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Cocaine facilitates PKC maturation by upregulating its phosphorylation at the activation loop in rat striatal neurons *in vivo*

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Abstract

Newly synthesized protein kinase C (PKC) undergoes a series of phosphorylation to render a mature form of the enzyme. It is this mature PKC that possesses the catalytic competence to respond to second messengers for activation and downstream signaling. The first and rate-limiting phosphorylation occurs at a threonine residue in the activation loop (AL), which triggers the rest maturation processing of PKC and regulates PKC activity in response to cellular stimulation. Given the fact that PKC is enriched in striatal neurons, we investigated the regulation of PKC phosphorylation at the AL site in the rat striatum by the psychostimulant cocaine *in vivo*. We found that PKC was phosphorylated at the AL site at a moderate level in the normal rat brain. Acute systemic injection of cocaine increased the PKC-AL phosphorylation in the two striatal structures (caudate putamen and nucleus accumbens). Cocaine also elevated the PKC-AL phosphorylation in the medial prefrontal cortex. The cocaine-stimulated PKC phosphorylation in the striatum is rapid and transient. A reliable increase in PKC phosphorylation was seen 7 min after drug injection, which declined to the normal level by 1 h. This kinetics corresponds to that seen for another striatum-enriched protein kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase, in response to cocaine. This study suggests a new model for exploring the impact of cocaine on protein kinases in striatal neurons. By modifying PKC phosphorylation at the AL site, cocaine is believed to possess the ability to alter the maturation processing of the kinase in striatal neurons *in vivo*.

Keywords

protein kinase C; ERK; dopamine; stimulant; caudate; nucleus accumbens; prefrontal cortex; addiction

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1. Introduction

Protein kinase C (PKC) is a family of enzymes that modify protein function through phosphorylating serine and threonine residues on modified proteins. This family consists of at least ten isozymes (Mellor and Parker, 1998). Based on their regulatory domain compositions which determine the linkage to different second messengers, these isozymes are divided into three subfamilies: conventional (or classical), novel, and atypical (Nishizuka, 1995; Mellor and Parker, 1998; Newton, 2009). Conventional PKC (cPKC) isoforms include α , β II and the alternatively spliced β I different only in the last 43 residues, and γ isoforms. They require Ca^{2+} , diacylglycerol (DAG), and a phospholipid such as phosphatidylserine for activation. Novel PKC (nPKC) isoforms (δ , ϵ , η , and θ) are activated by DAG, although Ca^{2+} is not required for their activation. Thus, both cPKC and nPKC respond to the same phospholipase C (PLC)-dependent signaling pathway. Atypical PKC (aPKC) isoforms (ζ and ι/λ), on the other hand, require neither Ca^{2+} nor DAG signals for activation. In mammalian cells, including neurons, PKC is one of most ubiquitous multifunctional kinases involved in the modification of a variety of substrate proteins related to diverse cellular and synaptic activities (Dempsey et al., 2000; Geraldles and King, 2010; Rosse et al., 2010).

While PKC phosphorylates numerous proteins, PKC itself is a substrate of other kinases and is subject to the phosphorylation-dependent modification (Newton, 2003). Indeed, phosphorylation is a key mechanism controlling the maturation of PKC. Through three ordered phosphorylation, the kinase gains its maturation, i.e., catalytic competence (Newton, 2003). Specifically, newly synthesized unphosphorylated PKC initiates its maturation processing by associating with a membrane compartment, where it is first phosphorylated by a membrane-bound kinase, phosphoinositide-dependent kinase-1 (PDK-1), at an activation loop (AL) site: threonine 497 (T497) for PKC α , T500 for PKC β , and T514 for PKC γ . This in turn triggers sequential autophosphorylation at two C-terminal sites in the turn loop and the hydrophobic loop via an intramolecular mechanism. The phosphorylation at these sites renders the maturation (catalytic competence) of the enzyme. Mature PKC species is then released to the cytosol where it responds to Ca^{2+} /DAG second messengers, thereby translocating to the membrane to trigger downstream signaling (Keranen et al., 1995; Parekh et al., 2000; Newton, 2003). Apparently, the early phosphorylation is a prerequisite step priming PKC for later activation. Central among the sequential phosphorylation is the AL site phosphorylation, which acts as the first and rate-limiting step to control the maturation of PKC.

PKC is expressed in broad areas of mammalian brains (Tanaka and Saito, 1992). In the striatum, a key structure in limbic reward circuits essential for drug addiction, PKC is enriched in both the dorsal striatum/caudate putamen (CPu) and the ventral striatum/nucleus accumbens (NAc) (Saito et al., 1989; Yoshihara et al., 1991; Naik et al., 2000). Accumulative data have demonstrated a pivotal role of PKC in drug action (Newton and Ron, 2007; Lee and Messing, 2008; Olive and Newton, 2010), although the direct effect of the psychostimulant cocaine on the maturation processing of PKC is unknown at present. In this study, we examined the possible impact of cocaine on PKC maturation in the rat striatum *in vivo*. We monitored the PKC phosphorylation at the specific AL site as an index of PKC maturation. To analyze phosphorylated PKC (pPKC) at the AL site, we chose an antibody that immunoreacts primarily with PKC γ -T514. Additionally, the antibody may cross-react with PKC α and PKC β when they are phosphorylated at the AL residue homologous to T514 of human PKC γ . For this reason, we referred to the antibody as an anti-pPKC-AL antibody. Total cellular levels of PKC were also examined to determine whether cocaine alters the abundance of PKC proteins.

2. Results

2.1. Specificity of antibodies

We first carried out dephosphorylation assays with striatal tissue to confirm the specificity of the phospho- and site-specific antibody against pPKC at the AL site. In rat striatal whole-cell homogenates, we detected a single and strong band close to 75 kDa in Western blot (Fig. 1A), similar to the appropriate molecular weight of cPKC observed from rat brain extracts or cultured rat hippocampal neurons (Tejero-Diez et al., 1995). This band almost disappeared in striatal homogenates that were pretreated with λ -phosphatase (Fig. 1A). When the same membrane was stripped and re-probed with an antibody against total PKC (tPKC), a tPKC band was recognized at a position corresponding to phosphorylated species of PKC (Fig. 1B). Subsequent immunoblot with an anti-actin antibody showed consistent actin immunoreactivity among all lanes (Fig. 1C), confirming equal protein loadings in each lane. These data support the phosphorylation specificity of the pPKC-AL antibody.

2.2. Effects of cocaine on PKC-AL phosphorylation in the striatum

We next investigated the effect of cocaine on PKC-AL phosphorylation. We subjected rats to a single i.p. injection of cocaine at different doses. We then sacrificed rats 7 min after drug injection. The targeted brain sites were removed for immunoblot analysis of changes in PKC-AL phosphorylation. In total protein homogenates from the CPu, cocaine (40 mg/kg) significantly increased PKC-AL phosphorylation. As shown in Fig. 2A, basal levels of pPKC-AL proteins were elevated in cocaine-treated rats compared to saline-treated rats ($168.0 \pm 17.8\%$ of saline, $P < 0.05$). Similar to the CPu, pPKC-AL levels in the NAc were increased following cocaine administration ($213.1 \pm 29.8\%$ of saline, $P < 0.05$) (Fig. 2A). Total cellular PKC proteins remained unchanged in cocaine-treated rats relative to saline-treated rats (Fig. 2A). Thus, the increase in levels of pPKC proteins results from an increased phosphorylation reaction rather than an increased abundance of total proteins. In addition to PKC, we tested another common protein kinase as a positive control. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are sensitive targets of cocaine and were markedly increased in phosphorylation in the striatum after acute cocaine administration (Valjent et al., 2000; Zhang et al., 2002; 2004; Sun et al., 2007). Consistent with these studies, cocaine substantially increased phosphorylated ERK1/2 (pERK1/2) proteins in both the CPu ($259.1 \pm 32.6\%$ of saline, $P < 0.05$) and NAc ($324.0 \pm 34.8\%$ of saline, $P < 0.05$) (Fig. 2B). In contrast, total ERK1/2 (tERK1/2) proteins were not altered in these regions (Fig. 2B). In separate experiments, we tested the effect of cocaine at a lower dose. Cocaine at 10 mg/kg did not induce a significant increase in PKC-AL phosphorylation in the CPu and NAc (Fig. 2C). Cocaine at this dose increased ERK1/2 phosphorylation in the CPu, although the increase in the NAc did not reach a statistically significant level (Fig. 2D). These data demonstrate that cocaine at a dose of 40 mg/kg can enhance PKC-AL phosphorylation in striatal neurons.

2.3. Effects of cocaine on PKC-AL phosphorylation in the cortex

The medial prefrontal cortex (mPFC) is another forebrain site of dopamine projection. The mesocorticolimbic pathway is an important part of the brain reward system implicated in psychostimulant action (Tzschentke, 2000; Steketee, 2003). We therefore examined the mPFC site for PKC-AL phosphorylation in response to cocaine. Following an acute injection of cocaine (40 mg/kg, i.p.; 7 min), pPKC-AL levels in the mPFC were increased in cocaine-treated rats compared to saline-treated rats ($194.2 \pm 24.5\%$ of saline, $P < 0.05$), while tPKC levels were not altered (Fig. 3A). In contrast to pPKC, pERK1/2 levels showed an insignificant change after cocaine injection ($117.8 \pm 28.3\%$ of saline, $P > 0.05$) (Fig. 3B). Total ERK1/2 expression was not altered in cocaine-treated rats (Fig. 3B). These results

indicate that the mPFC is another sensitive dopamine-innervated forebrain site where PKC-AL phosphorylation is regulated by cocaine.

2.4. Time-course of cocaine-stimulated PKC-AL phosphorylation

To determine the temporal property of the cocaine-stimulated PKC-AL phosphorylation, we administered a same dose of cocaine (40 mg/kg, i.p.) to rats, and we then sacrificed rats at different time points (7, 15, or 60 min after drug injection) for immunoblot analysis of PKC-AL phosphorylation. At 7 min, as expected, cocaine caused a robust increase in pPKC-AL proteins in the CPu (Fig. 4A). This increase persisted at 15 min and declined at 60 min. In fact, at 60 min, no significant increase in pPKC-AL phosphorylation was observed in cocaine-treated rats (Fig. 4A). In the NAc, the cocaine-stimulated PKC-AL phosphorylation was consistently detected at 7 min (Fig. 5A). A reliable increase in AL phosphorylation was also detected at 15 min. At 60 min, cocaine did not induce a statistically significant increase in pPKC-AL proteins. At all time points surveyed, tPKC proteins in both the CPu and NAc remained a minimal change in cocaine-treated rats relative to saline-treated rats. Similar to PKC, ERK1/2 phosphorylation showed a rapid and transient change in response to cocaine. In the CPu, increased ERK2 phosphorylation was seen at 7 and 15 min, but returned to the normal level at 60 min (Fig. 4B). In the NAc, a rapid increase in ERK2 phosphorylation was seen at an early time point (7 min), but not at two later time points (15 and 60 min) (Fig. 5B). These results indicate that cocaine induces a rapid and reversible increase in PKC-AL phosphorylation.

3. Discussion

The first step of PKC maturation is the AL phosphorylation. In this study, we examined the impact of cocaine on PKC-AL phosphorylation in key regions of rat forebrain reward circuits. We found that acute injection of cocaine increased PKC-AL phosphorylation in the striatum, including both the CPu and NAc. Cocaine also increased PKC-AL phosphorylation in the mPFC. The cocaine-stimulated PKC-AL phosphorylation was a rapid and transient event, similar to the kinetics of the upregulated ERK1/2 phosphorylation in response to cocaine. Together, our results demonstrate that the maturation processing of PKC is subject to the regulation by a psychoactive agent. Acute cocaine administration is able to robustly enhance the maturation-related phosphorylation of PKC in striatal neurons *in vivo*.

The balance of phosphorylation and dephosphorylation is a fundamental mechanism for regulating most mammalian protein kinase activity. PKC is among protein kinases that are actively regulated by phosphorylation. Specifically, after synthesis, PKC activity is regulated by two coordinated mechanisms: 1) phosphorylation-dependent maturation; and 2) direct activation by second messengers (Newton, 2003). The former precedes the latter and primes the enzyme ready to respond to second messengers. Among a series of sequential phosphorylation events, the first phosphorylation at the AL site is essential. In this step, the newly synthesized PKC is targeted to the membrane to allow the binding of membrane-bound PDK-1 to the C-terminus of PKC, which promotes phosphorylation at the AL site. After the phosphorylation, PDK-1 is released. The liberated C-terminus of PKC is then autophosphorylated via an intramolecular mechanism. The fully phosphorylated PKC is released into the cytosol, where they remain inactive until interacted by second messengers. For cPKC, Ca^{2+} and DAG second messengers recruit the enzyme to the plasma membrane, leading to activation of the enzyme and downstream signaling. In this study, we found that cocaine increased AL phosphorylation of PKC. This probably reflects an accelerated maturation of PKC in response to cocaine, which may lead to upregulation of PKC activity. In fact, elevated AL phosphorylation was accompanied by increased enzymatic activity in response to a variety of experimental manipulations (Cat et al., 2006; Peng et al., 2006; Kuri et al., 2007; Lin et al., 2007; Podar et al., 2007). Of note, the phospho-specific antibody used

in this study reacts with PKC γ . It may also cross-react with other PKC isoforms. The cross-reactive nature of the antibody needs to be further characterized experimentally. Thus, accurate implications of our results to PKC isoforms relies on further evaluation of the antibody cross-reactivity.

A large number of reports have documented an important role of PKC in neurochemical and behavioral effects of drugs (Lee and Messing, 2008). Early pharmacological studies show that the PKC inhibitor Ro31-8220 blocked amphetamine-mediated dopamine release in rat striatal slices (Kantor and Gnegy, 1998) and, when injected into the NAc, inhibited acute hyperlocomotion responses to amphetamine (Browman et al., 1998). A recent study confirmed that Ro31-8220 infused into the NAc attenuated amphetamine-stimulated dopamine overflow in the NAc (Loweth et al., 2009). Thus, acute amphetamine stimulates dopamine release and behavioral activity via a PKC-dependent mechanism. In a sensitization model in response to repeated drug exposure, intra-NAc injection of the PKC inhibitor bisindolymaleimide I attenuated the expression of behavioral sensitization to cocaine in rats (Pierce et al., 1998). Similarly, the PKC inhibitor chelerythrine suppressed the development of dopamine release sensitization to methamphetamine in mice (Narita et al., 2004). Thus, PKC participates into the neurochemical and behavioral sensitization to repeated stimulant administration. PKC is also believed to contribute to the rewarding effect of stimulants as measured using the conditioned place preference (CPP) procedure. Intracerebroventricular or intra-NAc injection of chelerythrine reduced the cocaine- or methamphetamine-stimulated CPP, respectively (Cervo et al., 1997; Narita et al., 2004). Similarly, Intra-amygdala injection of the PKC inhibitor NPC 15437 abolished the expression of CPP for cocaine (Lai et al., 2008). Intra-NAc injection of NPC 15437 blocked CPP for amphetamine (Aujla and Beninger, 2003).

It is currently unclear which PKC isozyme regulates stimulant action. In the case of morphine, PKC γ seems to be essential. This isoform was selectively upregulated in its expression in the limbic forebrain in morphine-conditioned mice, while other isozymes (α , β I and β II) were not (Narita et al., 2001). In loss of function experiments, PKC γ knockout mice showed a lack of CPP for morphine (Narita et al., 2001). In addition to PKC γ , studies with PKC ϵ knockout mice demonstrated the role of this isozyme. No CPP was induced in response to low doses of ethanol in PKC ϵ knockout mice, and instead conditioned place aversion was induced (Newton and Messing, 2007). PKC ϵ knockout mice also self-administered less ethanol (Olive et al., 2000). Of note, PKC is a regulator for many cellular and synaptic activities, and has numerous downstream substrates. Among these substrates are dopamine transporters and glutamate receptors which are the major central sites of action of psychostimulants. Available data show that PKC-mediated phosphorylation of these substrates is highly sensitive to cocaine (Cervinski et al., 2005; Wang et al., 2006; Bakshi et al., 2009; Kim et al., 2009; Zahniser and Sorkin, 2009). Thus, by regulating phosphorylation and thus function of these substrates and others, PKC is deemed to contribute substantially to neural plasticity and drug addiction.

4. Experimental procedures

4.1. Animals

Adult male Wistar rats weighing 225–275 g (Charles River, New York, NY) were individually housed in a controlled environment at a constant temperature of 23°C and humidity of 50 $\pm$ 10% with food and water available *ad libitum*. The animal room was on a 12-h/12-h light/dark cycle. Rats were allowed 5–6 days of habituation to the animal colony. All animal use and procedures were in strict accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

4.2. Systemic drug injection

Rats received an intraperitoneal (i.p.) injection of saline or cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO). The dose of cocaine was calculated as the salt. A high dose range of cocaine (40 mg/kg) was chosen because the drug at this dose caused typical motor stimulation and a reliable change in posttranslational modifications of proteins (phosphorylation and palmitoylation) in the striatum (Liu et al., 2006). Age-matched rats received an acute injection of saline (1 ml/kg) and served as controls.

4.3. Protein extraction

Rats were anesthetized with Equithesin (9 ml/kg, i.p.) and decapitated at time points indicated. Brains were quickly removed and cut into coronal sections. The CPU, NAc, and mPFC were dissected separately from sections. These regions were dissected into an 1.5 ml microtube containing ice-cold homogenization buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithioerythritol, 1% NP-40, 1% sodium deoxycholate, 1% SDS, a protease inhibitor cocktail (Thermo Scientific), and a phosphatase inhibitor cocktail (Thermo Scientific). Protein concentrations were determined and adjusted for Western blot analysis.

4.4. Western blot analysis

Western blot was performed as described previously (Yang et al., 2006; Mao et al., 2009). Briefly, the equal amount of protein was separated on SDS NuPAGE Novex 4–12% gels (Invitrogen, Carlsbad, CA). Proteins were transferred to the polyvinylidene fluoride membrane (Invitrogen) and blocked in blocking buffer (5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween 20) for 1 h. The blots were washed and incubated in the blocking buffer containing a primary rabbit antibody against phosphorylated species of PKC at the AL (T514) site (Abcam, Cambridge, MA; Cat. #: ab5778;), total conventional PKC (Santa Cruz Biotechnology, Santa Cruz, CA; Cat. #: sc-10800), phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) (Cell Signaling, Beverly, MA), total ERK1/2 (Cell Signaling), or actin (Santa Cruz Biotechnology) usually at 1:1000 overnight at 4°C. This was followed by 1 h incubation in a horseradish peroxidase-linked secondary antibody against rabbit (Jackson ImmunoResearch Laboratory, West Grove, PA) at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ), and captured into Kodak Image Station 2000R. Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) and MagicMark XP Western protein standards (Invitrogen) were used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software (Van Dolah et al., 2011). Same membranes were usually stripped and reprobed with next antibodies.

4.5. Dephosphorylation assays

The specificity of pPKC antibody was assessed in dephosphorylation assays. The striatum was dissected from brains removed from anesthetized rats. The dissected striatum was homogenized in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, and a protease inhibitor cocktail (EDTA-free). The homogenate was treated with the protein phosphatase, λ -phosphatase (Santa Cruz; Cat. #: sc-200312), to dephosphorylate proteins. To this end, 80 μ l homogenates (3.4 μ g/ μ l) were treated with 20 μ l 5X phosphatase buffer (final concentration: 20 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol, 0.01% BRIJ 35, and 2 mM MnCl_2) containing 800 units of λ -phosphatase for 1.5 h at 30°C. Dephosphorylation reaction was terminated by adding 33 μ l 4X LDS sample loading buffer. Control samples (80 μ l) were treated with only 20 μ l 5X phosphatase buffer without λ -phosphatase for 1.5 h at 30°C, followed by addition of 33 μ l 4X LDS. Both control and

dephosphorylated samples were boiled and subject to SDS-PAGE analysis (48 µg/well) and probed with pPKC-AL antibodies.

4.6. Statistics

The results are presented as means $\pm$ S.E.M., and were evaluated using a one-way analysis of variance followed by a Bonferroni (Dunn) comparison of groups using least squares-adjusted means or two-tailed unpaired Student's t-test. Probability levels of < 0.05 were considered statistically significant.

Highlights

1. Cocaine increased PKC phosphorylation in the rat nucleus accumbens and caudate putamen in a dose-dependent manner *in vivo*.
2. Cocaine also upregulated PKC phosphorylation in another limbic reward region, medial prefrontal cortex.
3. Cocaine-stimulated PKC phosphorylation was rapid and transient in a time-course study.
4. Since PKC phosphorylation is required for maturation of the kinase, our data identify the maturation processing as a specific step targeted by cocaine to modify PKC activity.

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Figure 1. Specificity of rabbit phospho- and site-specific antibodies

(A) A representative immunoblot with a rabbit anti-pPKC-AL antibody. Note that strong bands of approximately 75 kDa (A) were detected from the control rat striatal homogenates but not from homogenates treated with λ -phosphatase (λ -PP). (B) A representative immunoblot with a rabbit anti-PKC antibody. (C) A representative immunoblot with a rabbit anti-actin antibody. Striatal homogenates (80 μ l, 4 μ g/ μ l) were treated with λ -phosphatase (1,000 units) for 90 min at 30°C, followed by SDS-PAGE analysis.

Figure 2. Effects of cocaine on phosphorylation of PKC and ERK1/2 in the rat striatum
 (A and B) Effects of cocaine (Coc) at 40 mg/kg or saline (Sal) on PKC-AL (A) and ERK1/2 (B) phosphorylation in the CPu and NAc. Representative immunoblots are shown above to the quantified data. Note that cocaine significantly increased PKC-AL and ERK1/2 phosphorylation in both the CPu and NAc, while it did not alter tPKC and tERK1/2 abundance in these regions. (C and D) Effects of cocaine (10 mg/kg) or saline on PKC-AL (C) and ERK1/2 (D) phosphorylation in the CPu and NAc. Rats received a single dose of cocaine (10 or 40 mg/kg, i.p.) and sacrificed 7 min after injection for immunoblot analysis. Data are presented as means $\pm$ SEM ($n = 4-5$ per group). * $P < .05$ versus saline.

Figure 3. Effects of cocaine on phosphorylation of PKC and ERK1/2 in the rat mPFC
(A) Effects of cocaine (Coc) or saline (Sal) on PKC-AL phosphorylation in the mPFC. **(B)** Effects of cocaine or saline on ERK1/2 phosphorylation in the mPFC. Representative immunoblots are shown above to the quantified data. Note that cocaine significantly increased PKC-AL in the mPFC, while it did not alter ERK1/2 phosphorylation in this region. Rats received a single dose of cocaine (40 mg/kg, i.p.) and sacrificed 7 min after injection for immunoblot analysis. Data are presented as means $\pm$ SEM (n = 4 per group). * P < .05 versus saline.

Figure 4. Time-dependent effects of cocaine on phosphorylation of PKC and ERK2 in the rat CPu

(**A**) Time-dependent effects of cocaine on PKC-AL phosphorylation. (**B**) Time-dependent effects of cocaine on ERK2 phosphorylation. Note that cocaine induced a rapid and transient increase in PKC-AL and ERK2 phosphorylation in the CPu. Rats received a single dose of cocaine (40 mg/kg, i.p.) and sacrificed at different time points (7, 15, or 60 min) after drug injection for immunoblot analysis. Data are presented as means $\pm$ SEM ($n = 4-6$ per group).

* $P < .05$ versus saline.

Figure 5. Time-dependent effects of cocaine on phosphorylation of PKC and ERK2 in the rat NAc

(**A**) Time-dependent effects of cocaine on PKC-AL phosphorylation. (**B**) Time-dependent effects of cocaine on ERK2 phosphorylation. Note that cocaine induced a rapid and transient increase in PKC-AL and ERK2 phosphorylation in the NAc. Rats received a single dose of cocaine (40 mg/kg, i.p.) and sacrificed at different time points (7, 15, or 60) min after drug injection for immunoblot analysis. Data are presented as means $\pm$ SEM ($n = 4-6$ per group).

* $P < .05$ versus saline.