hydroxybenzylamine or *N-n*-alkyl phenylethylamine derivatives were moderately active as inhibitors of both uptake systems, possessing IC_{50} values of between 29 μM and 75 μM against [^{3}H]GABA uptake, and between 2.8 μM and 4.3 μM against [^{3}H]5-HT uptake (Fig. 1; Table 1).

It is probable that the ability of these *N-n*-alkyl derivatives to interact with both [^{3}H]GABA and [^{3}H]5-HT uptake systems relates to the surfactant-like properties of these highly lipophilic compounds and reflects a non-specific interaction with neuronal cell membranes. Evidence to support this suggestion comes from the fact that whereas 5-HT itself is a potent inhibitor of [^{3}H]5-HT uptake while being inactive against [^{3}H]GABA uptake, the *N-n*-nonyl (12) derivative of 5-HT inhibited both uptake systems (Table 1).

In summary, our results indicate that in addition to their ability to inhibit [^{3}H]GABA uptake in the brain, a series of *N-n*-alkylbenzylamine and *N-n*-alkylphenylethylamine derivatives were also moderately active at inhibiting central [^{3}H]5-HT uptake mechanisms. These results extend the significance of our initial observations concerning the O-sulphate of (6). Although low degrees of selectivity were observed with some compounds (7, 8, 11), these differences are too small to encourage the search for selectively acting drugs by this strategy. Thus attempts to improve the CNS penetrability of specific inhibitors of neurotransmitter uptake processes through the addition of *N-n*-alkyl side chains may result in the formation of non-specific inhibitors of CNS uptake processes.

Department of Pure and Applied Chemistry
University of Strathclyde
Thomas Graham Building
295 Cathedral Street
Glasgow G1 1XL, U.K.

COLIN J. SUCKLING
ROBIN J. BRECKENRIDGE
SUKHVINDERS S. BANSAL

* Author to whom all correspondence should be addressed.

Neuroscience Research Centre
Merck Sharp and Dohme Research Laboratories
Terlings Park
Eastwick Road
Harlow
Essex CM20 2QR, U.K.

LINDSEY C. WILLIAMS
KEITH J. WATLING*
LESLIE L. IVERSEN

REFERENCES

1. D. R. Curtis, in *GABA-Neurotransmitters* (Eds. P. Krogsgaard-Larsen, J. Scheel-Kruger and H. Kofod), p. 17. Munksgaard, Copenhagen (1979).
2. B. S. Meldrum, *Clin. Neuropharm.* **6**, 293 (1982).
3. L. L. Iversen and J. S. Kelly, *Biochem. Pharmac.* **24**, 933 (1975).
4. D. L. Martin, in *GABA in Nervous System Function* (Eds. E. Roberts, T. N. Chase and D. B. Tower), p. 347. Raven Press, New York (1976).
5. D. R. Curtis, C. J. A. Game and D. Lodge, *Exp. Brain Res.* **25**, 413 (1976).
6. W. D. Matthews, G. P. McCafferty and P. E. Setter, *Neuropharmacol.* **20**, 561 (1981).
7. M. J. Croucher, B. S. Meldrum and P. Krogsgaard-Larsen. *Eur. J. Pharmac.* **89**, 217 (1983).
8. R. J. Breckenridge, S. H. Nicholson, A. J. Nicol, C. J. Suckling, B. Leigh and L. L. Iversen, *Biochem. Pharmac.* **30**, 3045 (1981).
9. C. A. Dewar, C. J. Suckling and C. Higgins, *J. Chem. Res.* (s) 335, (M) 3801 (1979).
10. H. N. Rydon and J. C. Tweddle, *J. chem. Soc.* 3499 (1955).
11. A. Stoll, F. Trexler, J. Peyer and A. Hofmann, *Helv. chim. Acta* **38**, 1452 (1955).
12. P. Bucort, J. Vallas and R. Jolly, *Chem. Abs.* **54**, 13018 (1960).
13. P. Krogsgaard-Larsen and G. A. R. Johnston, *J. Neurochem.* **25**, 797 (1975).

Biochemical Pharmacology, Vol. 34, No. 23, pp. 4177–4179, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

Regulation of cerebrovascular γ-glutamyltranspeptidase by adrenergic and cholinergic agonists

(*Received* 4 *October* 1984; *accepted* 20 *May* 1985)

Cerebral microvascular endothelium, the constituent cell of the blood–brain barrier, is enriched in the enzyme γ-glutamyltranspeptidase [(5-glutamyl)-peptide:amino acid 5-glutamyltranspeptidase, EC 2.3.2.2] [1, 2]. Elevated levels of this enzyme are found in several other cell types where amino acid transport is a prominent feature [3, 4]. Additionally, uptake of amino acids has been correlated with the level of transpeptidase activity [5]. These data suggest that γ-glutamyltranspeptidase plays a role in the regulation of amino acid uptake and transport. The factors that may modulate enzyme activity are unknown, though the demonstration of glial cell induction of cerebral endothelial transpeptidase indicates the importance of external factors [1]. Recent studies have demonstrated the presence of muscarinic cholinergic receptors on cerebral endothelial cells [6] and have shown that muscarinic and β-adrenergic receptors interact to determine the level of endothelial cAMP, a purported mediator of cerebrovascular permeability [7]. It has been also postulated that acetylcholine plays a role in amino acid transport [8] in non-nervous tissue. In this report, we test the hypothesis that adrenergic and/or cholinergic agonists, either independently or jointly, modulate the activity of γ-glutamyltranspeptidase in cerebral endothelium.

Methods

A microvessel preparation consisting primarily of capillary segments was isolated from the cerebral cortices of groups of twenty-four male Sprague–Dawley rats, as previously described [9]. The final microvessel pellet was resuspended in Dulbecco's modified essential medium containing 10% fetal calf serum supplemented with 20% dimethyl sulfoxide and stored in liquid nitrogen until use. Microvessels were quick-thawed and resuspended in phosphate-buffered saline (1 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM NaCl, 10 mM NaH_2PO_4, pH 7.4) (PBS). An alternate buffer, Hanks' balanced salt solution (137 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 0.6 mM Na_2HPO_4 6 mM glucose, pH 7.4 (HBSS), initially selected, yielded basal enzyme levels 60% lower than those obtained in PBS. Although the mechanism whereby incubation in PBS results in higher enzyme activity is unknown, the use of

HBSS was discontinued and all experiments were performed in PBS. The incubation mixture contained 1 mg microvessel protein and appropriate agents (carbachol, isoproterenol, atropine, propranolol) at concentrations ranging from 0.01 μM to 1 mM in a total volume of 1 ml. Cells were incubated for 10 min at 37°, and the reaction was terminated by the addition of 1 ml of ice-cold PBS and centrifugation. The microvessels were resuspended in cold PBS and assayed for γ-glutamyltranspeptidase activity according to the method of Caspers and Diglio [10]. A unit of enzyme is defined as the amount of γ-glutamyltranspeptidase activity catalyzing the formation of 1 nmole *p*-nitroaniline/min at 37°. Cell protein was determined by the method of Lowry *et al.* [11] using bovine serum albumin as a standard. The data represent the means of three to seven separate experiments performed in triplicate. Statistical significance was determined by one-way analysis of variance.

Results and discussion

The γ-glutamyltranspeptidase activity of unstimulated cerebral microvessels in PBS is 19.3 ± 4 units/mg. Addition of the β-adrenergic agonist isoproterenol increased the level of enzyme activity from 17% (0.1 μM) to approximately 50% (100 μM) above control (Fig. 1). The stimulatory effect of isoproterenol on microvascular transpeptidase was blocked in the presence of 10 μM propranolol (Table 1). Incubation with a cholinergic agonist at a maximal concentration of 100 μM did not alter significantly the basal level of transpeptidase activity (18.2 ± 5 units/mg). However, concurrent incubation with carbachol (100 μM) and isoproterenol prevented the increase in transpeptidase activity elicited by isoproterenol alone ($P < 0.001$) (Table 1). At lower carbachol concentrations (0.01 to 100 μM), cholinergic inhibition of the stimulation of isoproterenol was proportionally less (0–90%), with 50% inhibition occurring at 1–10 μM. The muscarinic antagonist atropine sulfate (10 μM) blocked the inhibition of enzyme activity evoked by carbachol (Table 1).

These data indicate that γ-glutamyltranspeptidase activity is modulated by cholinergic–adrenergic agonists.

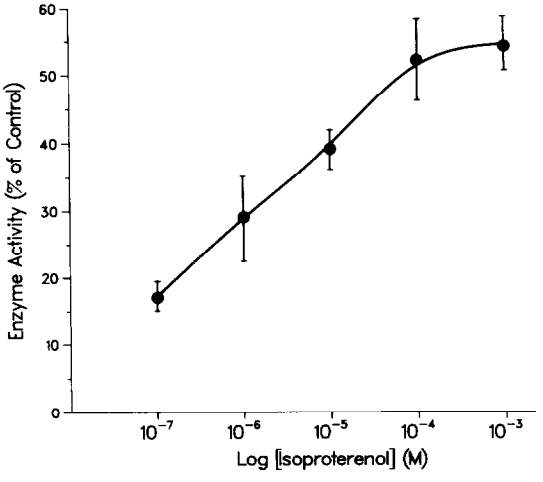

Fig. 1. Stimulation of microvascular γ-glutamyltranspeptidase activity by various concentrations of isoproterenol. Results are the difference between control and stimulated enzyme activity expressed as percent of control. The data represent the means ± standard deviation from three separate experiments.

* Address correspondence to: Dr. Paula Grammas, Department of Pathology, Room 9255 Scott Hall, Wayne State University School of Medicine, 540 East Canfield, Detroit, MI 48201.

Table 1. γ-Glutamyltranspeptidase activity in cerebral microvessels

Additions	Activity (units/mg)
None	19.3 ± 4
Carbachol (100 μM)	18.2 ± 5
Isoproterenol (100 μM)	26.2 ± 7*
Isoproterenol + propranolol (10 μM)	19.5 ± 1
Carbachol + isoproterenol	16.8 ± 4
Carbachol + isoproterenol + atropine (10 μM)	28.9 ± 3

Values are means ± standard deviation from three to five separate experiments.

* Significant change from control and from carbachol + isoproterenol ($P < 0.001$).

While the inverse coupling of cholinergic receptor activation and enzyme inhibition was not significant, it completely prevented the increase in transpeptidase activity evoked by isoproterenol. The mechanism whereby this receptor interaction occurs is unknown. In this regard one can speculate that enzyme activity may be related to the level of intracellular cAMP since this nucleotide is also dually regulated in a similar manner [7]. Alternatively, since direct evidence linking transpeptidase activity to adenylate cyclase is lacking, γ-glutamyltranspeptidase may be regulated by a secondary mediator independent of the adenylate cyclase–cAMP pathway.

There is evidence that neurotransmitters in non-nervous tissue may perform a variety of functions including nutrient transport [8]. Data from the human placenta indicate that placental plasma membrane vesicles possess muscarinic receptors that, in response to acetylcholine stimulation, alter amino acid transport [12]. We suggest that the homeostatic function of cerebral endothelium in the maintenance of brain milieu may be influenced by cholinergic–adrenergic agonists. On the basis of evidence that γ-glutamyltranspeptidase is intimately linked to amino acid transport in several tissues, including cerebral endothelium [13], one can speculate that cholinergic–adrenergic modulation of transpeptidase activity might regulate blood–brain amino acid transport. This hypothesis is supported by the presence of adrenergic and cholinergic effectors in the cerebral microvasculature [14, 15]. Further experiments to directly link amino acid transport to cholinergic–adrenergic regulation are currently in progress.

In summary, stimulation of adrenergic receptors in cerebral capillaries increased γ-glutamyltranspeptidase activity, whereas concurrent muscarinic activation prevented this effect. Cholinergic agonists alone did not alter significantly basal enzyme activity. The possible significance of transpeptidase modulation by cholinergic–adrenergic agonists regarding amino acid transport is discussed.

Acknowledgements—The authors gratefully acknowledge the contributions of Denise Bessert, Terri Janasik and Joel Mathews. The support of Dr. Ray K. Brown is greatly appreciated. Supported in part by United States Public Health Services Grant HL 23603 from the Heart, Lung and Blood Institute, National Institutes of Health, and by the Petroleum Research Fund administered by the American Chemical Society.

Department of Pathology
Wayne State University School of Medicine
Detroit, MI 48201; and
Department of Chemistry
The University of Detroit
Detroit, MI 48221, U.S.A.

PAULA GRAMMAS*
MARY LOU CASPERS
CLEMENT A. DIGLIO
FILIBERTO GIACOMELLI
JOSEPH WIENER

REFERENCES

1. B. M. Djuricic and B. B. Mrsulja, *Brain Res.* **138**, 561 (1977).
2. L. DeBault and P. A. Cancilla, *Science* **207**, 653 (1980).
3. A. Meister, *Science* **180**, 33 (1973).
4. G. V. Marathe, B. Nash, R. H. Haschemeyer and S. S. Tate, *Fedn Eur. Biochem. Soc. Lett.* **107**, 436 (1979).
5. J. Vina, I. R. Puertes, J. B. Montoro and J. R. Vina, *Fedn Eur. Biochem. Soc. Lett.* **159**, 119 (1983).
6. P. Grammas, C. A. Diglio, B. H. Marks, F. Giacomelli and J. Wiener, *J. Neurochem.* **40**, 645 (1983).
7. P. Grammas, C. A. Diglio, F. Giacomelli and J. Wiener, *J. Neurochem.* **44**, 1732 (1985).
8. B. V. R. Sastry and C. Sadavongvivad, *Pharmac. Rev.* **30**, 65 (1979).
9. C. A. Diglio, P. Grammas, F. Giacomelli and J. Wiener, *Lab. Invest.* **46**, 544 (1982).
10. M. L. Caspers and C. A. Diglio, *Biochim. biophys. Acta* **803**, 1 (1984).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol Chem.* **193**, 265 (1951).
12. J. O. Olubadewo and B. V. R. Sastry, *J. Pharmac. exp. Ther.* **204**, 433 (1978).
13. P. A. Cancilla and L. E. DeBault, *J. Neuropath. exp. Neurol.* **42**, 191 (1983).
14. F. M. Lai, S. Underfriend and S. Spector, *Proc. natn. Acad. Sci. U.S.A.* **72**, 4622 (1975).
15. C. Estrada, E. Hamel and D. N. Krause, *Brain Res.* **266**, 261 (1983).

Biochemical Pharmacology, Vol. 34, No. 23, pp. 4179–4180, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

Lack of inhibition of glucuronidation in isolated rat hepatocytes by diethyl ether anesthesia

(*Received* 9 *February* 1985; *accepted* 28 *May* 1985)

Diethyl ether anesthesia has been shown to decrease the glucuronidation of both exogenous and endogenous compounds in rats [1, 2]. The inhibition of glucuronidation by diethyl ether is caused by a depletion of uridine diphosphate glucuronic acid (UDPGA), an essential cofactor for glucuronidation [3–5]. A 10-min exposure to diethyl ether results in a decrease in UDPGA levels of 90–95% [5, 6]. However, when animals are allowed to recover from the anesthesia, the UDPGA concentration returns to a control value within 1 hr after exposure. All the previous studies [1, 3–5] determined UDPGA levels in tissue homogenates. Therefore, it was of interest to determine if diethyl ether anesthesia decreases UDPGA levels and thus glucuronidation in another hepatic preparation, isolated hepatocytes, used to study this conjugation pathway. Diethyl ether anesthesia was compared to urethane anesthesia, as urethane has been shown to decrease UDPGA levels only slightly in rats [4, 5].

Male Sprague–Dawley rats were anesthetized with urethane (approximately 1.5 g/kg) i.p. or diethyl ether by continuous inhalation. Hepatocytes were isolated by a modification of the procedure described by Eacho and Weiner [7]. The abdominal cavity was opened, the inferior vena cava was ligated above the renal vein, and the chest cavity was opened. An incision was made in the right atrium through which the inferior vena cava was canulated. A retrograde perfusion of the liver was initiated with a Ca^{2+}-free Krebs–Ringer bicarbonate buffer containing 5.5 mM glucose and 0.5 mM ethyleneglycolbis(aminoethyl-ether)tetra-acetate (EGTA). Livers were perfused until relatively cleared of blood and then perfused with a Krebs–Ringer bicarbonate buffer containing 4.75 mM Ca^{2+} and 0.5 mg/ml collagenase at a flow rate of 17–20 ml/min for 20 min. Cells were diluted to a final concentration of 2×10^6 cells/ml with Krebs–Ringer bicarbonate buffer containing 1% bovine serum albumin.

Glucuronide formation of *p*-nitrophenol (*p*-NP) was determined as previously described [8]. The level of UDPGA in hepatocytes was determined enzymatically by the method of Ullrich and Bock [9]. Aliquots (2 ml) of cell suspension containing approximately 2×10^6 cells/ml were removed at 0, 15 and 30 min after the addition of *p*-NP and then boiled for 2 min. After heating, the suspensions were cooled on ice and centrifuged for 15 min at 10,000 *g*. UDPGA was determined in the supernatant fluid by means of the 4-methylumbelliferone-glucuronyl transferase assay [10].

A comparison of the effects of urethane and diethyl ether anesthesia, used in the preparation of isolated hepatocytes, on glucuronide formation is shown in Fig. 1. No significant difference was found between urethane and diethyl ether in the amount of *p*-NP glucuronide formed. Similarly, UDPGA levels were not found to be significantly different between the two anesthetics at each time interval (Table 1). UDPGA concentrations of 3.10 ± 0.24 and 3.20 ± 0.21 nmoles/10^6 cells were found at 30 min in hepatocytes of rats anesthetized with urethane or diethyl ether respectively. These levels are within the range of previous studies [9, 11, 12]. When calculated on a basis of 127×10^6 cells/g liver (wet wt) [13], a level of 0.41 μmole/g liver was obtained. This level is in agreement with that found in the studies *in vivo* [14, 15] and in perfused livers [14, 16].

A possible explanation for the failure of diethyl ether to decrease UDPGA levels in hepatocytes as it does in tissue homogenates may relate to the differences in metabolic capabilities and thus the capacity for synthesizing UDPGA in these preparations. Once hepatic UDPGA levels have been depleted by diethyl ether, the hepatocyte is disrupted in the preparation of liver homogenates and therefore is incapable of subsequent formation of UDPGA. Conversely, hepatocytes retain their full spectrum of biochemical capabilities during isolation and incubation. Therefore, because glucose, a precursor of UDPGA, is present at physiological levels in most incubation buffers [17] and all the enzymes necessary for the synthesis of UDPGA are available, hepatocytes can resynthesize UDPGA and thus prevent depletion. Furthermore, since the liver must be perfused and the cells then incubated, filtered, centrifuged, and diluted to the appropriate concentration, there is sufficient time (approximately 1.5 hr) after cessation of exposure to diethyl ether for UDPGA levels to return to normal values. This conclusion is supported by the previously mentioned study in which animals anesthetized with diethyl ether were allowed to recover, and UDPGA levels rapidly returned to control concentrations by 1 hr after exposure [5]. Similarly, Aune *et al.* [18] examined the direct effects of ether on the metabolism of paracetamol in isolated hepatocytes and found that, when the concentration of ether is maintained throughout the incubation, the formation of glucuronide is reduced 70%.

Although not significantly different, there was a trend for UDPGA levels from rats anesthetized with ether to be slightly higher than those from animals anesthetized with