibody. Ratios of the optical densities of pNG, pCaMKII, or pPKC to that of β-actin were calculated for each sample. Histograph data, expressed as mean ± S.E.M., were constructed from the representative figure shown and three other experiments. $^{**}p<0.01$, $^{***}p<0.001$ compared to the saline control group.

Enhanced activities of PKC and CaMKII are hallmarks for the opioid tolerance and dependence state [4,7,13–15,24,26,30,32]. We confirmed in the current study that both PKC and CaMKII were activated in the acute mouse model of opioid tolerance and dependence. Moreover, the on-set of the activation of these kinases showed some temporal correlation with the development of opioid tolerance and dependence. It is not entirely clear how these protein kinases are activated in opioid tolerance and dependence. We tested in this initial study if activation of PKC and CaMKII can be regulated by a common factor. Neurogranin has been proposed to interact with both PKC and CaM [1,6,17,18]. PKC phosphorylates Ng at Ser-36, decreasing the latter's affinity to CaM [18]. Ser-36 is located within the IQ domain of Ng that binds to CaM. Therefore, PKC phosphorylation and CaM binding are in a competition; phosphorylated Ng cannot bind to CaM. At low Ca^{2+}, such as in resting neurons, Ng binds to CaM, effectively concentrating CaM at specific reservoir sites, which can release free CaM in response to increasing Ca^{2+} and PKC activity [10,31]. Association of Ng with CaM also accelerates the dissociation of Ca^{2+} from the C-terminal lobe of CaM [5].

Based on these proposed roles for Ng, we hypothesize that Ng may serve as a common regulator synchronizing the activities of CaMKII and PKC in opioid tolerance and dependence. We tested the hypothesis by simultaneously monitoring the phosphorylation status of Ng, PKC, and CaMKII in a mouse acute model of tolerance and dependence. To our knowledge, this is the first report demonstrating increased phosphorylation of neurogranin in morphine tolerance and dependence. The effect apparently correlated with the changes in the activities of PKC and CaMKII, and with the development of opioid tolerance and dependence.

In addition to regulating the concentration of free cytosolic CaM, phosphorylated Ng can also stimulate the G-protein coupled phosphoinositide to release Ca^{2+} from intracellular stores such as endoplasmic reticulum [21]. Thus, phosphorylation of Ng increases Ca^{2+} as well as Ca^{2+}–CaM to stimulate CaMKII and PKC. This may be one mechanism for the up-regulation of CaMKII and PKC activities in opioid tolerance and dependence. Opioids have been reported to increase intracellular Ca^{2+} in various cell types [19,22] This may also explain why Ng can function as an upstream modulator of PKC [9]. Mice lacking Ng exhibited decreased activation of PKC by phorbol 12-myristate 13-acetate (PMA) [31]. Ng-null mice also showed decreased forskolin-stimulated activation of cAMP dependent protein kinase (PKA), decreased phosphorylation of cAMP response element-binding protein (CREB), and decreased phosphorylation of mitogen-activated protein (MAP) kinase [9,31]. PKA, CREB and MAP kinase are phosphorylated and/or activated after treatment with

opioids [16,23,27]. Our data support the hypothesis that neurogranin may potentially function as a common regulator synchronizing the activities of CaMKII and PKC in opioid tolerance and dependence. Recently, we found that down-regulation of Ng by antisense deoxyoligonucleotides attenuated the development of opioid tolerance [25].

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