

Calcium-Mediated Second Messengers Modulate the Expression of Behavioral Sensitization to Cocaine¹

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Accepted for publication April 15, 1998 This paper is available online at <http://www.jpet.org>

ABSTRACT

To assess the influence of calcium channel antagonists on the expression of behavioral sensitization to cocaine, the L-type calcium channel antagonist diltiazem or the N-type calcium channel antagonist ω -conotoxin GVIA was microinjected into the medial nucleus accumbens before a systemic cocaine challenge injection among rats that were previously treated with daily systemic saline or cocaine injections. The results indicated that both of these drugs attenuated the expression of behavioral sensitization to cocaine. Among saline-pretreated rats, diltiazem did not influence the behavioral response to an acute injection of cocaine, whereas ω -conotoxin significantly impaired acute cocaine-induced behavioral hyperactivity. A second series of experiments assessed the influence of protein kinases on the expression of behavioral sensitization to cocaine. Inhibitors of calcium/calmodulin-dependent protein kinase II (KN-93, N-[2-[[[3-(4'-chlorophenyl)-2-propenyl]meth-

ylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxybenzenesulfonamide phosphate), protein kinase A (H-89, N-[2((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide) or calcium-dependent protein kinase C (bisindolymaleimide I, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) were microinjected into the medial nucleus accumbens before a challenge injection of cocaine among rats repeatedly administered either saline or cocaine. None of the kinase inhibitors influenced the behavioral response induced by cocaine in saline-pretreated rats. Among cocaine-sensitized animals, the microinjection of KN-93 or bisindolymaleimide I blocked the expression of behavioral sensitization to cocaine, whereas H-89 had no effect. Taken together, these results indicate that neuronal calcium, acting *via* calcium-dependent kinases, promotes the expression of behavioral sensitization to cocaine.

A growing body of evidence indicates that calcium and calcium-mediated second messenger systems play an important role in the expression of behavioral sensitization to psychostimulants. The expression of the sensitized behavioral response to amphetamine or cocaine is inhibited by the systemic injection of an L-type calcium channel antagonist, whereas the acute behavioral response to these psychostimulants is unaffected (Pani *et al.*, 1990; Karler *et al.*, 1991; Martin-Iverson and Reimer, 1994). One of the main neurochemical alterations associated with the expression of behavioral sensitization is an enhancement in the ability of psychostimulants to increase extracellular dopamine in the nucleus accumbens and striatum (Kalivas and Stewart, 1991; Robinson and Berridge, 1993; Pierce and Kalivas, 1997b). *In vivo* microdialysis experiments revealed that the sensitized dopamine release in the nucleus accumbens in-

duced by amphetamine becomes calcium-dependent among animals sensitized to a psychostimulant. Thus, substitution of magnesium for calcium in the microdialysis buffer eliminated the sensitized portion of the amphetamine-induced increase in extracellular dopamine in the nucleus accumbens of rats sensitized to this stimulant (Warburton *et al.*, 1996). Similarly, incorporating an L- or N-type calcium channel antagonist or an inhibitor of CaM-KII into the microdialysis buffer blocked the augmented dopamine release in the nucleus accumbens induced by the local administration of amphetamine among rats sensitized to repeated injections of cocaine (Pierce and Kalivas, 1997a). The systemic administration of an L-type calcium channel antagonist also blocked the sensitized increase in extracellular dopamine in the neostriatum produced by cocaine in cocaine-sensitized rats (Pani *et al.*, 1990). Taken together, these results indicate that calcium and calcium-mediated second messenger systems influence the expression of behavioral sensitization by eliminating the sensitized increase in dopamine in the nucleus accumbens and striatum of animals treated repeatedly with psychostimulants.

Received for publication January 22, 1998.

¹This work was supported by the Washington State Alcohol and Drug Abuse Program, United States Public Health Service Grants MH40817 and DA03906 and Research Career Development Award DA00158 (P.W.K.), as well as a National Alliance for Research on Schizophrenia and Depression Young Investigator Award and USPHS grant DA11168 (R.C.P.).

ABBREVIATIONS: ANOVA, analysis of variance; CaM-KII, calcium/calmodulin-dependent protein kinase II; PKA, protein kinase A; PKC, protein kinase C.

The present experiments were designed to verify that perturbations in calcium signaling in the nucleus accumbens influence the behavioral expression of sensitization to cocaine. Among animals pretreated with either cocaine or saline, the influence of the microinjection of a calcium channel antagonist into the medial nucleus accumbens on the behavioral hyperactivity produced by a systemic injection of cocaine was assessed. In addition, drugs that block the activity of CaM-KII, PKA or PKC were administered into the nucleus accumbens before the assessment of the behavioral expression of sensitization to cocaine.

Materials and Methods

Subjects. Male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were individually housed with food and water available *ad libitum*. A 12/12-hr light/dark cycle was used with the lights on at 7:00 a.m. All cocaine injections and behavioral testing were performed during the light cycle.

Repeated cocaine or saline treatment. The day before the start of the experiment, all rats (weighing 250–350 g) were habituated to the photocell boxes (Omnitech Electronics, Columbus, OH) for 3 hr. On the first treatment day, all animals were habituated to the photocell boxes for 1 hr. Following habituation, animals received either cocaine (15 mg/kg i.p.) or saline (1.0 ml/kg i.p.) and behavior (horizontal photocell beam breaks) was monitored for 2 hr. On days 2 to 6, cocaine rats received daily injections of cocaine (30 mg/kg, i.p.) while control animals received saline in their home cages. On the seventh day, all animals were again habituated to the photocell boxes for 1 hr followed by the administration of cocaine (15 mg/kg i.p.) or saline and behavior was monitored for 2 hr postinjection. Thus, approximately half of the subjects received 7 administrations of cocaine while the remainder were injected daily with saline.

Surgery. Two weeks after the last repeated daily injection of cocaine or saline, the rats were anesthetized with Equithesin (3.0 ml/kg) and mounted in a stereotaxic apparatus. Cannulae (14 mm, 26 gauge) were implanted bilaterally 2 mm dorsal to the shell of the nucleus accumbens (1.0 mm A/P; .8 mm M/L; 6.0 mm D/V relative to bregma; Paxinos and Watson, 1986) and cemented in place by affixing dental acrylic to three stainless steel screws tapped into the skull.

Microinjections. One week after surgery (*i.e.*, 21 days after the end of the repeated cocaine or saline regimen), the rats were habituated to the photocell cell apparatus (Omnitech Electronics) for 1 hr. Following this adaptation period, the obturators were removed from the microinjection guide cannulae and replaced by injection needles (33 gauge stainless steel), which extended 2 mm below the tips of the guide cannulae into the nucleus accumbens shell. In all cases, the bilateral microinjections were made over 60 sec in a volume of 0.5 μ l/side; the injector was left in place for 30 sec to allow the drug or vehicle to diffuse from the tips of the cannulae.

The saline-pretreated rats received a microinjection of either the L-type calcium channel antagonist diltiazem (10 nmol/0.5 μ l), the N-type calcium channel antagonist ω -conotoxin (0.005 nmol/0.5 μ l), the CaM-KII inhibitor KN-93 (10 nmol/0.5 μ l), the PKA inhibitor H-89 (10 nmol/0.5 μ l), the PKC inhibitor bisindolymaleimide I (10 nmol/0.5 μ l) or the vehicle (0.9% saline or 10% DMSO) used to dissolve these drugs. Cocaine (15 mg/kg, i.p.) was injected 10 min after the microinjection and the rat was returned to the photocell cage for a 2-hr behavioral testing period. The animals in the repeated cocaine group underwent a 3- to 4-day microinjection regimen. On each day the rats received a microinjection of either diltiazem (1 or 10 nmol/0.5 μ l), ω -conotoxin (0.0005, 0.005 or 0.05 nmol/0.5 μ l), KN-93 (1 or 10 nmol/0.5 μ l), H-89 (1 or 10 nmol/0.5 μ l), bisindolymaleimide I (1 or 10 nmol/0.5 μ l) or the vehicle in which each of these drugs was dissolved. For the cocaine-pretreated rats, the drug and vehicle injections were counterbalanced over the 3- to 4-day micro-

injection regimen. Ten minutes after the microinjection, 15 mg/kg cocaine was injected (i.p.) and behavioral activity was monitored for 2 hr.

Drugs. Cocaine was obtained as a gift from the National Institute on Drug Abuse. All other drugs were purchased from Calbiochem (San Diego, CA). Cocaine, diltiazem and ω -conotoxin were dissolved in 0.9% sterile saline. The kinase inhibitors were dissolved in 10% DMSO. All drug doses were expressed as the salt form. The doses of the calcium channel antagonists and KN-93 were based on doses that effectively blocked the amphetamine-induced sensitized increase in accumbal dopamine when the drugs were applied *via* a microdialysis probe (Pierce and Kalivas, 1997a). The doses of H-89 and bisindolymaleimide I used in this study were ~0.4 to 4 times the IC₅₀ values calculated from *in vitro* experiments (Chijiwa *et al.*, 1990; Toullec *et al.*, 1990). It should be noted that both H-89 and bisindolymaleimide I have nonspecific inhibitory PKA and PKC activity, respectively. However, H-89 effectively inhibits PKC at a dose ~660 times more potent than the highest dose used in the current study (Chijiwa *et al.*, 1990), whereas a dose of bisindolymaleimide I ~200 times greater than the highest used in the current study is required to inhibit PKA (Toullec *et al.*, 1990).

Histology. Following the behavioral experiments, rats were given an overdose of pentobarbital (<100 mg/kg i.p.) and perfused intracardially with phosphate-buffered saline followed by 10% formalin. The brain was removed and stored in 10% formalin for at least 1 week. The brains then were blocked, and coronal sections (100 μ m) were taken at the level of the nucleus accumbens with a vibratome. The sections were mounted on gelatin-coated slides and stained with cresyl violet. Probe and cannula placements were determined according to the atlas of Paxinos and Watson (1986) by an individual unaware of the subjects' behavioral response.

Results

Effect of calcium channel antagonists. As shown in figure 1, the microinjection of the L-type calcium channel antagonist (diltiazem) or the N-type calcium channel antagonist (ω -conotoxin) dose-dependently impaired the expression of cocaine behavioral sensitization. In both experiments, the behavioral response to cocaine after a vehicle microinjection was significantly greater than the cocaine-induced behavioral hyperactivity recorded on day 1. The highest dose of diltiazem (10 nmol/0.5 μ l) produced a significant decrease in the behavioral effect of cocaine relative to the response observed after a vehicle microinjection. Similarly, microinjection of the two highest doses of conotoxin (0.005 and .05 nmol/0.5 μ l) produced significant reductions in the behavioral response to cocaine. In addition, the highest dose of conotoxin completely blocked behavioral sensitization to cocaine since the behavioral response to cocaine was not significantly greater than the effect of cocaine observed on day 1.

The influence of intra-accumbal microinjections of diltiazem (10 nmol/0.5 μ l) or ω -conotoxin (0.005 nmol/0.5 μ l) on the acute behavioral response to 15 mg/kg cocaine measured in saline-pretreated rats is shown in table 1. Although diltiazem did not attenuate the behavioral response to cocaine, ω -conotoxin produced a significant decrease in cocaine-induced behavioral hyperactivity.

Effect of inhibitors of CaM-KII, PKA and PKC. As shown in figure 2, the CaM-KII (KN-93) and PKC (bisindolymaleimide I) inhibitors blocked the expression of behavioral sensitization to cocaine in a dose-dependent manner; the PKA inhibitor (H-89) had no effect. In all three experiments,

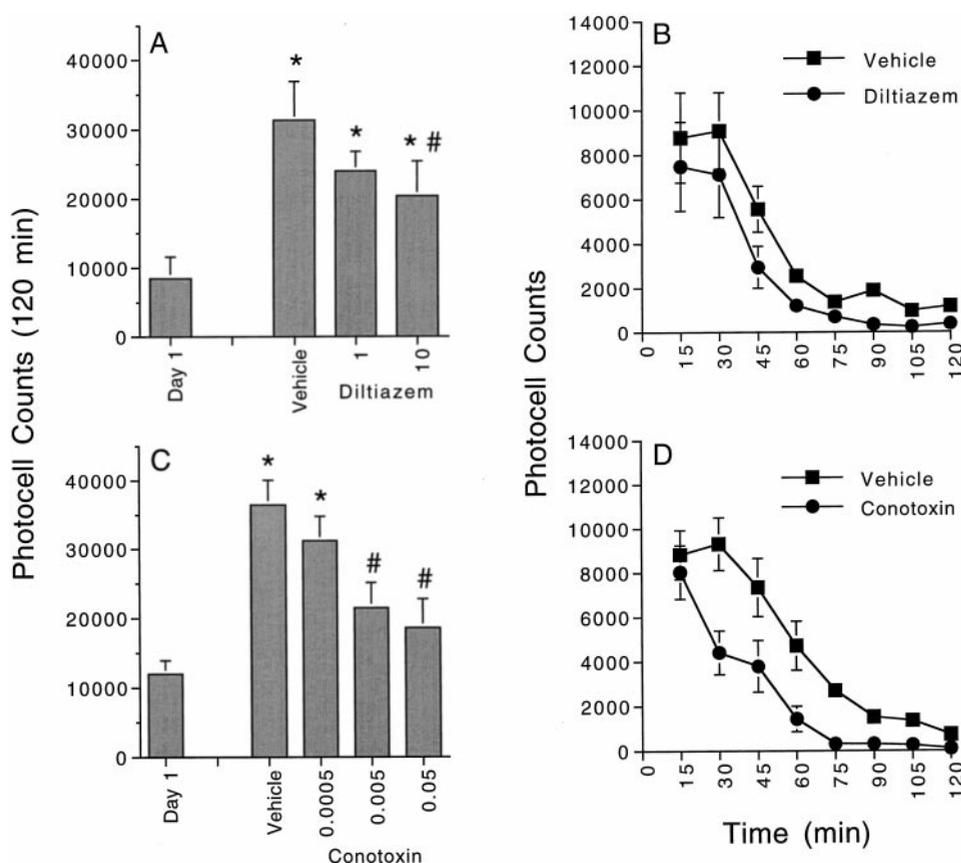


Fig. 1. Effect of the microinjection of L-type (diltiazem, $n = 8$) or N-type (ω -conotoxin, $n = 10$) calcium channel antagonists into the shell of the nucleus accumbens on the expression of behavioral sensitization to cocaine. The data presented in A and C represent the total photocell counts recorded over the 120 min after the injection of cocaine. These data were analyzed with separate one-way repeated measures ANOVAs. The analyses of the data revealed significant main effects of treatment for diltiazem [$F(4,28) = 5.46$, $P < 0.0022$] and ω -conotoxin [$F(5,45) = 7.19$, $P < .0001$]. The time courses of the hyperactive behavioral response induced by cocaine after a challenge injection of vehicle or the highest doses of diltiazem or ω -conotoxin are represented in B and D, respectively. *Significant difference from day 1 (Fisher's LSD). #Significant difference from vehicle (Fisher's LSD)

TABLE 1

Effect of the microinjection of an L-type calcium channel antagonist, diltiazem, an N-type calcium channel antagonist, ω -conotoxin, a CaM-KII inhibitor, KN-93, a PKA inhibitor, H-89, a PKA inhibitor, bisindolymaleimide I or vehicle (0.9% saline or 10% DMSO) into the shell of the nucleus accumbens on the acute behavioral response elicited by cocaine.

Three weeks after 7 daily saline injections, one of these drugs or its vehicle was microinjected into the shell of the nucleus accumbens 10 min before the systemic injection of cocaine (15 mg/kg). Behavior was recorded over the 120-min period after the cocaine injection. The data are presented as mean horizontal photocell beam breaks with standard error in parentheses. Of the five drugs tested, only conotoxin significantly influenced the behavioral response elicited by cocaine.

Drug/Vehicle	<i>n</i>	Behavioral Activity ^a	Statistics ^b
Diltiazem (10 nmol)	8	27115 (7109)	$t(12) = 0.69$, $P < .501$
Saline	6	19683 (7922)	
Conotoxin (0.005 nmol)	6	8340 (1470)	$t(10) = 3.47$, $P < .006$
Saline	6	29023 (5776)	
KN-93 (10 nmol)	7	16686 (4078)	$t(10) = 0.28$, $P < .788$
DMSO	5	18769 (6954)	
H-89 (10 nmol)	8	17972 (6033)	$t(13) = 0.63$, $P < .539$
DMSO	7	22504 (3370)	
Bisindolymaleimide I (10 nmol)	8	12832 (3685)	$t(14) = 0.57$, $P < .580$
DMSO	8	15855 (3866)	

^a The total horizontal behavioral activity (photocell beam breaks) recorded during the 120-min period after the injection of cocaine (15 mg/kg, i.p.).

^b Unpaired *t* test.

behavioral sensitization to cocaine was observed 21 to 23 days after the repeated cocaine injection regimen in that the behavioral response to cocaine recorded after a vehicle microinjection into the shell of the nucleus accumbens was significantly greater than day 1. While the highest dose of bisindolymaleimide I completely blocked the sensitized behavioral response, the capacity of KN-93 to antagonize behavioral sensitization was only partial.

The influence of intra-accumbal microinjections of KN-93 (10 nmol/0.5 μ l), H-89 (10 nmol/0.5 μ l) or bisindolymaleimide

I (10 nmol/0.5 μ l) on the behavioral hyperactivity induced by 15 mg/kg cocaine in saline-pretreated rats is shown in table 1. None of these kinase inhibitors influenced the acute behavioral response to cocaine as reflected by the lack of significant differences between the behavioral responses recorded in rats that received a microinjection of the kinase inhibitors and the 10% DMSO solution in which these drugs were dissolved.

Histology. As shown in figure 3, all of the microinjection sites were in the medial limb of the shell of the nucleus

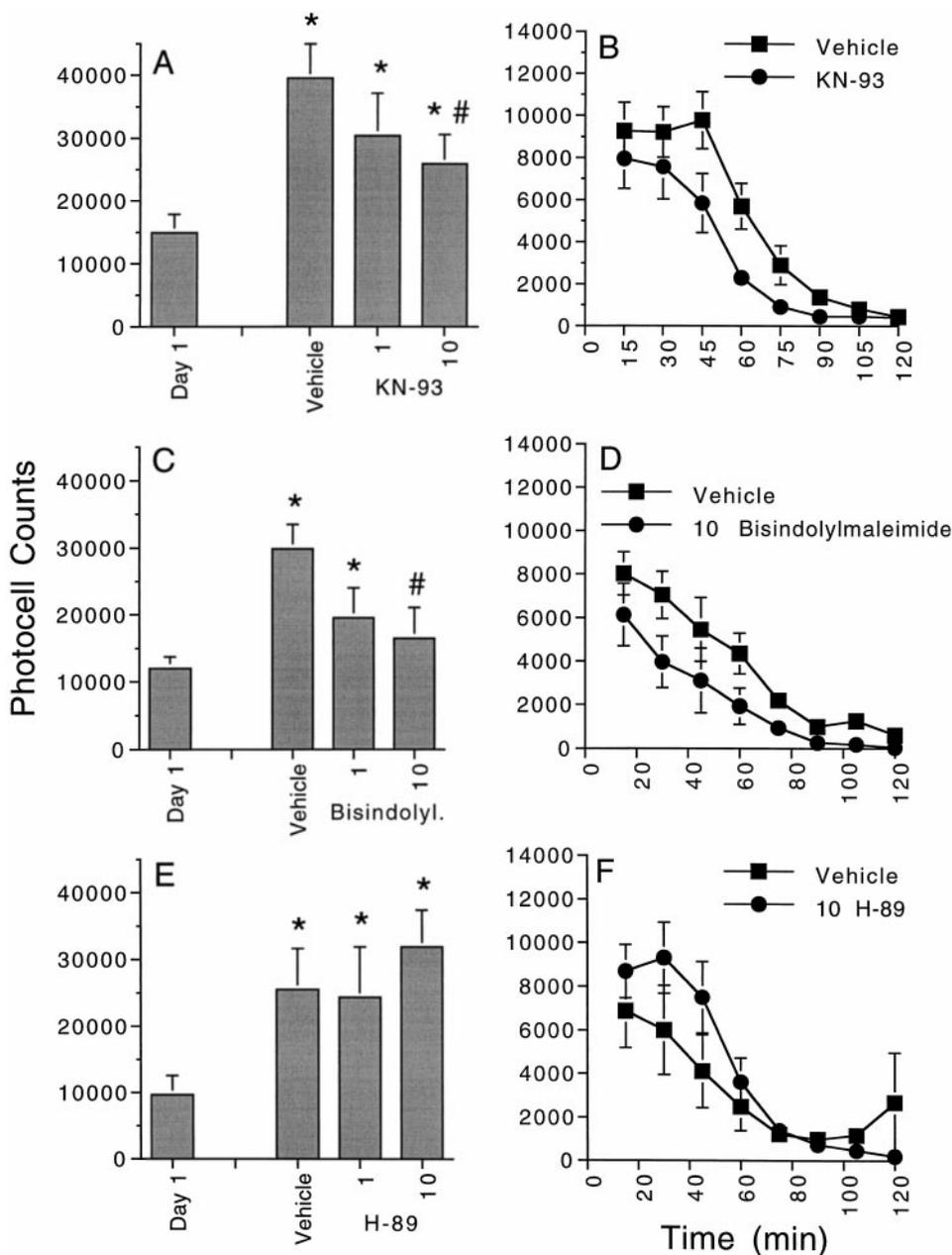


Fig. 2. Effect of microinjection of CaM-KII (KN-93, $n = 10$), PKC (bisindolymaleimide I, $n = 10$) or PKA (H-89, $n = 8$) inhibitors into the nucleus accumbens shell on the expression of behavioral sensitization to cocaine. The data presented in A, C and E represent the total photocell counts recorded over the 120 min after the injection of cocaine. These data were analyzed with separate one-way repeated measures ANOVAs. The analyses revealed significant main effects of treatment with KN-93 [$F(4,36) = 5.32, P < .0018$] and bisindolymaleimide I [$F(4,36) = 3.47, P < .017$] data (see A and C). There was a marginally significant main effect of treatment with the H-89 data shown in E [$F(4,28) = 2.53, P < .063$]. The time courses of the hyperactive behavioral responses induced by cocaine after a challenge injection of vehicle or the highest doses of KN-93, bisindolymaleimide I or H-89 are represented in B, D and F, respectively. *Significant difference from day 1 (Fisher's LSD). #Significant difference from vehicle (Fisher's LSD)

accumbens or on the border between the medial shell and the core of the nucleus accumbens.

Discussion

The data in this study indicate that the blockade of L- and N-type calcium channels in the nucleus accumbens impairs the expression of behavioral sensitization to cocaine. Likewise, inhibition of CaM-KII and PKC blocked the expression of sensitization. Although the L-type calcium channel antagonist and the inhibitors of CaM-KII and PKC did not influence the acute behavioral response to cocaine, the N-type calcium channel antagonist significantly impaired cocaine-induced behavioral hyperactivity in saline-pretreated rats. This suggests that N-type calcium channels may not play a selective role in the expression of behavioral sensitization to cocaine. Taken together, these results are consistent with the hypothesis that increases in cytosolic calcium result in an

enhancement in calcium-mediated second messengers in the dopaminergic projections to the ventral forebrain that play a critical role in the expression of behavioral sensitization to cocaine (Gnegy *et al.*, 1997; Pierce and Kalivas, 1997a).

One of the main supports for the dopamine hypothesis of behavioral sensitization is the enhanced release of dopamine in the nucleus accumbens and striatum of rats sensitized to amphetamine-like psychostimulants (Kalivas and Stewart, 1991; Robinson and Berridge, 1993; Pierce and Kalivas, 1997b). Recent evidence indicates that this enhanced increase in dopamine in sensitized rats is blocked by the administration of L- or N-type calcium channel antagonists as well as a CaM-KII inhibitor (Pani *et al.*, 1990; Warburton *et al.*, 1996; Pierce and Kalivas, 1997a). Calcium and calcium-mediated second messengers could influence the sensitized increase in striatal/accumbal dopamine induced by cocaine and related dopamine reuptake blockers by blocking vesicu-

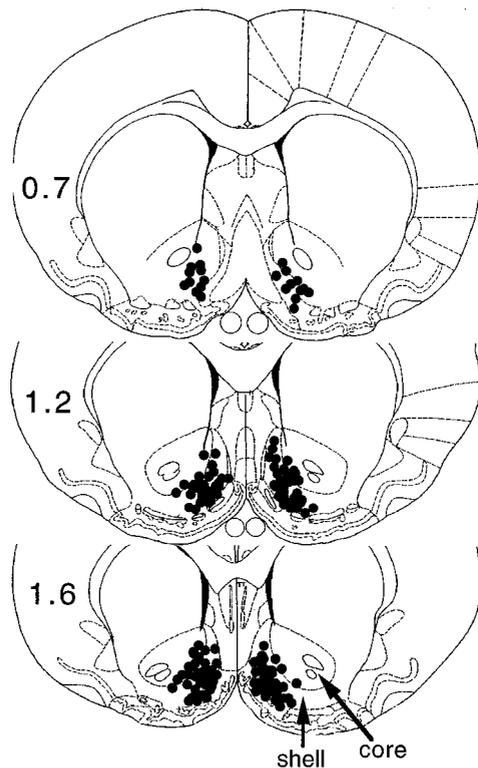


Fig. 3. A schematic diagram of brain sections from the atlas of Paxinos and Watson (1986) taken at the level of the nucleus accumbens (the numbers indicate millimeters anterior to bregma). The dots represent the location of the tips of the microinjection cannulae. Note that all of the microinjection placements were in the medial shell of the nucleus accumbens or on the border between the shell and the core.

lar exocytosis or impairing the activation of calcium-mediated second messengers.

The increase in extracellular dopamine induced by reuptake blockers requires the release of vesicular dopamine and is, therefore, dependent on the entry of calcium into the dopaminergic terminal *via* voltage-dependent calcium channels (Westerink *et al.*, 1989). Thus, the capacity of N-type calcium channel blockers to inhibit the behavioral effects of cocaine in both naive and cocaine-sensitized rats may be due to a direct effect on vesicular exocytosis. A growing body of evidence indicates that calcium influx through N-type, but not L-type, calcium channels is responsible for vesicular exocytosis (Hirning *et al.*, 1988; Miller and Freedman, 1984; Miller, 1987). For example, N-type calcium channel antagonists impair calcium influx into rat brain synaptosomes, whereas L-type calcium channel antagonists have no effect (Daniell *et al.*, 1983; Yamada *et al.*, 1993). Similarly, in synaptosomes or slices obtained from the rat striatum, N-type calcium channel antagonists attenuated potassium-stimulated release of tritiated dopamine, whereas L-type calcium channel antagonists had no influence on the excretion of tritiated dopamine (Carvalho *et al.*, 1995; Yamada *et al.*, 1993; Bowyer and Weiner, 1990). These results are consistent with the suggestion that N-type calcium channels are localized in nerve terminals, whereas L-type calcium channels are found in the cell bodies and proximal dendrites (Westenbroek *et al.*, 1990; Nowycky *et al.*, 1985). However, there may be L-type calcium channels on the terminals of dopaminergic afferents to the striatum since dopamine-

depleting lesions significantly reduce the quantity of L-type calcium channels in the striatum (Daniell *et al.*, 1983; but see also Sanna *et al.*, 1986). The putative terminal L-type calcium channels do not appear to influence dopamine release since blockade of L-type calcium channels does not influence the electrically stimulated release of dopamine in the striatum (Mitchell and Adams, 1993) or potassium-induced release of tritiated dopamine from cultures of mesencephalic dopamine neurons (de Erausquin *et al.*, 1992). Collectively, these findings indicate that L-type calcium channels may be expressed in the terminals of dopamine neurons in the basal forebrain, but calcium influx through these channels is not involved in rapid vesicular exocytosis. Thus, whereas calcium influx through N-type calcium channels appears to mediate vesicular exocytosis, the movement of calcium through L-type channels likely is involved in the regulation of calcium-dependent protein kinases. This conclusion is consistent with the present data, which indicate that blockade of L-type calcium channels, the inhibition of CaM-KII or the inhibition of PKC attenuate only the sensitized behavioral response to cocaine, whereas the antagonism of N-type calcium channels impairs both the acute and sensitized behavioral effect of cocaine.

A second potential mechanism through which calcium may influence sensitized dopamine release involves CaM-KII-induced phosphorylation of vesicular proteins. In preparation for exocytosis, vesicles must unbind from intracellular protein filaments and migrate into the active zone near the plasmallemal membrane where the calcium channels responsible for exocytosis are embedded. Vesicles are attached to cytoskeletal filaments via proteins known as synapsins, which must be phosphorylated for the vesicle to unbind (Burgoune and Morgan, 1995; Schweizer *et al.*, 1995); the primary kinase responsible for phosphorylating synapsin is CaM-KII (Lin *et al.*, 1990). Thus, calcium channel antagonists and CaM-KII inhibitors also can influence the release of accumbal dopamine by preventing dopamine vesicles from unbinding and moving into the active zone in preparation for exocytosis (see Pierce and Kalivas, 1997a). Recent evidence supports the proposal that the phosphorylation of synapsin I by CaM-KII underlies the influence of calcium and calcium-mediated second messengers on the expression of behavioral sensitization to psychostimulants. Sensitized release of dopamine was demonstrated in striatal synaptosomes of animals sensitized to amphetamine, and this effect was blocked by a CaM-KII inhibitor (Gnegy *et al.*, 1997). Consistent with this finding, increases in calmodulin, CaM-KII activity and site 3-phosphosynapsin I have been reported in striatal synaptosomes of amphetamine-sensitized rats (Gnegy *et al.*, 1997; Iwata *et al.*, 1996; Gnegy *et al.*, 1991). Taken together, these results support the hypothesis that neuronal calcium, acting specifically through CaM-KII, influences sensitized dopamine release in the basal forebrain of animals repeatedly treated with psychostimulants by altering the phosphorylation state of synapsin I.

The present results also demonstrate that the intra-accumbal administration of bisindolymaleimide I, an inhibitor of PKC, impairs the expression of behavioral sensitization to cocaine. This behavioral effect could be due to an effect of PKC inhibition on dopamine release since vesicular exocytosis can be stimulated by either calcium influx or the activation of PKC (Billiard *et al.*, 1997). The lack of an effect of

bisindolymaleimide I on the acute behavioral response to cocaine suggests that changes in PKC may play a specific role in the expression of cocaine behavioral sensitization. However, recent evidence indicates that there is no change in PKC activity in striatal synaptosomes prepared from animals sensitized to amphetamine (Gnegy *et al.*, 1997), which suggests that alterations in PKC activity do not contribute to the expression of behavioral sensitization to amphetamine. Taken together, these data suggest that there may be a divergence in the role of PKC in the expression of behavioral sensitization to cocaine and amphetamine.

In summary, the present data demonstrate that drugs that are capable of attenuating sensitized dopamine release in the nucleus accumbens and striatum of sensitized animals, such as calcium channel antagonists and a CaM-KII inhibitor (Gnegy *et al.*, 1997; Pierce and Kalivas, 1997a) also impair the behavioral expression of sensitization to cocaine. These data are consistent with the hypothesis that calcium influx into dopamine terminals may preferentially activate calmodulin/CaM-KII transduction and the subsequent phosphorylation of synapsin, which appears to contribute selectively to the enhanced release of dopamine in the nucleus accumbens of animals sensitized to psychostimulants. It should be noted that there may be changes in calcium-dependent protein kinases in other cells of the striatal complex. For example, among amphetamine-pretreated rats there is a decrease in the ability of calmodulin to potentiate D₁ receptor-mediated increases in striatal adenylyl cyclase activity (Roseboom *et al.*, 1990), which suggests that postsynaptic changes in calcium-dependent protein kinases also may influence the expression of behavioral sensitization. The role of calcium and calcium-mediated protein kinases in the expression of behavioral sensitization is clearly complex; further work is necessary to delineate the specific influences of presynaptic and postsynaptic changes in calcium-stimulated second messenger systems on the modulation of locomotor behavior by the nucleus accumbens and striatum.

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