10. B. Matkivics, A. Laszlo and L. Szabo, *Clin. Chim. Acta* **118**, 289 (1982).
11. D. P. Chanhan, P. H. Gupta, M. R. N. Nampoothiri, P. C. Singhal, K. S. Chugh and C. R. Nair, *Clin. Chim. Acta* **123**, 153 (1982).
12. T. P. Flynn, D. W. Allen, G. J. Johnson and J. P. White, *J. clin. Invest.* **71**, 1215 (1983).
13. J. M. C. Gutteridge, *FEBS Lett.* **128**, 343 (1981).
14. J. Stocks and T. L. Dormandy, *Clin. Chim. Acta* **27**, 117 (1970).
15. B. Halliwell, *FEBS Lett.* **92**, 321 (1978).
16. J. M. C. Gutteridge, *FEBS Lett.* **150**, 454 (1982).
17. M. R. Clemens and H. Remmer, *Blut* **45**, 329 (1982).
18. M. R. Clemens, H. Einsele, H. Frank, H. Remmer and H. D. Waller, *Biochem. Pharmac.* **32**, 3877 (1983).
19. E. Beutler, C. West and K. G. Blume, *J. lab. Clin. Med.* **88**, 328 (1976).
20. R. Henry, D. C. Cannon and J. W. Winkleman, in *Clinical Chemistry, Principles and Practice*, p. 1149. Harper and Row, New York (1974).
21. H. Frank, T. Hintze, D. Bimboes and H. Remmer, *Toxic. appl. Pharmac.* **56**, 337 (1980).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. G. W. Burton, K. U. Ingold and K. E. Thompson, *Lipids* **16**, 946 (1981).

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Thyroliberin (TRH), 6-methyl-5-oxothiomorpholinyl-3-carbonyl histidylproline amide and histidylproline diketopiperazine do not affect the release of [^{3}H]-acetyl choline and [^{3}H]-choline from rat brain tissue cubes

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Thyroliberin (L-pyroglutamyl-L-histidyl-L-proline amide, TRH) elicits a variety of behavioural changes [1] in addition to its role as a hypophysiotrophic hormone. The effect of TRH on locomotor activity, mediated by the mesolimbic dopaminergic pathway [2, 3] and its antagonism of drug-induced narcosis [4, 5] possibly mediated by the septal–hippocampal cholinergic pathway [6] have been particularly well studied.

Several studies have sought a biochemical basis for some of the TRH-induced behavioural changes. One of the most frequently reported results is that 10^{-5}–10^{-3} M TRH stimulates the release of dopamine from nucleus accumbens [3, 7] and striatal [8, 9] preparations. In view of the relatively high concentrations required, it is unlikely that TRH has a direct effect on dopamine release. TRH has been reported to stimulate the release of acetylcholine (ACh) from rabbit cerebral cortex [6]. It was decided, therefore, to test an alternative hypothesis that the prime effect of TRH is to stimulate ACh release, which in turn enhances DA release from the nucleus accumbens [10] and striatum [11]. In particular an effect of TRH on the release of ACh from the septum might provide a biochemical basis for the TRH-induced antagonism of pentobarbital and ethanol narcosis [6].

Few reports have appeared on the effect of TRH on cholinergic biochemistry. This study finds that TRH, its analogue 6-methyl-5-oxothiomorpholinyl-3-carbonylhistidyl proline amide (CG3703) and a metabolite histidylproline diketopiperazine (cyclo His-Pro) do not effect the release of either ACh or Ch from rat brain nucleus accumbens or septal tissue cubes.

Materials and methods

Male Wistar rats (250 ± 50 g) were decapitated, their brains rapidly removed and nuclei accumbens dissected according to the method of Horn *et al.* [12]. Septa were dissected by making a coronal section at the optic chiasma and a second section 3 mm caudal to the optic chiasma. The slice of tissue was placed rostral face uppermost and the septal region dissected as a cube (90–120 mg wet weight), with 2–3 mm^2 cross section, on the mid line, immediately ventral to the corpus callosum, and between the corpora striata.

Choline uptake and release. Tissue chops (0.2 mm × 0.2 mm) were obtained from each brain region, using a McIlwain tissue chopper, and incubated in Tris-buffered saline (50 mg tissue/ml) consisting of 20 mM Tris–HCl, pH 7.4 containing 120 mM NaCl; 5 mM KCl; 3.5 mM $NaHCO_3$; 2.5 mM $CaCl_2$; 1.2 mM KH_2PO_4; 1 mM $MgSO_4$; 20 mM glucose; 0.5 mM asorbic acid; 0.1 mM physostigmine; 50 μg/ml bacitracin and 0.17 μM [^{3}H]-choline chloride (10 μCi; sp. act 60 Ci/mmole) at 37° with shaking for 30 min. Uptake was terminated by centrifugation at 10,000 *g* for 10 min at 4°, the pellet washed three times in 5 ml Tris-buffered saline and resuspended in the same medium (600 μl) per nucleus accumbens or septum). Aliquots (600 μl) were transferred to Eppendorf microcentrifuge tubes and the brain fragments pelleted by centrifugation at 10,000 g for 2 min at 20°. The tubes were placed on ice and the supernatant removed by aspiration. Tris-buffered saline (600 μl) at 37°, containing either 5 mM KCl or 50 mM KCl, in the presence or absence of 0.1 mM TRH, 0.1 mM cyclo His Pro or 0.1 mM CG 3703, was added to each tube, which were then incubated, with frequent mixing, at 37° for 5 min. Release of [^{3}H]-compounds was terminated by cooling the tubes to 0° followed by centrifugation at 10,000 *g* for 2 min. Aliquots (400 μl) of each supernatant were lyophilized, the residue dissolved in 50 μl methanol containing 10 mM choline (Ch) and 10 mM acetylcholine (ACh) as standards, and the proportion of radioactivity in Ch and ACh determined by chromatography of 10 μl samples on cellulose TLC plates. The total radioactivity released was determined by removing 100 μl aliquots of the supernatant for liquid scintillation counting using toluene–Triton X-100 (2:1 v/v) containing 0.8% (w/v) PPO as scintillant. The remainder of the supernatant was removed, by aspiration, and the pellets were homogenized in methanol (150 μl) containing 10 mM Ch and 10 mM ACh, using a mini-drill with a Teflon-tipped pestle. The homogenate was stored at −20° for 30 min and the protein precipitate removed by centrifugation at 10,000 *g* for 2 min. The total radioactivity, extracted from the pellet, was measured by removing aliquots (100 μl) of the supernatant for liquid scintillation spectrometry. The proportion of tritium in Ch and ACh determined by chromatography of 10 μl aliquots of the supernatant on cellulose TLC plates.

Release of [^{3}H]Ch and [^{3}H]ACh was expressed as:

$$\frac{\text{cpm in supernatant}}{\text{cpm in Supernatant + cpm in pellet}} \times 100\%$$

Statistical significance was determined using the Student's *t*-test [13].

Chromatographic separation of Ch and ACh. Cellulose TLC plates were developed for approximately 6 hr, in butan-1-ol/water/ethanol/glacial acetic acid (8:3:2:1 by volume), dried under warm air and the position of Ch (R_f 0.43) and ACh (R_f 0.33) located by iodoplatinate reagent (20 vols 4.5% w/v KI in ethanol/water 1:1 v/v freshly mixed with 1 vol. 5% v/v chloroplatinic acid). Regions of the chromatogram corresponding to Ch and ACh were transferred to scintillation vials, containing 3 ml toluene–Triton X-100 scintillant. 1.4 ml H_2O was added and the particles of cellulose suspended in a stable gel by vigorous shaking.

Stability of ACh during lyophilization. Aliquots (400 μl) of 20 mM Tris-buffered saline, containing 0.2–0.8 μCi[^{14}C] ACh, were lyophilized in microtubes and the residues dissolved in 50 μl methanol, 10 mM Ch and 10 mM ACh. 10 μl Aliquots of the methanolic solution were chromatographed on cellulose TLC plates and the proportion of radioactivity in Ch and ACh determined as described above.

[^{3}H]-Choline chloride (60 Ci/mmole) and [^{14}C]-acetylcholine chloride (26.5 mCi/mmole) were purchased from Amersham International PLC, (Amersham, Bucks, U.K.). Thyroliberin and histidylproline diketopiperazine were purchased from Pierce and Warriner Ltd. (Cheshire, U.K.). Bacitracin was supplied by Sigma London Chemical Co. Ltd. (Poole, Dorset, U.K.). 6-Methyl-5-oxothiomorpholinyl-3-carbonyl-histidyl proline amide (CG3703) was a gift from Dr. L. Flohé, Grünenthal Gmbh, G.F.R. All other fine chemicals were supplied either by B.D.H. Chemicals Ltd., Poole, Dorset, U.K. or by Sigma London Chemical Co. Ltd.

Results and discussion

50 mM KCl stimulated the release of [^{3}H] ACh from tissue chop preparations of nucleus accumbens by 200% and septal preparations by 150% (Table 1).

This result is in agreement with other studies which reported that KCl stimulated the release of ACh from guinea pig cerebral cortical synaptosomes [14, 15] and rat hippocampal slices [16]. 50 mM KCl also stimulated the release of [^{3}H] Ch from tissue chop preparations of nucleus accumbens and septa, by 70% and 50% respectively (Table 1). This slight stimulation is in agreement with other workers who also found that KCl has only a minimal effect on the release of [^{3}H]Ch from synaptosomes [15, 17] or tissue slices [16]. 99.6 ± 0.04% (N = 3) of [^{14}C] ACh added to the supernatant, obtained by terminating the release assay, was recovered, unhydrolysed, after lyophilization. Thus the [^{3}H] Ch, recovered during release, is not due to hydrolysis of ACh during the isolation procedure. These results suggest that the effect of depolarising concentrations of KCl on the release of ACh from nucleus accumbens and septal tissue chops is similar to that reported for other brain regions.

0.1 mM TRH had no significant effect on the release of ACh or Ch, in the presence of absence of 50 mM KCl, from either preparation (Table 1). The failure of TRH to alter the release of ACH or Ch is unlikely to be due to degradation of the tripeptide, because the analogue CG3703, which is resistant to peptidases, which inactivate TRH [18], was also inactive (Table 1). Furthermore 0.1 mM cyclo His Pro, which is one of the main products of TRH metabolism, and has been reported to be more effective than TRH in the antagonism of ethanol narcosis [19] also had no effect on the release of [^{3}H]ACh or [^{3}H] Ch (Table 1).

Renaud *et al.* [20] found that TRH did not alter the uptake of [^{3}H] choline or the KCl stimulated release of [^{3}H] from synaptosomes prepared from guinea pig or rat cortices. The present study extends that work as TRH had no effect on the release of [^{3}H]ACh or [^{3}H]Ch from tissue chops of the nucleus accumbens and septum. Both these brain regions have been reported to exhibit high affinity binding sites for TRH and 3-methylhistidyl TRH [21] and to contain choline acetyltransferase [22, 23] which suggests that they contain cholinergic nerve endings.

In summary, thyroliberin (TRH), its metabolite histidylproline diketopiperazine and the analogue 6-methyl-5-oxothiomorpholinyl-3-carbonylhistidyl proline amide (CG3703) had no effect on either the spontaneous or K^+-stimulated release of [^{3}H]-acetylcholine or [^{3}H]-choline from tissue cubes of the nucleus accumbens or septal region

Table 1. Effect of KCl, TRH, cyclo His-Pro and CG3703 on the release of [^{3}H] choline and [^{3}H] acetylcholine from rat brain tissue chops

	Compound released*			
Addition	Acetylcholine	Stimulation % due to 50 mM KCl	Choline	Stimulation % due to 50 mM KCl
A. Nucleus Accumbens				
None	12.3 ± 4.6 (6)	—	15.8 ± 3.9 (6)	—
KCl (50 mM)	*38.4 ± 7.4 (6)	212	*26.6 ± 5.3 (6)	68
TRH (0.1 mM)	11.3 ± 2.6 (6)	N.S.	16.0 ± 4.4 (6)	N.S.
TRH + KCl	*39.2 ± 7.3 (6)	246	25.1 ± 5.6 (6)	56
CG3703 (0.1 mM)	9.7 ± 3.5 (3)	N.S.	12.7 ± 2.4 (3)	N.S.
CG3703 +KCl	*35.7 ± 9.9 (3)	268	26.6 ± 0.9 (3)	109
B. Septum				
None	9.9 ± 2.2 (6)	—	16.3 ± 0.9 (6)	—
KCl (50 mM)	*24.5 ± 4.8 (6)	147	*24.8 ± 2.6 (6)	52
TRH (0.1 mM)	11.0 ± 4.3 (6)	N.S.	18.0 ± 4.5 (6)	N.S.
TRH + KCl	*24.0 ± 8.0 (6)	118	†21.8 ± 5.2 (6)	21
CG3703 (0.1 mM)	9.6 ± 3.7 (5)	N.S.	16.5 ± 4.1 (5)	N.S.
CG3703 + KCl	*26.6 ± 6.7 (5)	177	*26.2 ± 5.7 (5)	59
Cyclo His-Pro (0.1 mM)	8.3 ± 2.1 (5)	N.S.	15.9 ± 2.8 (5)	N.S.
Cyclo His-Pro + KCl	*25.6 ± 9.8 (5)	208	*25.1 ± 3.5 (5)	58

* Results are means ± S.D. for release from (N) different preparations. Significantly different from release in the absence of 50 mM KCl: *0.001, † 0.005.

N.S. Not significantly different from release in the absence of the addition.

of rat brain. These results do not support the hypothesis that the TRH antagonism of drug-induced narcosis is mediated by modulating the release of ACh from cholinergic neurones which form synapses in the nucleus accumbens or septum.

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REFERENCES

1. G. G. Yarbrough, *Prog. Neurobiol.* **12**, 291 (1979).
2. D. J. Heal and A. R. Green, *Neuropharmacology* **18**, 23 (1979).
3. M. Miyamoto, S. Narumi, Y. Nagai, T. Shima and Y. Nagawa, *Jap. J. Pharmac.* **29**, 335 (1978).
4. G. R. Breese, J. M. Cott, B. R. Cooper, A. J. Prange, Jr., M. A. Lipton and N. P. Plotnikoff, *J. Pharmac. exp. Ther.* **193**, 11 (1974).
5. J. M. Cott, G. R. Breese, B. R. Cooper, T. S. Barlow and A. J. Prange, Jr., *J. Pharmac. exp. Ther.* **196**, 594 (1976).
6. G. G. Yarbrough, *Life Sci.* **33**, 111 (1983).
7. R. W. Kerwin and C. J. Pycock, *Br. J. Pharmac.* **67**, 323 (1979).
8. L. M. Shapiro, G. M. Bowes and P. F. T. Vaughan, *Life Sci.* **27**, 2099 (1980).
9. W. D. Horst and N. Spirt, *Life Sci.* **15**, 1073 (1974).
10. J. S. De Belleroche and I. M. Gardiner, *Br. J. Pharmac.* **75**, 359 (1982).
11. J. S. De Belleroche and H. F. Bradford, *Adv. Biochem. Psychopharmac.* **19**, 57 (1978).
12. A. S. Horn, A. C. Cuello and R. J. Miller, *J. Neurochem.* **22**, 265 (1974).
13. D. Colquhoun, in *Lectures on Biostatistics*. Clarendon Press, Oxford (1971).
14. S. Wonnacott, *J. Neurochem.* **34**, 1567 (1980).
15. S. Wonnacott and R. M. Marchbanks, *Biochem. J.* **156**, 701 (1976).
16. A. H. Mulder, H. I. Yamamura, K. J. Kuhar and S. H. Snyder, *Brain Res.* **70**, 372 (1974).
17. L. C. Murrin, R. N. De Haven and K. J. Kuhar, *J. Neurochem.* **29**, 681 (1977).
18. H. H. Hennies and L. FLohé, *Biochem. Pharmac.* **31**, 2430 (1982).
19. C. Prasad, T. Marsui and A. Peterkofsky, *Nature. (Lond).* **268**, 142 (1977).
20. L. P. Renaud, H. W. Blume, Q. J. Pitman, Y. Lamour and A. T. Tan, *Science* **205**, 1275 (1979).
21. R. L. Taylor and D. R. Burt, *J. Neurochem.* **38**, 1649 (1982).
22. D. M. Jacobowitz and M. Palkovits, *J. comp. Neur.* **157**, 13 (1974).
23. D. M. Jacobowitz and M. Palkovits, *J. comp. Neur.* **157**, 29 (1974).

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Precursor- and pool-dependent differential effects of ethanol on human platelet prostanoid synthesis

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Ethanol-induced changes in prostanoid synthesis occur both *in vitro* [1–4] and *in vivo* [5] and in a variety of tissues and species [6, 7]. Effects of ethanol on prostaglandin-stimulated adenylate cyclase activity are reported as well [1, 8, 9]. Chronic ethanol administration also inhibits the desaturases that convert *cis*-linoleic acid (cLA) to gamma-linolenic acid (GLA) and dihomo-gamma-linolenic acid (DGLA) to arachidonic acid (AA) in liver and brain [10, 11]. Many of these effects occur within physiologically relevant concentration ranges of ethanol.

These biochemical effects of ethanol on essential fatty acid (EFA) metabolism and prostanoid synthesis may underlie diverse *in vivo* effects of ethanol. Evidence supporting this is that in rodents manipulation of EFA metabolism and prostanoid synthesis with non-steroidal anti-inflammatory drug, EFA-deficient diets or with prostaglandin (PG) precursor EFA treatment alters behavioral, physiologic and pathologic effects of ethanol, including: sedation [12, 13], hyperactivity [14], hypothermia [15], withdrawal behavior [1, 13, 16] and the development of fatty liver measured by the accumulation of hepatic triglycerides [17, 18].

Further, treatment of human alcoholics with EFAs (10% GLA, 72% cLA) may reduce alcohol craving, improve liver function, and enhance impaired cognitive function [19].

Given the vast amounts of EFAs esterified to membrane phospholipids (much of which are available for prostaglandin synthesis), it is not clear how to account for the potent effects of dietary EFAs on the behavioral response to ethanol. Based on analysis of eicosanoid metabolism in rabbits fed an EFA-deficient diet, Crawford [20] has proposed two physiologically active pools for prostaglandin synthesis: (1) a "membrane" pool of esterified EFAs (not readily affected by diet) released upon phospholipase activation, and (2) a "metabolic" precursor pool of free EFAs (sensitive to dietary changes) which may play an important role in maintaining prostanoid levels under non-stimulated conditions. We have studied this concept in pulse-labeled platelets (in which nearly all labeled precursor EFAs are esterified to phospholipids) and in platelets incubated briefly with free (non-esterified) EFAs. We now report data which (1) may help to clarify the different roles of "membrane" vs "metabolic" EFAs in ethanol-stimulated prostanoid formation, and (2) may have implications for the design of therapeutic trials with EFA precursors.

Materials and Methods

Materials. [^{14}C]AA (5,8,11,14-eicosatetraenoic acid) and [^{14}C]DGLA (8,11,14-eicosatrienoic acid) (both 58 mCi/mmole) and [^{3}H]PGE_1 and [^{3}H]PGE_2 (both 28 Ci/mmole) were purchased from the New England Nuclear Corp., Boston, MA. TLC plates (LK5D silica gel plates, 20 cm ht.) were obtained from the Whatman Co., Clifton, NJ. Prostaglandin standards [PGE_1, PGE_2, thromboxane (TXB)] and all other reagents were from the Sigma Chemical Co., St. Louis, MO.

Methods. Platelet-rich plasma was prepared from citrated