Characterization of Muscarinic Cholinergic Receptors in Human and Dog Cerebral Arteries

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SUMMARY Various concentrations of acetylcholine (ACh) produced dose dependent relaxations of isolated, helical preparations of human cerebral arteries and these responses were blocked by atropine. The median effective concentration (EC_{50}) of ACh was 6.1 ± 0.2 × 10^{-5}M. ACh produced dual responses in isolated dog cerebral arteries. ACh in low concentrations produced relaxation, and contraction occurred when the concentration was raised to 1 × 10^{-5}M. The EC_{50} of ACh which produced relaxation, in dog cerebral arteries was 7.2 ± 0.2 × 10^{-5}M. Muscarinic cholinergic receptors in these arteries were analyzed directly using ^3H-QNB as the ligand. The specific ^3H-QNB binding to the arteries was saturable and of K_{d} = 1.5 nM and B_{max} = 93 fmol/mg protein in human cerebral arteries and K_{d} = 0.59 nM, B_{max} = 340 fmol/mg protein in dog cerebral arteries. Specific binding of ^3H-QNB was displaced by muscarinic cholinergic agents. K_{d} values and Hill coefficients were as follows: QNB, 1.0 × 10^{-5}M, 0.89; atropine, 1.1 × 10^{-5}M, 0.95; ACh, 0.8 × 10^{-5}M and 2.1 × 10^{-4}M, 0.54; carbachol, 1.2 × 10^{-5}M and 4.3 × 10^{-5}M, 0.33 in human cerebral arteries and QNB, 0.55 × 10^{-5}M, 0.85; atropine 0.9 × 10^{-5}M, 1.00; ACh, 0.9 × 10^{-5}M and 1.1 × 10^{-5}M, 0.43; carbachol 6.3 × 10^{-5}M and 1.1 × 10^{-5}M, 0.36 in dog cerebral arteries. Endogenous choline acetyltransferase (ChAT) activity was 1.6 ± 0.4 nmol/mg protein/hr in human cerebral arteries and 3.7 ± 0.1 nmol/mg protein/hr in dog cerebral arteries. These results suggest the presence of both cholinergic innervation and muscarinic receptors in both human and dog cerebral arteries. The difference in biochemical parameters in cerebral arteries from these two species would explain, in part, the difference in EC_{50} of ACh required to produce relaxation of these arteries.

INTRA-ARTERIAL INFUSION of acetylcholine (ACh) or cholinergic agonists increases cerebral blood flow and this increase is blocked by atropine.1-3 Histological studies revealed a rich innervation of cholinesterase-stained neurons in cerebral arteries of humans,4 monkeys5 and other species.6,7 Since development of a sensitive radioenzymatic assay for endogenous choline acetyltransferase (ChAT) activity,8,9 high ChAT activity was detected in cerebral arteries of rabbits,10 dogs11 cats12,13 and rat cerebral arteries.14 These findings suggest that cholinergic innervation reaches intraparenchymal vessels.13,14 Significant specie differences were noted in the response of cerebral blood vessels to ACh. In isolated cat cerebral arteries, various concentrations of ACh produced dual responses; low concentrations of ACh (<1 × 10^{-5}M) induced relaxation, and high concentrations of ACh (>1 × 10^{-5}M) induced contraction.15 Isolated dog cerebral arteries contracted in the presence of a low concentration of ACh (1 × 10^{-5}M), under certain experimental conditions.16 On the other hand, various concentrations of ACh induced only relaxation in human cerebral arteries.4 The median effective concentration (EC_{50}) of ACh required to induce relaxation was about 10 times higher in human than in cat cerebral arteries.4,15 These differences may be due to the characteristics of muscarinic cholinergic receptors in cerebral arteries, as both relaxation and contraction were blocked by low concentrations of atropine.

The present study was undertaken to characterize muscarinic cholinergic receptors in human and dog cerebral arteries, using a radioligand binding assay. ChAT activity as a marker of cholinergic innervation was measured and the responses to exogenously applied ACh in isolated helical preparations of these cerebral arteries were also examined.

Materials and Methods

Human and Dog Cerebral Arteries

Cerebral arteries (mainly basilar artery, middle cerebral artery, anterior cerebral artery and Willis's ring) of twelve patients were carefully removed at autopsy between 1 hr and 2 hr after death. The causes of death in these 30 to 77-year-old patients (8 males and 4 females) were 2 with lung cancers, 3 each with renal cancer, cecum cancer, pancreatic cancer, hepatoma, 2 from traffic accidents and 4 with strokes. Dogs of either sex, weighing 10 to 15 kg, were anesthetized with pentobarbital sodium (25 mg/kg, i.v.), and exsanguinated from the common carotid arteries. The brains were removed, the cerebral arteries excised and kept in the same manner as the human tissues.

Responses to Exogenous ACh

The isolated cerebral arteries were prepared as helically cut arterial strips (2 mm in width and 15 mm in length) and kept in a Krebs-Henseleit solution at 37°C. All experiments were performed in a water-jacketed glass bath at 37°C and a control perfusion pressure of 70 mm Hg was maintained with a peristaltic pump. Isometric responses were recorded on Grass model 79 strain gauge transducers and amplified using Grass model 7P3D amplifiers.
length). Ligatures were placed on both ends of the strips and one end was attached to a tissue holder and the other to a strain gauge force-displacement transducer (Nihonkoden, Tokyo, Japan) connected to a polygraph (San-ei Instrument, Tokyo, Japan) on which isometric tension changes were recorded. Each strip was then placed in a 20 ml tissue organ bath containing physiological salt solution of the following composition (mM) NaCl 120; KCl 4.7; MgSO4 1.2; KH2PO4 1.2; CaCl2 2.5; NaHCO3 25 and glucose 10. The solution was maintained at 37 ± 0.5°C and bubbled with 95% O2 and 5% CO2 (pH 7.4) throughout the experiments. An initial 1.5 g tension was applied to each strip and an equilibration period of 120 min was allowed before commencement of experiments.

Binding Assay

The arteries were minced with scissors and homogenized in 10 volumes of ice-cold 50 mM sodium phosphate buffer (pH 7.4) with a glass homogenizer. The homogenates were filtered through two layers of gauze, re-homogenized at a setting of 10 on a Polytron homogenizer with 20 s burst, the homogenates centrifuged at 1,000 x g for 10 min and the supernatant carefully removed and centrifuged at 100,000 x g for 60 min. The resulting pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.4). Protein concentration was determined by the method of Lowry et al.17

3H-QNB bindings were performed by incubating aliquots of the cerebral artery homogenates at a temperature 37°C for 60 min in 250 μl of sodium phosphate buffer, containing 3H-QNB in the absence or presence of high concentrations of atropine (1 μM). The binding in the presence of 1 μM atropine was termed "nonspecific" and was subtracted from that obtained in the absence of 1 μM atropine "total binding", to obtain the binding termed "specific binding". The assay was terminated by addition of 3 ml of the ice-cold buffer and rapid filtration through Whatman GF/B glass fiber filters under suction. After washing twice with 3 ml of the buffer, the filters were dried in an oven, transferred to counting vials and 8 ml of scintillation fluid added. Radioactivity was counted in a Packard Tri-Carb scintillation spectrometer (Model 3255).

Scatchard analysis was performed according to Bennet.18

ChAT Assay

ChAT activity in the 1000 x g supernatant fraction of the human cerebral arteries was measured according to the procedure of Fonnum et al.8 as modified by Florence and Bevan.19 Incubation was carried out for 12 minutes at 37°C.

Chemicals and Reagents

3H-QNB (36 Ci/mmol) and 14C-acetyl-coenzyme A (58 mCi/mmol) were obtained from New England Nuclear, Boston, Mass., and cold QNB was a gift from Dr. S. Spector, Roche Institute of Molecular Biology, Nutley, N.J. All other chemicals were of reagent grade and were obtained commercially.

Statistical Evaluations

The results given in the text are means ± S.E. of N experiments. The regression lines were calculated by a non-linear least square method using a computer program. The K, values for displacement of 3H-QNB binding by cholinergic agents were determined from concentration-response curves.

Results

Responses to Exogenous ACh

Responses to exogenously applied ACh were examined in isolated helical preparations of human and dog cerebral arteries. In human cerebral arteries, ACh induced relaxations, in a dose dependent manner (figs. 1, 2). Contractions were not observed in any preparation tested (N = 12). The EC50 of ACh was 6.1 ± 0.2 x 10^-6M. These relaxations were blocked by atropine (1 x 10^-7 M). In dog cerebral arteries, ACh first induced relaxation when the concentration was lower than 1 x 10^-7M and a higher concentration of ACh (1 x 10^-5M) led to contraction (figs. 1, 2). The EC50 of ACh required to induce relaxation was 7.2 ± 0.2 x
Concentration of ACh (log M) -9 -8 -7 -6 -5 -4

FIGURE 2. Dilatory effect of ACh on isolated human and dog cerebral arteries. Twelve human cerebral arteries (6 middle cerebral, 4 posterior cerebral, 1 anterior cerebral, 1 basilar) were tested. Points represent the means ± S.E. for 12 experiments in human cerebral arteries (○); and 5 experiments in dog cerebral arteries (●). EC50s were 6.1 ± 0.2 × 10⁻⁶M in human cerebral arteries (N = 12) and 7.2 ± 0.2 × 10⁻⁷M in dog cerebral arteries (N = 5), respectively.

3H-QNB Binding

Specific 3H-QNB binding increased linearly with increasing concentrations of the homogenates over the range of 30 fg to 150 fg protein per assay, in both human and dog cerebral arteries (data not shown). The binding assay was thus performed with less than 150 μg of protein per assay. The binding of 3H-QNB was time dependent and equilibrium was achieved within 30 min (data not shown).

Saturability of 3H-QNB Binding

Specific binding of increasing concentrations of 3H-QNB (0.2 to 6 nM) was saturable in the homogenate of human (N = 6) and dog cerebral arteries (N = 4) (fig. 3). Scatchard analyses indicated a single class of binding sites with an apparent equilibrium dissociation constant (Kd) and a maximum binding capacity (Bmax) (fig. 4). The Kd and Bmax were 1.5 nM and 93 fmol/mg protein in case of human cerebral arteries. The Kd and Bmax were 0.59 nM and 340 fmol/mg protein in the dog cerebral arteries.

Specificity of 3H-QNB Binding

The specificity of 3H-QNB binding was studied using cholinergic agents (fig. 5, table 1). Displacement curves for 3H-QNB binding indicated that Hill coefficients of muscarinic cholinergic agonists (ACh and carbachol) were smaller than 1.0 and those of muscarinic cholinergic antagonists (atropine and QNB) were close to 1.0. Ki values and Hill coefficients are summarized in table 1. Nicotinic cholinergic agents (nicotine and cytisine) partially inhibited the
MUSCARINIC RECEPTORS ON CEREBRAL ARTERY

FIGURE 5. The displacement of specific 3H-QNB binding to human cerebral arteries by cholinergic agonists and antagonists. Each point represents the mean of at least three separate experiments. (○); QNB, (△); atropine, (♦); ACh, (▲); carbachol.

binding but only in high concentrations in case of human cerebral arteries. The Ki values of nicotine and cytisine were $3.5 \pm 1.8 \times 10^{-3}$M and $5.7 \pm 4.1 \times 10^{-5}$M, respectively.

ChAT Activity in Cerebral Arteries

ChAT activities in the human and dog cerebral arteries were $1.6 \pm 0.4$ nmol/mg protein/hr in the former ($N = 6$) and $3.7 \pm 0.1$ nmol/mg protein/hr in the latter ($N = 6$).

Effects of Postmortem Delay in Freezing Tissue After Death on $^3$H-QNB Binding and ChAT Activity

Dog cerebral arteries were studied under two conditions: (A) tissue frozen immediately after death and stored, and (B) tissue frozen 2hr after death and stored. $^3$H-QNB binding at the concentration 0.45 nM was $137 \pm 10$ fmol/mg protein (N = 4) at the condition (A) and $128 \pm 6$ fmol/mg protein (N = 4) at the condition (B), respectively. ChAT activity was $3.7 \pm 0.1$ nmol/mg protein/hr (N = 6) at the condition (A) and $3.6 \pm 0.2$ nmol/mg protein/hr (N = 4) at the condition (B), respectively. The effects of 2 hrs postmortem delay in freezing the tissue on $^3$H-QNB binding and ChAT activity were not significant.

Discussion

Using a radioligand binding assay technique, we demonstrated the existence of muscarinic cholinergic receptors in human cerebral arteries and characterized their nature, compared with findings in dog cerebral arteries. Specific $^3$H-QNB binding in human and dog cerebral arteries was saturable. Scatchard and Hill plot analyses of the data indicated that $^3$H-QNB binding sites are of a single population. Muscarinic cholinergic drugs inhibited the binding at low concentrations. Displacement curve and Hill coefficients by muscarinic cholinergic agents indicated two classes of binding sites for agonists, as their Hill coefficients were smaller than 1.0 and one class binding site for antagonists, as their Hill coefficients were close to 1.0. These results are consistent with the previous reports on $^3$H-QNB binding sites in rat brain,19 guinea pig ileum,20 dog portal vein21 and bovine pial arteries.22 ChAT, the synthesizing enzyme of ACh, is useful marker of cholinergic innervation of presynaptic sites.8-9 As we detected ChAT activity in both human and dog cerebral arteries, these arteries seem to possess functional cholinergic innervation. ChAT activity of $3.7 \pm 0.1$ nmol/mg protein/hr in dog cerebral arteries is similar to the value given in the literature.11 In cerebral arteries, levels of ChAT activity appear to correlate with high affinity choline uptake and evoked ACh release.10,11 ChAT activity in human cerebral arteries was about half as high as that in dog cerebral arteries. Delay in freezing the tissue obtained at autopsy may have some effect on the ChAT activity in human cerebral arteries, however, the effect is probably not significant, since the ChAT activity was confirmed to be stable within 2 hrs in dog cerebral arteries and it is stable up to 72 hrs in the mouse brain.22 Lower ChAT activity in human cerebral arteries suggests that the density of cholinergic innervation may be lower than that in dog cerebral arteries.

Table 1  Hill Coefficients and Ki Values for Cholinergic Agonists and Antagonists of $^3$H-QNB Binding to Human and Dog Cerebral Arteries

<table>
<thead>
<tr>
<th>Drug</th>
<th>Human (N = 3)</th>
<th>Dog (N = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hill coefficient</td>
<td>Ki (nM)</td>
</tr>
<tr>
<td>QNB</td>
<td>0.89±0.12</td>
<td>1.0±0.2×10^{-9}</td>
</tr>
<tr>
<td>Atropine</td>
<td>0.95±0.09</td>
<td>1.1±0.6×10^{-8}</td>
</tr>
<tr>
<td>ACh</td>
<td>0.54±0.07</td>
<td>0.8±0.6×10^{-8}</td>
</tr>
<tr>
<td>Carbachol</td>
<td>0.33±0.02</td>
<td>1.2±0.2×10^{-7}</td>
</tr>
</tbody>
</table>

Ki was determined from the equation $Ki = IC_{50}(1 + S/K_D)$, where $S$ is the concentration of $^3$H-QNB used in the assay.
QNBP binding to human cerebral arteries may also be influenced by delay in freezing the tissue obtained at autopsy, however, 3H-QNB binding to human brain is reported to be unchanged up to 51 hrs of the postmortem course. The 3H-p-aminoclonidine and 3H-N-methylisocapoline binding to rat brain were gradually decreased within 24 hrs after death. We obtained human cerebral arteries within 2 hrs of death and confirmed in dogs that such a delay does not have a significant effect on 3H-QNB binding. Thus, the difference in 3H-QNB bindings between human and dog cerebral arteries is probably due to species differences rather than to postmortem effects on the receptors. Pharmacodynamic studies revealed that human cerebral arteries react to vasoactive agents or neural stimuli in a manner different from that of cerebral arteries from other species. These species differences were given attention mainly with regard to adrenergic receptors. Regarding cholinergic innervation of human cerebral arteries, Edvinsson et al reported that unlike cat cerebral arteries, contractile responses were not obtained with various concentration of ACh, and that the concentration of ACh required to produce relaxation in human cerebral arteries was about 10 times as large as that in cat cerebral arteries. In the present study, we also found that ACh induced only relaxation in the human cerebral arterial preparations and that the EC50 of ACh was about 10 times higher, as compared with that of dog cerebral arteries. One possible explanation for this low responsiveness is the low affinity and density of muscarinic cholinergic receptors, in the human cerebral arteries. It was reported that ACh and ChAT activities in middle cerebral and basilar arteries in cats and dogs exist heterogeneously. Muscarinic cholinergic receptors may also exist heterogeneously at the arteries of anterior and posterior circulations. Further studies are underway to clarify regional differences in the receptors.

In the present study on the isolated dog cerebral artery, ACh induced dual responses and in other studies, repetitive administration of ACh (1 x 10-7 M) led to contraction. On the other hand, ACh induced only relaxation in the human cerebral artery. Such a difference in the mechanical responses induced by ACh between dog and human cerebral arteries cannot be explained by differences in biochemical parameters of muscarinic receptors. Some populations of the muscarinic cholinergic receptors in the arteries were considered to locate on the vascular endothelium, as the relaxation to ACh disappeared after removal of the endothelium. Estrada and Krause reported that muscarinic cholinergic receptors have two classes of binding sites for agonists, in bovine cerebral arteries, and suggested that the binding site with a higher affinity for agonists on the endothelium mediates relaxation and that the other binding site with a lower affinity mediates contraction. The finding that higher concentrations of ACh induced contraction rather than relaxation in dog cerebral arteries seems to support this hypothesis. In human cerebral arteries, however, muscarinic cholinergic receptors also have two classes of binding sites for agonists, and only relaxation was produced by the administration of ACh. Further studies should clarify the physiological meaning of multiple binding sites for agonists and single binding sites for antagonists.

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Isolated Murine Brain Mitochondrial Oxidative Phosphorylation

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SUMMARY Glucagon has been shown to increase further the enhanced tolerance for hypoxia of mice with elevated blood ketones and to stimulate ketone utilization by rat brain slices, suggesting that glucagon may affect brain metabolism. In addition to stimulating gluconeogenesis, glucagon alters the metabolism of mitochondria isolated from liver and heart. This study was designed to test whether glucagon can act directly and selectively on brain mitochondrial substrate oxidation. Mitochondria were isolated from normal murine brains using differential centrifugation through Ficoll gradients. Glucagon (3.6 μM) stimulated respiration in the presence of glutamate, and glutamate plus beta-hydroxybutyrate, but not in the presence of glutamate plus malate, succinate or beta-hydroxybutyrate alone. With glutamate as the substrate the hormone significantly increased State 3 oxygen consumption rates from control values of 91 mol O2/mol of cytochrome aa3/min to 117 mol O2/mol aa3/min (p < 0.0001), and also increased State 4 rates slightly but significantly. Glucagon did not change mitochondrial respiratory control ratios, but increased estimated rates of ATP synthesis from 434 (control) to 597 mols ADP consumed/mol aa3/min (p < 0.0001). The data indicate that in addition to stimulating State 3 oxygen consumption rates in the presence of BHB as a substrate. These substrate-specific effects were not altered when respiration was studied in the presence of postmitochondrial supernatant or exogenous 3',5'-cyclic AMP, indicating that glucagon, in addition to its systemic effects, may increase hypoxic tolerance by modifying brain metabolism of ketones. The results also support the postulate that glucagon's substrate-specific stimulation of mitochondrial oxidative phosphorylation, although not by direct enhancement of ketone oxidation, may play a role in cerebral protection against hypoxic damage in instances when circulating hormone levels are high.

PREVIOUS STUDIES from our laboratory demonstrated that, when mice were placed in an hypoxic environment, those with elevated blood ketones survived up to five times longer than controls. Butanediol induced ketosis is also associated with a reduced neurologic deficit in the hypoxic rat. Furthermore, this enhanced hypoxic tolerance in mice was potentiated by exogenous glucagon (GG). In vitro studies on rat brain slices we found that GG stimulates the incorporation of the ketone beta-hydroxybutyrate (BHB) into carbon dioxide both in the presence or absence of added glucose. In addition, Harris et al have reported a stimulatory effect of GG on liver mitochondrial respiratory rates in the presence of BHB as a substrate. These results suggest an hypothesis that GG, in addition to its systemic effects, may increase hypoxic tolerance by modifying brain metabolism of ketones.

The ability of glucagon (GG) to alter cell metabolism is widely recognized, but its mechanisms and sites of action remain to be fully elucidated. For example, acute GG treatment of intact rats increases hepatic mitochondrial respiration and calcium fluxes. Suzuki reported that intravenous injection of GG in rats in-
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