Effect of NIK-247 on Basal Concentrations of Extracellular Acetylcholine in the Cerebral Cortex of Conscious, Freely Moving Rats

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ABSTRACT—We studied the effect of orally administered NIK-247 (9-amino-2,3,5,6,7,8-hexahydro-1*H*-cyclopenta[*b*]quinoline monohydrochloride monohydrate) on basal extracellular acetylcholine (ACh) concentrations in the rat cerebral cortex using microdialysis without the addition of cholinesterase inhibitor to the perfusion fluid and radioimmunoassay for ACh. In addition, the effect of oral administration of NIK-247 on acetylcholinesterase (AChE) activity in rat cerebral cortex was determined. The mean basal ACh content in the perfusate from the cerebral cortex of freely moving rats was 123.2±21.8 fmol/30 min (n=7). NIK-247 (2.5-10.0 mg/kg, p.o.) increased the ACh content of the perfusate in a dose-dependent manner. NIK-247 at 10 mg/kg significantly increased the ACh content in the perfusate from 0.5 to 2.5 hr after administration, and the maximum increase was attained at 1 hr after administration. 9-Amino-1,2,3,4-tetrahydroacridine (5 mg/kg, p.o.) and physostigmine (0.5 mg/kg, i.p.) significantly increased the ACh content in the perfusate from 1 to 2 hr and from 0.5 to 1.5 hr after administration, respectively. AChE activities in the cerebral cortex were about 32% and 12% below the control value at 1 hr and 3 hr after administration of NIK-247 at 10 mg/kg, respectively. These findings demonstrate that NIK-247 increases extracellular ACh concentration and inhibits AChE activity in the cerebral cortex after oral administration, and they suggest that NIK-247 facilitates central cholinergic transmission.

Keywords: NIK-247, Acetylcholine concentration (basal extracellular), Cerebral cortex microdialysis (rat), Acetylcholinesterase activity, Radioimmunoassay

Deficits in the central cholinergic system, such as decreased acetylcholine (ACh) content and choline acetyltransferase (CAT) activity in the nucleus basalis of Meynert, have been reported in patients with clinically diagnosed Alzheimer's disease (1-4). NIK-247 (9-amino-2,3,5,6,7,8-hexahydro-1*H*-cyclopenta[*b*]quinoline monohydrochloride monohydrate) has been reported to inhibit acetylcholinesterase (AChE) (5) and to improve learning and memory performance in animal studies (6-10). Oral administration of NIK-247 increases ACh content in the brain and increases ACh efflux from brain slices (5, 11). These findings indicate that NIK-247 increases ACh concentration in the cholinergic synaptic cleft via inhibition of AChE and activates central cholinergic transmission. For precise determination of the effect of NIK-247 on central cholinergic function, changes in extracellular ACh concentrations at cholinergic nerve endings must be measured under basal conditions without the use of cholinesterase (ChE) inhibitor in the perfusion fluid.

The intracerebral microdialysis procedure permits estimation of cholinergic neuronal activity in discrete regions of the brain by measurement of ACh content in the perfusate. Since ACh released from nerve endings is hydrolyzed rapidly by AChE, extracellular concentrations of ACh under physiological conditions are very low. Due to difficulties associated with measurement of small amounts of ACh using conventional methods, most microdialysis studies have required the addition of physostigmine (PHY), a ChE inhibitor, to the perfusion fluid to increase the recovery of ACh in the perfusate. However, perfusion of ChE inhibitor induces artificial activation of cholinergic neurons and thus makes interpretation of the effects of drugs difficult (12-14).

Recently, effects of some drugs on extracellular ACh concentrations have been reported using the microdialysis procedure without the use of ChE inhibitor in the perfusion fluid (12-17). However, in those studies, the effects of only i.p. injection of drugs were examined (15-17). In

the present study, we investigated the effects of orally administered NIK-247 on the extracellular ACh concentration in the cerebral cortex of conscious, freely moving rats using a microdialysis procedure without addition of ChE inhibitor to the perfusion fluid and radioimmunoassay (RIA) for ACh (12). The effects of NIK-247 were compared with those of 9-amino-1,2,3,4-tetrahydroacridine (THA) and PHY. We also studied the effects of oral administration of NIK-247 on AChE activity in rat cerebral cortex.

MATERIALS AND METHODS

Animals

Male Wistar rats (Nihon SLC, Shizuoka; 9 weeks of age) were housed at a temperature of 23±3℃, humidity of 50±15% and a 12 hr light/12 hr dark cycle (lights on 8:00, off 20:00) for at least 1 week before the experiment.

Drugs

NIK-247 was supplied from Nikken Chemicals Co., Ltd. (Omiya), THA hydrochloride monohydrate and PHY salicylate from Sigma (St. Louis, MO, USA), [^{3}H]-ACh chloride (2.85 TBq/mmol) from Amersham (Buckinghamshire, UK) and bovine γ-globulin from Miles (Kankakee, IL, USA). All other chemicals were of reagent grade and obtained from commercial sources.

NIK-247 and THA were dissolved in distilled water and administered by gavage. PHY was dissolved in 0.9% physiological saline and injected i.p.

Surgery

Rats were anesthetized with pentobarbital-Na (50 mg/kg, i.p.) and positioned in a stereotaxic apparatus (SM-15S; Narishige, Tokyo). The skull was exposed and a hole drilled for implantation of a microdialysis probe. The microdialysis probe (3.0-mm membrane and 0.5-mm diameter; CMA/12; Carnegie Medicin, Stockholm, Sweden) was implanted into the left cerebral cortex at 40° from the vertical axis, to a depth of 3.5 mm from the cortical surface and at 2.0 mm anterior and 2.0 mm lateral to the bregma, according to the rat brain stereotaxic coordinates indicated in the atlas of Paxinos and Watson (18), and permanently secured by dental cement to bone screws. Following surgery, rats were housed individually in home cages. Placement of the probe within the cerebral cortex was confirmed by visual inspection of the probe track at the end of the experiment.

Microdialysis

Three days after implantation, the microdialysis probe was connected to a 2-channel swivel (Eicom, Kyoto) and Ringer's solution (147 mM NaCl, 4.0 mM KCl and 2.3 mM $CaCl_2$) was perfused by using a microperfusion pump (Model 22; Harvard, South Natick, MA, USA) at the rate of 2 μl/min. The perfusate was discarded during the first hour of perfusion and then collected at 30-min intervals into tubes containing 35 μl of 0.01 N acetic acid in an ice-water bath.

After three fractions had been collected, rats were administered distilled water (5 ml/kg, p.o.), saline (2 ml/kg, i.p.), NIK-247 (2.5, 5.0 or 10.0 mg/kg, p.o.), THA (5.0 mg/kg, p.o.) or PHY (0.5 mg/kg, i.p.).

Determination of ACh by RIA

The amount of ACh in the perfusate was measured by RIA using the method of Kawashima et al. (12). RIA was performed with rabbit antiserum raised against choline hemiglutarate-bovine serum albumin conjugates and [^{3}H]-ACh with a specific activity of 2.85 TBq/mmol (Amersham). This assay is specific for ACh, and its levels of cross-reactivity with choline, phosphatidylcholine and phosphorylcholine are each less than 0.012%. The sensitivity of the assay was 3 pg/tube (about 20 fmol/tube). For the RIA buffer, 0.15 M Tris-HCl, pH 7.4, was used. A 100-μl portion of [^{3}H]ACh chloride (about 9,000 dpm) in 0.15 M Tris-HCl buffer was added to a tube containing a mixture of 100 μl of diluted antiserum (1:1,000) in 0.4% γ-globulin and 100 μl of 0.15 M Tris-HCl buffer, pH 7.4, and the perfusate was collected for 30 min. The contents of the tube were incubated overnight at 4℃. The antibody-bound [^{3}H]ACh was separated by an ammonium sulfate method, and the radioactivity of the antibody-bound fraction was then determined by a liquid scintillation counter (LSC3000; Aloka, Tokyo). The same volume of perfusion fluid served as a blank.

The amount of ACh in the perfusate was calculated by subtracting the blank value from that for the sample. Contents of ACh in the perfusate are shown as fmol/30 min (means±S.E.).

AChE activity

Animals were sacrificed by decapitation at 0.5, 1, 3 or 6 hr after the oral administration of NIK-247 at doses of 3 or 10 mg/kg. The cerebral cortex was rapidly dissected out. AChE activity was determined using the method of Zusho (19). Briefly, the cortex was homogenized in 10 volumes of 50 mM phosphate buffer (pH 7.4). The pH of a 4 ml portion of the homogenate in a reaction cell was monitored using a pH-electrode during continuous stirring at 37℃. After the pH had stabilized, a 20-μl portion of 600 mM ACh in 50 mM phosphate buffer was added to the cell. Changes in pH due to hydrolysis of ACh were recorded continuously. AChE activity was calculated from the time required to reach the end of the hydrolysis reaction and expressed as μmol/g wet tissue/min.

Statistical analyses

Two-way analysis of variance followed by Dunnett's multiple comparison test or Student's *t*-test was used for comparison of findings of the control and drug-treated groups. P values <0.05 were considered to indicate statistical significance.

RESULTS

Effects of NIK-247, THA and PHY on extracellular ACh concentrations

Using RIA, it was possible to determine the amount of ACh in the perfusate from the cerebral cortex of conscious, freely moving rats for at least 6.5 hr without the addition of ChE inhibitor to the perfusion fluid (Figs. 1-3). Basal ACh content in the perfusate prior to administration of vehicle was 123.2±21.8 fmol/30 min (n=7), and it was stable throughout the experiment. Basal ACh content in the perfusate was not influenced by the handling required for administration of distilled water (p.o.) or physiological saline (i.p.) (Figs. 1-3).

Oral administration of NIK-247 increased the ACh content of the perfusate in a dose-dependent manner (Fig. 1). The increase by NIK-247 at doses of 5 and 10 mg/kg was statistically significant compared with the control. The maximum increases, observed at 1 hr after the administration of NIK-247 at 5 and 10 mg/kg, were 2.5 and 4.7 times the control level, respectively. NIK-247 at 10 mg/kg significantly increased ACh content between 0.5-2.5 hr after administration; however, ACh content in the perfusate returned to the control levels within 3.5 hr of administration.

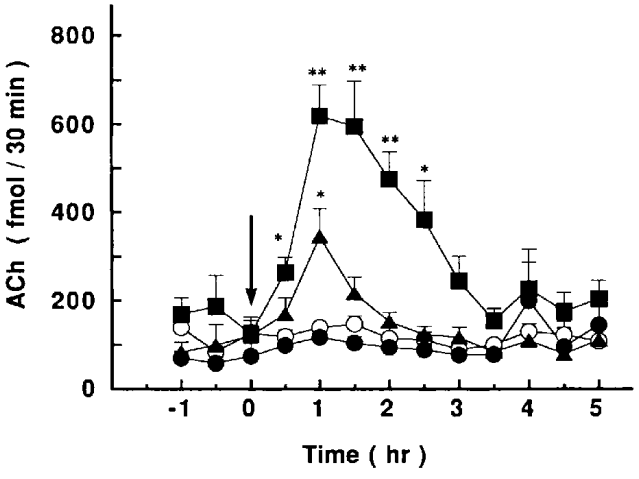

Fig. 1. Effect of NIK-247 on the ACh content in the perfusate from the cerebral cortex of conscious, freely moving rats. Vehicle (D.W., 5 ml/kg) or NIK-247 was administered orally (indicated by arrow). ○, control (vehicle) (n=7); ●, 2.5 mg/kg (n=6); ▲, 5.0 mg/kg (n=7); ■, 10.0 mg/kg (n=6). Values are means±S.E. *P<0.05, **P<0.01 vs control.

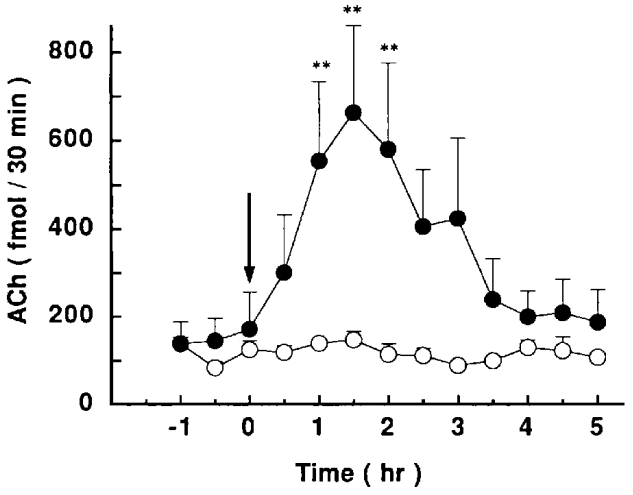

Fig. 2. Effect of THA on the ACh content in the perfusate from the cerebral cortex of conscious, freely moving rats. Vehicle (D.W., 5 ml/kg) or THA was administered orally (indicated by arrow). ○, control (vehicle) (n=7); ●, 5.0 mg/kg (n=7). Values are means±S.E. **P<0.01 vs control.

THA (5 mg/kg, p.o.) significantly increased ACh content in the perfusate from 1 to 2 hr after administration, and the peak effect (4.5 times control level) was noted at 1.5 hr after administration (Fig. 2). The ACh content in the perfusate returned to the control levels by 3.5 hr after administration.

PHY (0.5 mg/kg, i.p.) significantly increased the ACh content in the perfusate from 0.5 to 1.5 hr after administration. The maximal increase (4.9 times the control level) was observed at 1 hr after administration (Fig. 3). The

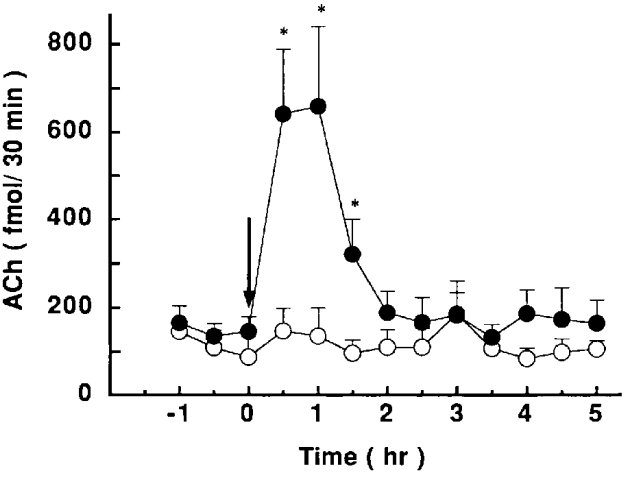

Fig. 3. Effect of PHY on the ACh content in the perfusate from the cerebral cortex of conscious, freely moving rats. Vehicle (0.9% saline, 2 ml/kg) or PHY was injected i.p. (indicated by arrow). ○, control (vehicle) (n=6); ●, 0.5 mg/kg (n=7). Values are means±S.E. *P<0.05 vs control.

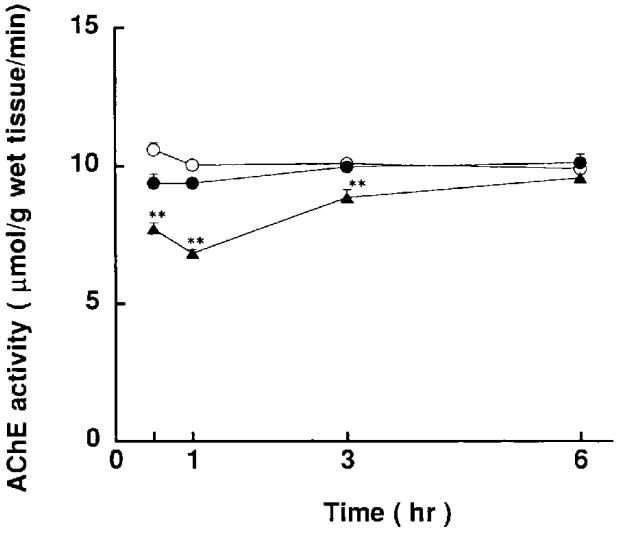

Fig. 4. Effect of NIK-247 on the AChE activity in the cerebral cortex of rats. Vehicle (D.W., 5 ml/kg) or NIK-247 was administered orally at 0 hr. ○, control (vehicle) (n=5-15); ●, 3.0 mg/kg (n=5-6); ▲, 10 mg/kg (n=5-6). Values are means±S.E. **P<0.01 vs control.

ACh content in the perfusate returned to the control level 2.0 hr after administration (Fig. 3).

Several mild signs of cholinergic stimulation, such as salivation and tremor, were observed during about the first hour after administration of NIK-247 (10 mg/kg, p.o.), THA (5 mg/kg, p.o.) or PHY (0.5 mg/kg, i.p.). The signs induced by NIK-247 and THA was milder than those induced by PHY. These observations were consistent with those reported by Yoshida and Suzuki (10).

Effect of NIK-247 on AChE activity

We first studied the effect of NIK-247 on AChE activity in the homogenate of rat cerebral cortex. NIK-247 inhibited AChE activity in a dose-dependent fashion, and the 50% inhibitory concentration (IC_{50}) of NIK-247 was found to be 1×10^{-6} M. This value of IC_{50} corresponded well with that reported previously (5).

We then studied the effect of oral administration of NIK-247 on AChE activity in the homogenate of the cerebral cortex. AChE activity in rat cerebral cortex in the control group was 10.04±0.16 μmol/g wet tissue/min. NIK-247 administered orally at 10 mg/kg significantly inhibited AChE activity in rat cerebral cortex (Fig. 4). NIK-247 at 10 mg/kg induced maximal inhibition (32%) at 1 hr after administration, and significant inhibition of AChE activity lasted for 3 hr.

DISCUSSION

Due to limitations in sensitivity for the determination of ACh, most intracerebral microdialysis studies of the effects of drugs on extracellular ACh concentration have been performed with the addition of a ChE inhibitor to the perfusion fluid to increase the recovery of ACh (20, 21). However, the results obtained with such an addition do not necessarily reflect the effects of drugs under basal conditions (12-14). In fact, Messamore et al. (22) reported that the systemic injection of physostigmine or heptylphysostigmine unexpectedly reduced the extracellular concentrations of ACh when the microdialysis probe was perfused with Ringer's solution containing physostigmine. These findings indicate that determination of the extracellular concentration of ACh by microdialysis under basal conditions without the addition of ChE inhibitor to the perfusion fluid is necessary for studying effects of drugs that influence central cholinergic function. In the present study, we therefore measured ACh content in the perfusate from the cerebral cortex of conscious, freely moving rats without addition of ChE inhibitor to the perfusion fluid. It is generally accepted that the ACh content in the perfusate reflects the extracellular concentration directly adjacent to the probe membrane. Accordingly, the increases in ACh content induced by NIK-247, THA and physostigmine observed in this study can be ascribed to increases in extracellular ACh concentration at the site of dialysis.

Under the experimental conditions we used, basal ACh in the perfusate was 123.2±21.8 fmol/30 min and stable throughout the 6.5-hr experimental period (Fig. 1). This value is similar to that for the cerebral cortex reported by Xu et al. (15).

NIK-247 has previously been reported to increase basal and K^+-stimulated ACh efflux from rat brain slices (11). In the present study, oral administration of NIK-247 and THA increased the extracellular concentration of ACh in the cerebral cortex of conscious, freely moving rats (Figs. 1 and 2). The degree of increase induced by NIK-247 at 10 mg/kg was almost equal to that induced by THA at 5 mg/kg. These results are consistent with the finding that the AChE inhibitory activity of NIK-247 in vitro is about one-third that of THA (5). The increase in extracellular concentration of ACh induced by PHY was rapid compared with that by NIK-247 and THA, probably because PHY was injected i.p. The effect of PHY peaked at 0.5-1 hr and disappeared within 2 hr after administration. These findings are consistent with those of Messamore et al. (17).

Oral administration of NIK-247 at 10 mg/kg significantly inhibited AChE activity in the cerebral cortex. Maximal inhibition was observed at 1 hr and significant inhibition persisted for at least 3 hr after administration. The effect of NIK-247 on extracellular ACh concentration was consistent to a certain extent with that on AChE-activity. However, the duration of the increase in extracellular

ACh concentration induced by NIK-247 was shorter than its AChE-inhibitory effect. Similarly, Messamore et al. (17) reported some discrepancies between ACh content in the perfusate and AChE inhibition determined ex vivo following systemic injection of ChE inhibitors. These findings indicate that extracellular ACh concentration determined under basal conditions by microdialysis is a more reliable index of functional activity than AChE activity for determination of the effects of drugs on central cholinergic transmission.

4-Aminopyridine (4-AP), a K^+ channel inhibitor, has been reported to increase the release of ACh from cholinergic nerve endings (23). In addition to AChE inhibition, NIK-247 and THA have K^+ channel inhibitory activity, and their IC_{50} values are 1×10^{-3} M and 5×10^{-4} M, respectively (24, 25). These findings suggest that AChE inhibition can not be used to accurately predict extracellular ACh concentration after systemic administration of NIK-247 and THA.

In conclusion, the findings of this study demonstrate that oral administration of NIK-247 inhibits AChE activity and increases extracellular ACh concentration in the cerebral cortex of conscious, freely moving rats, and they suggest that NIK-247 facilitates central cholinergic transmission. NIK-247 is thus a promising candidate for use in palliative therapy of Alzheimer's disease.

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