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Pharmacological Profile of a Novel Neuronal Calcium Channel Blocker Includes Reduced Cerebral Damage and Neurological Deficits in Rat Focal Ischemia

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BARONE, F. C., W. J. PRICE, P. JAKOBSEN, M. J. SHEARDOWN AND G. FEUERSTEIN. *Pharmacological profile of a novel neuronal calcium channel blocker includes reduced cerebral damage and neurological deficits in rat focal ischemia.* PHARMACOL BIOCHEM BEHAV 48(1) 77–85, 1994.—Excessive calcium entry into depolorized neurons contributes significantly to cerebral tissue damage following ischemia. Therefore, blocking voltage-operated calcium channels on nerve cells should provide significant neuroprotection in ischemia. We now report on a novel neuronal calcium channel blocker, NNC 09-0026, in terms of its selective effects on neuronal calcium current and its efficacy in reducing infarct size and neurological deficits in a rat model of focal stroke. In the present studies, the effects of NNC 09-0026 on neuronal calcium influx, calcium channel binding, and cardiovascular parameters were determined. Also, phencyclidine, NNC 09-0026, or vehicle were administered IV to rats subjected to permanent middle cerebral and common carotid artery occlusions. Infarct volumes and contralateral forepaw and hindlimb neurological deficits were assessed at 24 and 48 h after onset of stroke. NNC 09-0026 exhibited a pharmacological profile suggesting selectivity at neuronal calcium channels. It inhibited potassium-stimulated calcium uptake into rat synaptosomes with an IC_{50} of 13 μM. Voltage-operated calcium currents measured from cultured rat dorsal root ganglion cells using the patch clamp technique were blocked by 43% at 10 μM ($p < 0.05$). The compound showed only weak effects on smooth muscle from the guinea pig taenia coli and was relatively inactive at displacing nitrendipine and ω-conotoxin in receptor-binding studies. Single, bolus injections of NNC 09-0026 as high as 10 mg/kg IV produced only 12% reduction in heart rate and a 28% decrease in blood pressure. Phencyclidine, 1.5 or 3 mg/kg, IV bolus 30 min pre- and 24 h postocclusion, reduced infarct volume by 46% and 52% ($p < 0.05$) and decreased neurological deficits in a parallel manner. Phencyclidine did produce significant neurobehavioral side effects in all animals. NNC 09-0026, 30 mg/kg, IV administered slowly over a 1-h period beginning 30 min postischemia, reduced infarct volume by 45% and neurological deficits ($p < 0.05$). No obvious neurobehavioral side effects were produced by NNC 09-0026. These data indicate that NNC 09-0026 is a relatively selective neuronal calcium channel blocker that can provide significant neuroprotection in a rat model of focal ischemia.

Neuronal calcium channels Neuroprotection Focal ischemia Middle cerebral artery occlusion
Phencyclidine NNC 09-0026 Calcium currents Neurological deficits

CALCIUM is a critical regulator and second messenger of many cell functions, yet in high intracellular concentrations it has been argued that calcium mediates cell damage and death (39). Loss of cellular calcium homeostasis appears to account for neuronal death in several different pathophysiological states such as brain ischemia, hypoglycemia, and epileptic sei-

Animals were housed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (DHEW [DHHS] Publication No. [NIH] 85-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205). Procedures using lab animals were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals, plc.

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zures (44). Indeed, neuronal loss associated with high levels of excitatory amino acid neurotransmitters released during ischemia (8,19,20) apparently is due to excessive calcium influx (13,38). These recent data strongly suggest that the "excitotoxic hypothesis of neuronal death" and the "calcium hypothesis of neuronal damage" are coupled (14), and that activation of voltage-operated calcium channels (VOCCs) and receptor-operated calcium channels both contribute to neuronal damage.

The primary evidence for a role of excitotoxicity in mediating ischemic neuronal injury derives from data showing that glutamate receptor antagonists in models of cerebral focal ischemia (1,10,12,26,34,42,43,47) can reduce neuronal injury. In focal ischemia, increased excitatory neurotransmitter release (20) results in an inability to maintain normal membrane potential (44) and produces cellular depolorization and increased calcium influx via VOCCs (25,40). The role of calcium channel (i.e., VOCC) blockade in neuroprotection has been reviewed recently (16). Several VOCCs display characteristic electrophysiologic and pharmacological differences (27). The L-type channel is important for excitation-response coupling in cardiac muscle, smooth muscle, and neurons, and is blocked by dihydropyridines (i.e., nifedipine, nimodipine, etc.) and other organic VOCC blockers (i.e., diltiazem and verapamil). The N-type channel is important in neuronal transmission and is blocked by ω-conotoxin (16,27). The L-type VOCC has been studied most extensively, and available data suggest that the molecular biology and tissue diversity of VOCCs may provide achievement of tissue selectivity (45). However, the contribution of VOCC antagonist selectivity (i.e., for vascular vs. neuronal tissue) to protection from cerebral ischemia has not been defined. The lack of selectivity of available VOCC antagonists may explain their variable efficacy in cerebral ischemia/stroke [for review see (16)].

The novel Novo Nordisk compound (−)-*trans*-1-butyl-4-(4-dimethylaminophenyl)-3-(4-trifluoromethylphenoxymethyl) pyperidine dihydrochloride (NNC 09-0026) is one of a series of pyperidines identified to be relatively potent on neuronal VOCCs without cardiovascular effects. This compound has been shown to be neuroprotective in severe transient forebrain ischemia (22,42).

The purpose of the present series of experiments was to examine the efficacy of NNC 09-0026 on neuronal calcium influx, smooth muscle function, cardiovascular variables, and neuroprotection from focal ischemic damage leading to brain infarction and neurological deficits. The effects of NNC 09-0026 [synthesized at Novo Nordisk, Denmark (22)] on focal ischemia were compared to the NMDA noncompetitive receptor antagonist phencyclidine (1-(1-phenylcyclohexyl) piperidine hydrochloride) (Fig. 1) which was previously shown to convey neuroprotection from focal ischemia in the rat (9,10).

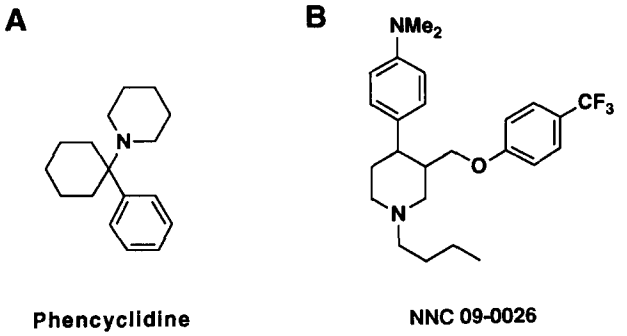

FIG. 1. Chemical structures of phencyclidine and NNC 09-0026.

MATERIALS AND METHODS

Neuronal Calcium Influx/Currents

Synaptosomal calcium influx. Adult male Wistar rats were decapitated and the cerebral cortex removed and homogenized in 20 ml of ice-cold 0.32-M sucrose using a glass homogenizer with a Teflon pestle. All subsequent steps for isolation of synaptosomes were conducted at 0–4°C (28,37). The homogenate was centrifuged at 1000 × *g* for 10 min and the resulting supernatant was centrifuged again at 18 000 × *g* for 20 min. The pellet then was resuspended in 0.32-M sucrose (10 ml per g of original tissue) with a Teflon pestle. A 50-μl aliquot of the crude synaptosomal suspension was added to glass tubes containing 0.625 ml of NaCl buffer (136 mM NaCl, 4 mM KCl, 0.35 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM tris(hydroxymethyl)aminomethane (Tris) HCl, 12 mM glucose, pH 7.4) and 25 μl of solutions containing NNC 09-0026 or nifedipine (dissolved in 48% ethanol to give final concentrations of 0.1, 0.3, 1, 3, and 10 μg/ml). Tubes (run in duplicate) were preincubated for 30 min on ice and then for 6 min at 37°C in water bath.

Calcium influx (uptake) (29) was inititated by incubating (15 s) 0.4 ml of $^{45}CaCl_2$ (specific activity = 29–39 Ci/g; 0.5 μCi/assay) in 145 mM NaCl for nondepolarized samples and in 145 mM KCl for depolarized samples. Uptake was terminated by rapid filtration through GF-C glass fiber filters which then were washed three times with 5 ml of a cold solution containing 145 mM KCl, 7 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM Tris HCl, pH 7.4. The amount of radioactivity on the filter disc was then determined by liquid scintillation spectrometry. Controls for depolarized and nondepolarized samples were included. A 25–75% inhibition of stimulated uptake was obtained to calculate an IC_{50} value (i.e., the concentration in μM which inhibited 50% of stimulated uptake of ^{45}Ca in depolarized samples, corrected for basal uptake in nondepolarized samples). The IC_{50} value was estimated from dose-response curves by computer.

Patch clamp calcium currents. Dorsal root ganglions from 8–10-day-old rats were treated with 1.25% colagenase for 13 min, 37°C, and dissociated by trypsination (2.5% trypsin, 60 min, 37°C (24). After adding DNase, ganglia were titurated and filtered through a nylon gauze (100 μm mesh) and 500 μl of the cell culture were inoculated (plated) onto poly L-ornithine-coated cover slips. Cells then were cultured in HAMF14 media + 10% horse serum and used for recordings 2 h later.

Cover slips containing the plated dorsal root ganglia (DRG) cells were transferred to a tyrodes solution containing (in mmol/l) NaCl 145, KCl 2.5, HEPES 10, glucose 10, $CaCl_2$ 1.5, and $MgCl_2$ 1.2 with pH adjusted to 7.4. The cover slip then was placed under a microscope and a whole cell patch obtained using a micropipette with a resistance of approximately 3 M ohm (15). The pipette contained a solution which supplied the calcium channels with necessary ATP and also blocked other currents—that is, the intracellular solution consisted of (in mmol/l) CsCl 140, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) 10, EGTA 10, $MgCl_2$ 4, NaATP 5, with pH adjusted to 7.2 (tetraethylammonium hydroxide).

After the whole cell patch configuration was established,

the external solution was changed to a calcium current external solution containing (in mM) TEA Cl 130, glucose 10, HEPES 10, $MgCl_2$ 1, $BaCl_2$ 10, with pH adjusted to 7.3 (TEA/OH). The measured current was carried by Ba^{++}, which substituted for Ca^{++}. After necessary adjustments for series resistance and membrane capacitance, the cell was maintained at −80 mV and every 15 s depolarized to 0 mV for 100 ms. This activated the calcium channels, and an inward current carried by barium ions was recorded. This procedure was carried out 1–2 min before the external solution was exchanged with the same solution now containing 10 μM of nimodipine or ω-conotoxin and/or NNC 09-0026, and the magnitude of the current was again recorded for at least another 3 min and the percent inhibition of the control currents (i.e., plateau currents) determined in duplicate.

Calcium Channel Binding

Additional data for nitrendipine binding were obtained for comparative purposes from NOVASCREEN® (Baltimore).

Functional Smooth Muscle Activity

Strips of taenia coli, 1.5–2.0 cm in length, were dissected from the caecum of male guinea pigs and suspended in 10-ml organ baths containing Ca^{2+} free K^+ depolarizing Tyrode solution, maintained at 35°C and gassed with 95% O_2 and 5% O_2. The composition of the Tyrode solution was (in mM) NaCl 97, KCL 49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, and glucose 5.5. The mechanical activity of muscles was measured using an HSE isometric transducer, connected via an HSE bridge amplifier to a potentiometric recorder.

Cumulative dose-response curves were obtained to $CaCl_2$ (30–1430 μM) by increasing the Ca^{2+} concentration at 3-min intervals in logarithmic increments. A 20-min washout period was allowed between curves. Several control curves were completed before the compound was evaluated after a preincubation of 20 min. Contractions were calculated as a percentage of the maximum response of the tissue. The compound effect was expressed as the dose ratio, calculated by computer as the ratio of the concentration of Ca^{2+} producing a 50% maximal contraction in the presence and absence of the antagonist.

Cardiovascular System

Adult male Wistar rats were anesthetized with pentobarbital (65 mg/kg, IP) and surgically prepared with a catheter into the right jugular vein to allow the IV administration of test compounds. A second catheter from the carotid artery was connected to a blood pressure transducer. The trachea was cannulated to facilitate respiration. Heart rate, measured using an EKA pulse rate meter, and blood pressure were recorded on a dual channel recorder and measured continuously. Each rat then was treated with IV bolus injections of compound (i.e., 0.1, 1.0, and 10 as 2.5 mg/kg dissolved in dimethyl sulfoxide (DMSO) : cremophore CL : isotonic saline [10 : 5 : 85]) at 10-min intervals. Data were expressed as mean percent decreases ± SEM normalized to basal (preinjection) levels. Comparisons were made using a one-way analysis of variance (ANOVA) followed by the Fisher least significant difference multiple comparison approach (46).

Focal Ischemia

Fisher F-344 rats, allowed free access to food and water and weighing 250–350 g, were anesthetized with halothane. Surgical procedures, with body temperature maintained at 37°C, were similar to that described previously in this strain (10,11). Briefly, right middle cerebral artery occlusion (MCAO) using electrocoagulation (Aspen Labs Inc., Littleton, CO; MF180 Electrosurgical Unit) was performed at the level of the inferior cerebral vein (2–4). The right common carotid artery was exposed and occluded (CCAO; double ligated then cut). The right femoral artery was cannulated for drug or vehicle administration. Animals were allowed to recover from anesthesia under a heating lamp to maintain normal body temperature and then returned to their cages.

At 24 and 48 h after MCAO a neurologic examination was performed. Each animal was classified using a neurological grade (7) into one of four grades. The grades of 0 (no observable deficit), 1 (any amount of consistent contralateral forelimb flexion), 2 (reduced resistance to lateral push toward the paretic, contralateral side), or 3 (circling behavior toward the paretic side) basically defined the degree of contralateral hemiparalysis that occurred as a consequence of focal ischemia and the associated ipsilateral hemispheric infarct. A hindlimb placement test (2–4) was performed for each rat. In this test the rat is held facing away from the edge of a table and the contralateral hindlimb is pulled over the edge of the table and extended downward. A normal response seen in nonsurgically treated animals or ipsilateral to the cerebral surgery is an immediate placement of the hindlimb back onto the table, thus appropriately coordinating sensory/motor stimuli. An abnormal response is no limb placement/movement.

At 48 h animals were sacrificed by an overdose of sodium pentobarbital, their brains were removed, and seven coronal forebrain slices (2 mm thick) were made from the level of the olfactory bulbs to the cortical-cerebellar junction. Slices were immersed immediately in a 1% solution of triphenyltetrazolium chloride (TTC) in phosphate buffer at 37°C for 20–30 min (6) and then fixed by infiltration in 10% phosphate-buffered formalin. Both sides of each TTC-stained section were photographed in color using a Polaroid™ camera and analyzed for the quantification of ischemic damage using an image analysis system (Amersham RAS 3000; Loats Associates, Inc., Westminster, MD). Morphological changes following surgery were evaluated in the entire forebrain (total of 14 planar surfaces) for each animal as described previously (3,4). Hemispheric swelling, infarct size, and infarct volume were determined for each slide. Hemispheric swelling, which was expressed as the percent increase in size of the ipsilateral hemisphere over the contralateral hemisphere, was calculated as ipsilateral hemispheric area minus contralateral hemispheric area divided by contralateral hemispheric area multiplied by 100. Infarct size was expressed as the percent infarcted tissue in reference to the contralateral hemisphere and was calculated as infarct area divided by contralateral hemispheric area multiplied by 100. The total volume of infarction was also calculated by summation of the infarct area (mm^2) from all the brain planar images that were considered 1 mm thick.

Phencyclidine (0, 1.5, 3.0, and 6.0 mg/kg, IV) obtained from Sigma Chemical Co. (St. Louis) was dissolved in saline and administered as a bolus 30 min pre- and 24 h post-MCAO. NNC 09-0026 (30 mg/kg, IV) was dissolved in 1 ml of saline and administered slowly over 1 h beginning 30 min post-MCAO. Control animals received 1 ml of saline administered over 1 h beginning 30 min post-MCAO. All drug doses are expressed as the free base. Chemical structures of phencyclidine and NNC 09-0026 are depicted in Fig. 1.

Data were expressed as means ± SEMs. In the case of nonparametric data (i.e., the hindlimb placement test) the χ^2 test for independent samples was utilized (41). For all the

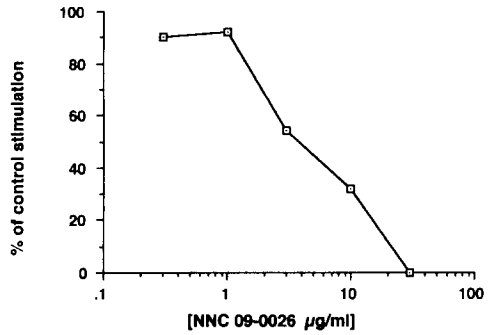

FIG. 2. Effect of NNC 09-0026 on potassium-stimulated $^{45}Ca^{2+}$ uptake into cerebellar granule cells (IC_{50} = 13 μM; procedure in text).

parametric data, comparisons were made using a one-way analysis of variance with Dunnett tests as appropriate follow-up analyses (46). Statistical significance was accepted when $p < 0.05$.

RESULTS

Calcium Influx/Currents

NNC 09-0026 exhibited a dose-dependent inhibition on potassium-stimulated Ca^{2+} uptake into rat cerebral cortex synaptosomes (Fig. 2) with an IC_{50} value of 13 μM. Nifedipine had an IC_{50} value of 90 μM in the same assay. NNC 09-0026 also had similar effects, in terms of potency, and blocked calcium currents by 43% at 10 μM using the patch clamp technique in rat DRG preparations. NNC 09-0026 also was able to block calcium currents even after pretreatment with nimodipine or ω-conotoxin in the DRG preparations. Since NNC 09-0026 blocked calcium influx in depolarized synaptosomes and calcium current in DRG cells in the presence of L- or N-type channel blockade, the compound can be characterized as a blocking both L- and N-type neuronal calcium channels (16,27,45).

Calcium Channel Binding and Function

NNC 09-0026 inhibited nitendipine binding with an IC_{50} value of 10 μM, which indicated that the compound was not very active on peripheral calcium channels. Known calcium channel blockers such as nifedipine have an IC_{50} of 0.5 nM (NOVASCREEN®). A weak effect on peripheral tissue was observed in the depolarized taenia coli smooth muscle preparation. At 10 μM, NNC 09-0026 produced a dose ratio of only 3.5. In smooth muscle, nifedipine has IC_{50}s in the nM range (5). As described recently, NNC 09-0026 is around 30 000-fold less active than peripherally active calcium channel blockers (42). These data indicate that this compound is not very active at peripheral calcium channels typically characterized as the smooth muscle L-type channel (27,45). These data do not, however, exclude potential effects at neuronal L-type calcium channels in the brain. Taken together, these in vitro data indicate that NNC 09-00026 is a selective blocker of neuronal calcium channels.

Cardiovascular System

Intravenous single bolus administrations of NNC 09-0026 caused a small decrease in blood pressure and heart rate (Table 1). After administration of vehicle, blood pressure slightly and transiently increased. Administration of 10 mg/kg NNC 09-0026 caused a short lasting drop in blood pressure followed by a transient increase above the baseline values. Heart rate changes significantly decreased only after administration of the 10-mg/kg dose. The effects of dihydropyridine calcium channel blockers at 100-fold lower doses delivered in the same manner are dramatic and sustained (5,31).

Phencyclidine on Focal Ischemia

The infarct produced by the combined MCAO and ipsilateral CCAO in F-344 rats produced a cortical infarction similar to but smaller than that described previously for spontaneously hypertensive rats following MCAO alone (3). Figure 3 illustrates the similar dose-related effects of phencyclidine on hemispheric infarct and infarct volume. At 1.5 and 3.0 mg/kg, IV dose treatments, a significant ($p < 0.05$) decrease in hemispheric infarct (42.8% and 54.5%) and infarct volume (45.8% and 52.4%) was observed, respectively. The 6.0 mg/kg IV treatment did not significantly reduce these variables. Phencyclidine did not significantly effect hemispheric swelling. The profiles for hemispheric infarcts and infarct areas presented from the anterior to the posterior forebrain are depicted in Fig. 4. The consistent reduction in infarction throughout the forebrains by the two effective dose (1.5 and 3.0 mg/kg) regimens can be identified. Figure 5 illustrates the comparable effects of phencyclidine on neurological deficits at 24 and 48 h postsurgery. At 48 h the 1.5 and 3.0 mg/kg (but not 6.0 mg/kg) phencyclidine dosing regimen significantly ($p < 0.05$) reduced the abnormal neurological grade (55.3% and 47.3%) and normalized the hindlimb placement (33.3% and 55.5%). Similar but nonsignificant effects were also observed at 24 h. Phencyclidine at all doses caused ataxia. At 1.5 mg/kg apparent increases in motor activity and ataxia were observed. At 3.0 mg/kg phencyclidine side effects were greater and animals exhibited increased ataxia, increased time to recover from anesthesia postsurgery, and unusual head movements. At 6.0 mg/kg side effects were most pronounced, with

TABLE 1

NNC 09-0026-INDUCED DECREASES IN BLOOD PRESSURE AND HEART RATE

		Doses (mg/kg)		
Measure	Vehicle	0.1	1.0	10.0
Blood Pressure+	0.3 ± 0.3	0.5 ± 0.5	2.2 ± 0.8	*28.3 ± 4.6
Heart Rate+	2.0 ± 0.5	1.5 ± 0.1	2.6 ± 0.5	*12.1 ± 0.2

+ = Percent, peak decreases from basal levels are depicted.
* = Significantly different from all other dose group values ($p < 0.01$; N = 3)

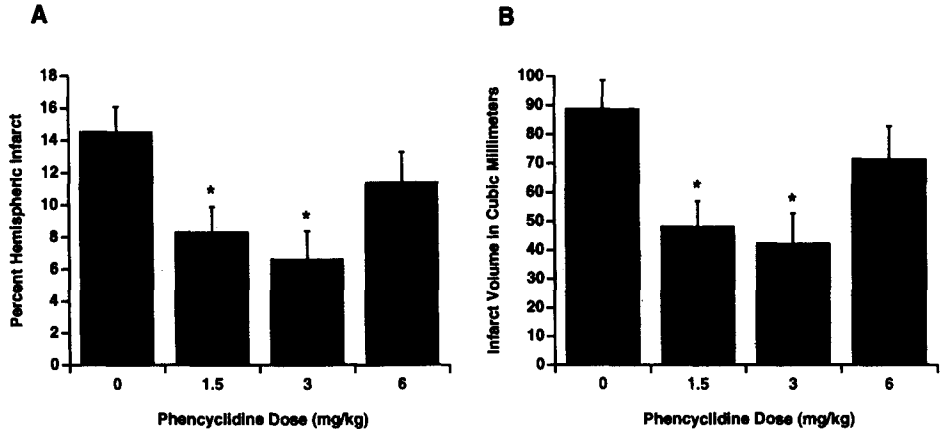

FIG. 3. Hemispheric infarct (A) and infarct volume (B) presented as a function of increasing phencyclidine doses administered as in text. Phencyclidine at 1.5 mg/kg IV (N = 15) and 3.0 mg/kg IV (N = 14), but not at 6.0 mg/kg IV (N = 15), significantly reduced cortical infarctions produced by permanent middle cerebral artery occlusion (MCAO) and ipsilateral common carotid artery occlusion (CCAO) compared to those in vehicle-treated animals (N = 31). *$p < 0.05$, ANOVA.

decreased motor activity and marked ataxia that lasted for greater than 1 h.

NNC 09-0026 on Focal Ischemia

Infarcts similar to those described previously were produced. Figure 6 illustrates the head to head comparison of 3.0 mg/kg phencyclidine regimen and 30 mg/kg NNC 09-0026 dosing regimens on hemispheric infarct and infarct volume. Both treatments significantly ($p < 0.05$) reduced hemispheric infarct (i.e., phencyclidine by 42.3% and NNC 09-0026 by 43.1%). Infarct volume also was significantly ($p < 0.05$) reduced by both treatments (i.e., phencyclidine by 41.1% and NNC 09-0026 by 44.7%). Neither compound significantly affected hemispheric swelling. The spatial profiles for hemispheric infarct and infarct areas are presented in Fig. 7, which

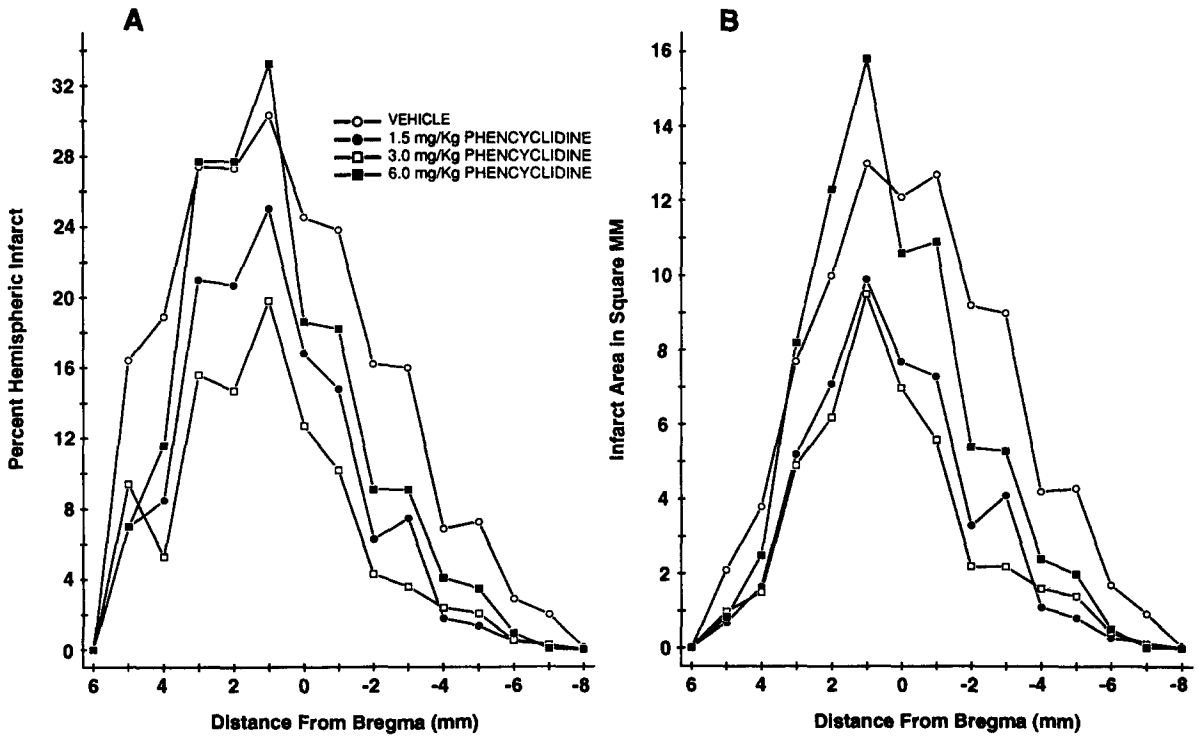

FIG. 4. Profiles of hemispheric infarcts (A) and infarct areas (B) presented as a function of the different forebrain section surfaces estimated at various distances from the skull landmark bregma for the same animals as in Fig. 3.

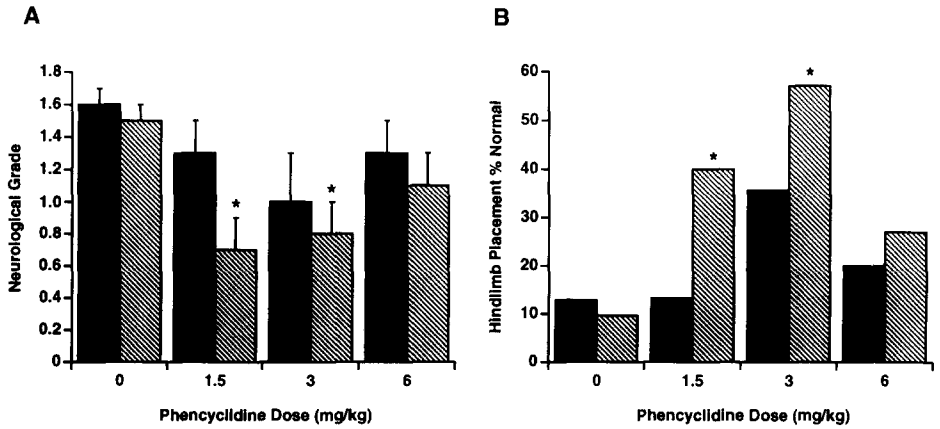

FIG. 5. Neurological grade (A) and hindlimb placement test (B) results presented as a function of increasing phencyclidine doses administered as in text for the same animals as in Figs. 3 and 4. Results 24 h and 48 h postsurgery are indicated by solid and striped bars, respectively. Phencyclidine at 1.5 and 3.0 mg/kg IV significantly reduced these neurological deficits produced by permanent middle cerebral artery occlusion (MCAO) and ipsilateral common carotid artery occlusion (CCAO) that paralleled reduced cortical infarctions as in Fig. 3. *$p < 0.05$, ANOVA.

clearly indicates reductions in infarction throughout the forebrain by both treatments. Figure 8 illustrates that NNC 09-0026 significantly ($p < 0.05$) reduced the abnormal neurological grade at 24 h postischemia, while phencyclidine tended to normalize both measures but its effect did not achieve statistical significance.

DISCUSSION

This study shows that both the noncompetitive NMDA receptor-associated calcium channel blocker phencyclidine and the neuronal selective voltage-operated calcium channel blocker NNC 09-0026 produced significant reductions in focal ischemic damage and neurological deficits. NNC 09-0026 was effective when administered postischemia as would be necessary in the acute treatment of human focal stroke. The profile of activities of NNC 09-0026 (i.e., blockage of neuronal calcium channels without significant cardiovascular liabilities) is clearly desirable as a clinical candidate in focal stroke (16). Since the compound appears to act at both neuronal N- and L-type channels, it may provide cerebral protection following MCAO by blocking both the neuronal terminal calcium influx that facilitates neurotransmitter release (i.e., excitotoxic amino acids such as glutamate) and the excessive influx of neuronal calcium, respectively, that are produced by the depolarization of tissue that is concommitant with focal ischemia (16,44). NNC 09-0026 also has been shown to be neuroprotective in the gerbil global ischemia model (22,42) using a dose similar to that in the present focal ischemia study, but delivered IP at multiple times postischemia. No effects were observed in the present study on hemispheric swelling that appears to be a consequence of hemispheric edema following focal ischemia (3,35).

Intrinsic variability in the MCAO model is an important consideration in the evaluation of drug effects (18). The infarct sizes in vehicle-treated rats in the present studies were similar to those reported by others in this strain of rat (11). The coefficient of variation in the present studies was 30-

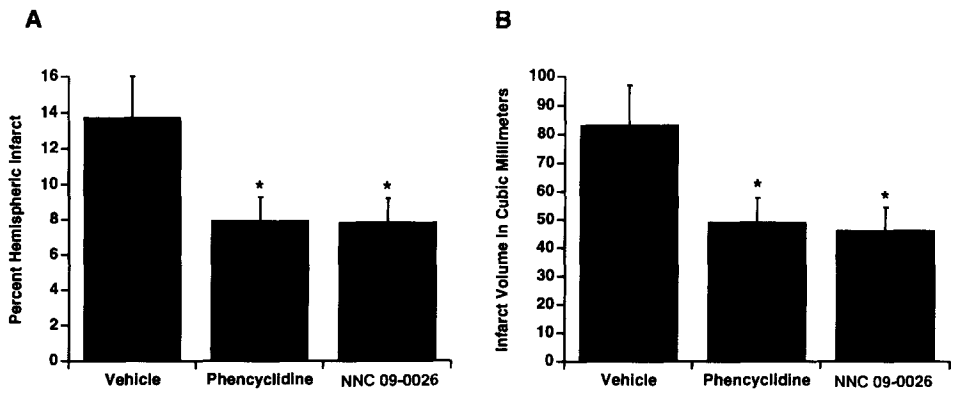

FIG. 6. Hemispheric infarct (A) and infarct volume (B) for vehicle ($N = 14$) and effective doses of phencyclidine ($N = 11$) and NNC 09-0026 ($N = 14$) as described in text. Phencyclidine and NNC 09-0026 significantly reduced cortical infarctions produced by focal ischemia. *$p < 0.05$, ANOVA.

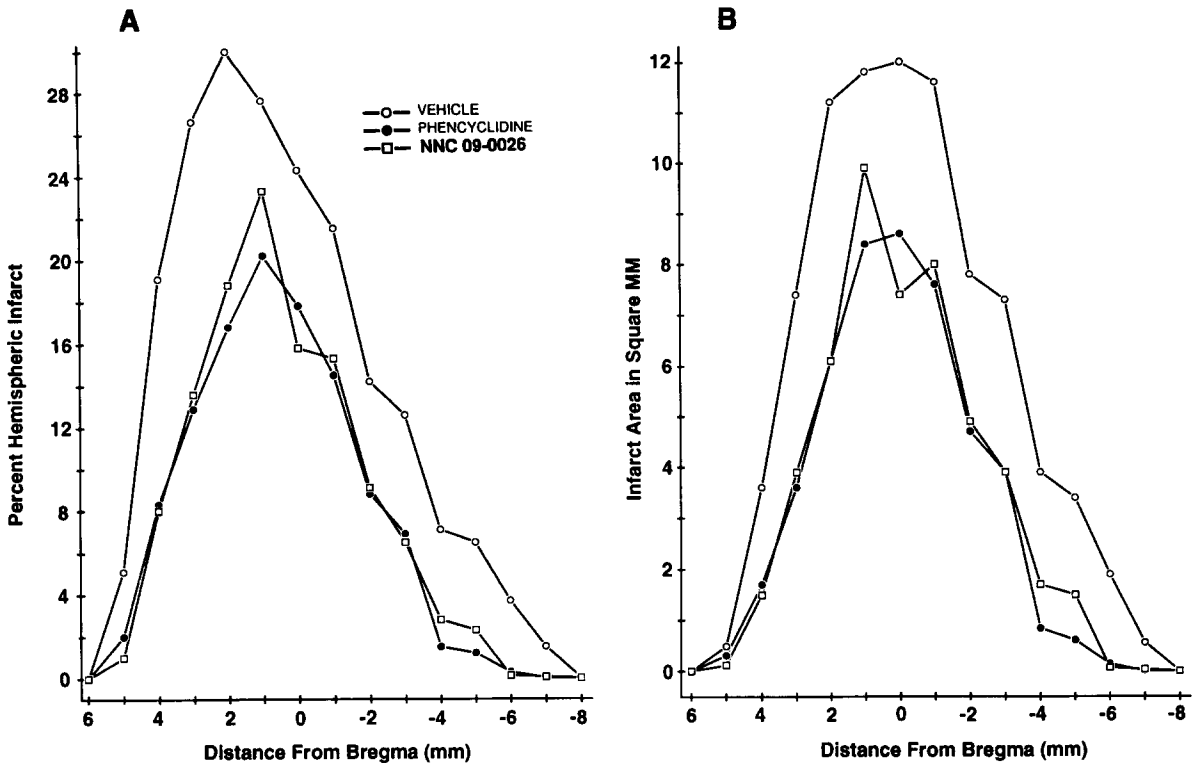

FIG. 7. Profiles of hemispheric infarcts (A) and infarct areas (B) presented as a function of different forebrain section surfaces estimated at various distances from the skull landmark bregma for the same animals as in Fig. 6.

50%, similar to that reported by others (10). Therefore, as described previously (10,36), the sample size in these experiments of 11–15 was necessary to obtain 80% power if infarct size would be required to accurately identify infarct size differences of 20–40%. The present information obtained in the two neurological tests indicates a similar outcome due to intrinsic variability, which again reflects the variability in cerebral tissue damage.

The results with phencyclidine indicate that this drug can be neuroprotective. These results are consistent with those of others that have shown a significant reduction in infarct size with pre- (9) or postphencyclidine (10) treatment. Also, the decrease in efficacy of phencyclidine at higher doses is not unusual for NMDA receptor-associated calcium ion channel-acting compounds (10). Phencyclidine does have a variety of other effects including the significant neurological/behavioral

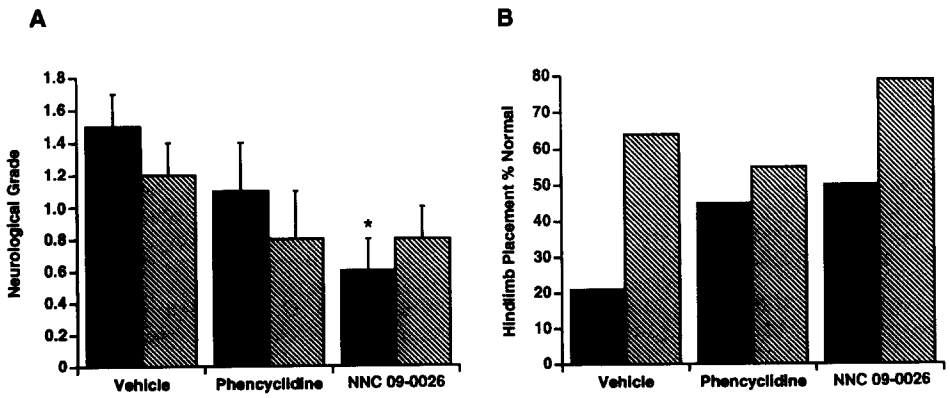

FIG. 8. Neurological grade (A) and hindlimb placement test (B) results for vehicle and effective doses of phencyclidine and NNC 09-0026 as described in text for the same animals as in Figs. 6 and 7. Results 24 h and 48 h postsurgery are indicated by solid and striped bars, respectively. Phencyclidine tended to reduce and NNC 09-0026 significantly reduced neurological deficits produced by focal ischemia. *$p < 0.05$, ANOVA.

effects (23) such as ataxia, sterotyped head movements, and decreased motor activity that were observed in the present study. Also, phencyclidine has broad pharmacological actions such as blockade of voltage-operated and neurotransmitter ion channels (23) and direct neurotoxicity (32,33) that could limit its efficacy and produce the observed inverted-U-shaped dose-response curve. The present study is the first to demonstrate that phencyclidine produces parallel, dose-related effects of both ischemic damage and neurological deficits in focal ischemia.

The comparison of effective doses of phencyclidine and NNC 09-0026 is important for several reasons. First, it replicated within a series of studies the effects of phencyclidine and established this compound as a reliable positive control in this stroke model. The smaller sample sizes utilized in this second study probably contributed to the more variable, less significant effects on infarct volume and neurological tests. Second, and most importantly, this is a clear demonstration of a selective neuronal calcium channel blocker (i.e., NNC 09-0026) having efficacy in a model of focal ischemia. It reduced both infarct size and neurological deficits without affecting hemispheric swelling or producing any overt neurological/behavioral side effects. In addition, in vitro data were provided as a basis for this drug's designation as a neuronal calcium channel blocker. The effect on inhibition of calcium uptake into potassium-depolarized synaptosomes and on voltage-dependent calcium currents in DRG culture taken together with the very small effect observed in the taenia coli preparation and on cardiovascular parameters clearly indicates that the compound has only very small effects on peripheral L-type calcium channels, but it appears to act significantly on neuronal calcium channels (27,42,45). By comparison, the variable effects observed with some of the other VOCC blockers in models of cerebral ischemia might be associated with less neuronal and more peripheral cardiovascular effects (16). Finally, this report also points to the variability in neurological tests from experiment to experiment and particularly for changes in measures for both tests over time where improvement might occur over the two (and perhaps more) days of observation. These findings underscore the importance of running matched controls in all studies of this type. The consistency of these and other (3) neurological tests must be evaluated in future studies. The importance of evaluating forelimb function in the MCAO model should be emphasized, since infarction usually centers on the motor cortex representing this control (30). The ability of the neurological grade utilized in the present study (7) to detect improved neurological outcome associated with the neuroprotective effects of other calcium antagonists has been reported (17,21). The present study also utilizes a test of hindlimb sensory-motor control that is affected by the resulting infarct (2–4). Certainly other neurological tests (3) need to be evaluated and new tests need to be developed for identifying drug effects on sensory-motor and learning performance in focal ischemia to extend results to the clinical setting (16).

In summary, the present results confirm that phencyclidine, a noncompetitive NMDA receptor antagonist, is neuroprotective in a rat model of focal ischemia. At the same doses providing reduced cerebral infarction, phencyclidine produces a parallel, significant reduction in neurological deficits on forelimb and hindlimb function using two tests that appear to provide an index for the neuroprotective effects of drugs of this model. Also, the pharmacological activity profile of a new, neuronal selective calcium channel blocker was presented. This new compound, NNC 09-0026, reduces neuronal calcium currents, has little effect on smooth muscle or cardiovascular measures, and exhibits neuroprotective efficacy in reducing cerebral tissue damage and neurological deficits following focal ischemia. This efficacy was demonstrated when the compound was administered after focal ischemia and without the neurological/behavioral side effects exhibited by phencyclidine, suggesting that this therapeutic approach could have utility in stroke.

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