Peptides, Vol. 11, pp. 939–944. © Pergamon Press plc, 1990. Printed in the U.S.A. 0196-9781/90 $3.00 + .00

Selective Cardiorespiratory Activity of an Iodinated Analog of Thyrotropin-Releasing Hormone (TRH)

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Received 20 February 1990

PAAKKARI, I., A. JÄRVINEN, S. VONHOF, P. T. MÄNNISTÖ, L. A. COHEN, V. M. LABROO AND G. FEUERSTEIN. *Selective cardiorespiratory activity of an iodinated analog of thyrotropin-releasing hormone (TRH).* PEPTIDES **11**(5) 939–944, 1990.—The biological activity of thyrotropin-releasing hormone (TRH) and its analogs 4(5)-I-Im-TRH and 2,4(5)-I_2-Im-TRH was assessed by means of their effects on: 1) the mean arterial pressure (MAP), 2) heart rate (HR), 3) ventilation minute volume (MV), 4) contractility of the rat duodenum, and 5) concentrations of thyrotropin (TSH) or prolactin (PRL) in serum. Also their binding to TRH-receptors in brain homogenates was studied. In urethane-anesthetized rats TRH ICV increased MAP, HR and MV. 4(5)-I-Im-TRH was equally as active as TRH on HR and MV but a significant elevation in MAP was observed only at a dose 100-fold to that of TRH. However, the maximal responses of 4(5)-I-Im-TRH and TRH did not differ. In conscious rats, TRH IA elevated MAP and HR but 4(5)-I-Im-TRH was active on MAP only. 2,4(5)-I_2-Im-TRH was devoid of cardiorespiratory activity. TRH dose-dependently inhibited the contractions of the rat duodenum while the iodinated analogs lacked such an activity. To induce a significant release of TSH several hundred times more of 4(5)-I-Im-TRH and over 1000 times more of 2,4(5)-I_2-Im-TRH were needed as compared to TRH. The iodoanalogs elevated PRL levels only at doses 2000-fold higher than those of TRH. The iodoanalogs displaced [^{3}H][3-Me-His^2]TRH ([^{3}H]MeTRH) from its binding sites at concentrations about 1000 times higher than those of TRH. Substitutions of the histidyl moiety of TRH in 4(5)-I-Im-TRH and 2,4(5)-I_2-Im-TRH resulted in substantial loss of the endocrine activity. While the di-iodinated analog was practically devoid of any biological activity the monoiodinated analog exerted similar cardiorespiratory activity to that of TRH.

Blood pressure Heart rate Minute ventilation volume Prolactin Receptor binding Smooth muscle Thyrotropin Thyrotropin-releasing hormone (TRH) TRH analog

EXOGENOUSLY administered thyrotropin-releasing hormone (TRH) not only releases thyrotropin (TSH) and prolactin (PRL) but it also elevates the mean arterial pressure (MAP), stimulates the heart rate (HR) and the ventilation, and exerts other central effects [for review, see (6, 21, 22)]. Several analogs have been synthesized in search of compounds that would selectively exert one or some of the effects of TRH. For example, the introduction of fluorine [4(5)-F-Im-TRH] into the imidazole ring of TRH results in prolonged cardiovascular and PRL-releasing activity (4,8). Analogs with a large electron-withdrawing substitution in the imidazole ring of the histidine moiety [4(5)-CF_3-Im-TRH or 2-CF_3-Im-TRH] show cardiovascular activity equal to that of TRH but exert increased PRL-releasing activity as compared to TRH (4,20). However, another analog with an electron-withdrawing substituent (4-NO_2-Im-TRH) showed no change from TRH in the cardiovascular properties, but had a decreased PRL-releasing capacity (4,20).

To evaluate further the structure activity relations of the histidine moiety of TRH, we have studied analogs with iodine substitution in the histidine ring. Iodine has about one-half of the electron-withdrawing capacity of the NO_2 group and it is slightly less bulky than the trifluoromethyl group. Cardiorespiratory, endocrine and smooth muscle effects of the iodoanalogs have been studied. Additionally, binding to central TRH receptors of the analogs was tested.

METHOD

Cardiovascular Experiments With Anesthetized Rats

Male Wistar rats (200–330 g) were kept in 12-hour light/dark

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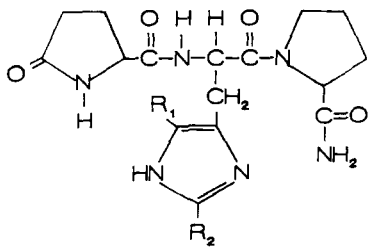

TRH $R_1 = H, R_2 = H$

4(5)-I-Im-TRH $R_1 = I, R_2 = H$

2,4(5)-I_2-Im-TRH $R_1 = I, R_2 = I$

FIG. 1. Structure formulas for TRH, 4(5)-I-Im-TRH and 2,4(5)-I_2-Im-TRH.

cycles and fed standard laboratory chow. The rats were anesthetized with urethane (1.5 g/kg IP). The trachea was cannulated for recording of the minute ventilation volume (MV) by means of a hot wire flow meter. The mean arterial pressure (MAP) and the heart rate (HR) were recorded from the left femoral artery at 1-min intervals. The colonic temperature was maintained at 37.0 ± 0.3°C using a thermistor probe controlled heating pad.

The rats were placed in a stereotaxic instrument for drug injections into the lateral ventricle (ICV) (coordinates from bregma in mm: rostral −5.3, lateral ±4.0 and vertical −1.8–2.0) (18). The injection site was verified at the end of the experiment by methylene blue staining. The technique for cannulation of the lateral ventricle and details of the computerized cardiorespiratory recording system have been described previously (14, 15, 17).

After the surgery MAP, HR and MV were monitored for 30 min before the administration of the drugs. All drugs were injected ICV in a volume of 10 μl within 10–15 seconds in a cumulative manner at 20-min intervals.

Cardiovascular Experiments With Conscious Rats

Male Sprague-Dawley rats (250–330 g) were anesthetized with ketamine (130 mg/kg) and acepromazine (1.3 mg/kg) 24 hours prior to the experiment and catheters were inserted into both femoral arteries for continuous measurement of MAP and HR (Narco 80) while the rats were conscious and freely moving. Drugs were injected into the arterial catheter (IA) in a volume of 100 μl. Each animal was given 3, 15 and 30 μmol/kg doses of the drugs at 1-hr intervals. MAP and HR samples were taken 1, 3, 5, 10, 15, 30 and 60 min after the drug administration.

TABLE 1

MAXIMAL CHANGES IN MAP, HR AND MV IN URETHANE-ANESTHETIZED RATS AFTER ICV INJECTIONS OF THE DRUGS

Treatment	MAP (mmHg)	HR (bpm)	MV (ml/min)
NaCl	4 ± 7	14 ± 6	−7 ± 8
NaI	1 ± 4	23 ± 13	6 ± 6
TRH	37 ± 7*	31 ± 4*	42 ± 10*
4(5)-I-Im-TRH	30 ± 6*	35 ± 7*	51 ± 6†
2,4(5)-I_2-Im-TRH	5 ± 8	34 ± 15	19 ± 9*

The maximal changes were observed with the highest dose of 1000 nmol/kg of TRH and both analogs. Number of animals was 6–7 in each group. Mean ± SEM are shown. *$p<0.05$ or †$p<0.01$ as compared to NaCl. The responses of the analogs and TRH that gained statistical significance as compared to NaCl did not differ significantly from each other.

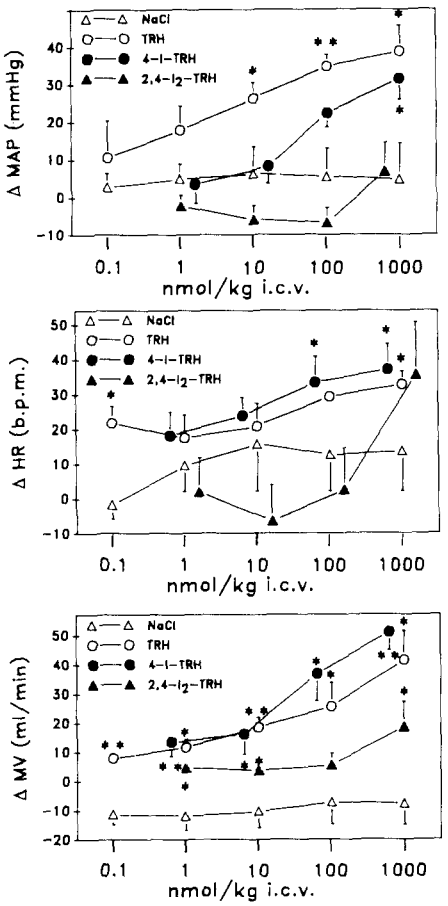

FIG. 2. The effects on the mean arterial pressure (MAP), the heart rate (HR) and the minute ventilation volume (MV) of TRH and the analogs after ICV administration in urethane-anesthetized rats. The responses of each drug were compared to that of saline (*$p<0.05$, **$p<0.01$ vs. NaCl). The responses of those doses of each drug that gained statistical significance as compared to saline were further compared with the response of 1000 nmol/kg dose of the same drug. According to this analysis TRH responses to doses of 10 nmol/kg or higher for MAP and HR and 0.1 nmol/kg or higher for MV did not differ statistically significantly from the responses of 1000 nmol/kg TRH, respectively. The maximal responses for 4(5)-I-Im-TRH were obtained with 1000 nmol/kg for MAP, 100 nmol/kg for HR and 10 nmol/kg for MV, respectively. Number of animals was 6 in each group.

Endocrine Experiments

For TSH and PRL studies, male Wistar rats were housed in individual cages for 5 days under conditions described above. The drugs were injected intraperitoneally (IP) in a volume of 1.0 ml and truncal blood was collected 15 min later for determination of TSH and PRL. At this time point the TSH response to TRH is

TABLE 2

MAXIMAL CHANGES IN MAP AND HR IN CONSCIOUS RATS AFTER IA ADMINISTRATION OF THE DRUGS

Dose	3 μmol/kg		15 μmol/kg		30 μmol/kg	
Treatment	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)
NaCl	2 ± 3	10 ± 10	3 ± 2	−11 ± 9	−3 ± 1	23 ± 11
TRH	2 ± 1	13 ± 7	13 ± 5*	37 ± 21	13 ± 2†	62 ± 13†
4(5)-I-Im-TRH	2 ± 2	9 ± 9	4 ± 2	−5 ± 12	8 ± 3*	11 ± 12
2,4(5)-I_2-Im-TRH	2 ± 2	3 ± 14	5 ± 1	1 ± 7	1 ± 1	10 ± 8

Number of animals was 7–14 in each group. Mean ± SEM are shown. *$p<0.05$ or †$p<0.01$ as compared to NaCl.

known to be maximal [(9), Männistö, unpublished observation]. Serum was separated by centrifugation and samples were stored at −20°C. Serum TSH and PRL concentrations were determined from duplicated samples (0.1 ml) by specific radioimmunoassays. The rat TSH and PRL kits were gifts from NIH. The TSH results are expressed in ng/ml of NIAMDD-TSH-RP-2 standard, which has a biological potency of 35 USP bovine units of TSH/mg in the McKenzie assay. The PRL results are expressed in ng/ml of NIAMDD-rPRL-RP-2, which has a biological potency of 30 IU/mg in the pigeon local crop sac assay of Nicoll. Intraassay coefficient of variation was less than 15%.

Smooth Muscle Experiments

Male Wistar rats (190–310 g) fasted 24 hours before decapitation. A piece of proximal duodenum 1 cm in length (wet weight 0.20–0.39 g) was excised. The tissue was placed in a chamber (5.0 ml) containing Krebs saline solution to record the isometric contractions of the longitudinal muscle.

The composition of Krebs saline solution was (in mM): NaCl 112.0, KCl 5.0, $NaHCO_3$ 25.0, NaH_2PO_4 1.0, $MgCl_2$ 0.5, $CaCl_2$ 2.5 and glucose 11.5. The solution was maintained at 37.0 ± 0.5°C and aerated with a mixture of 96% O_2 and 4% CO_2. Tissues were maintained under an optimal tension of 1.0 ponds. The optimal tension was derived from a series of preset basal tension levels from 0 to 1.5 ponds placed on the tissue that resulted in a maximal response when challenged with TRH.

For electrical stimulation electrodes were attached onto both ends of the preparation. Constant amplitudes of contractions were achieved at a stimulation rate of 1.0 Hz at 30–100 volts. An equilibrium period of 30 min preceded each experiment. The drugs were administered in a volume of 0.1 ml. After a contact time of 1.5 min for the drugs the electrical stimulation was switched off and the preparation was frequently washed with fresh Krebs saline solution during 15 min before the following drug administration. To ensure viability of the muscle preparation, responses to 10^{-9}–10^{-5} M TRH and 10^{-5} M bethanechol were checked at the end of each experiment.

The contractions of the duodenal tissue were recorded with an isometric transducer (FT 03C Force Displacement Transducer, Grass Instrument Co.) connected to a 7D polygraph (Grass Instrument Co.). The results were calculated as the mean of difference in the peak tension before and after the drug administration.

Receptor Binding

Male Sprague-Dawley rats (305–380 g) were decapitated and pituitary, hypothalamus, cortex and brainstem tissues were quickly separated on ice with a sharp blade. Anterior glands were pooled from 6 to 8 animals in each experiment whereas assays on the other brain regions were done using pooled tissue samples from 2 to 6 animals, respectively. All experiments were done on fresh tissue homogenates as previously described (26). Briefly, the tissue samples were homogenized in 15 ml of ice-cold sodium phosphate buffer (pH 7.4–7.5) for 20 seconds using an automated homogenizer (Polytron, Brinkman Instruments, setting 7). After addition of 10 ml buffer, the homogenate was centrifuged at

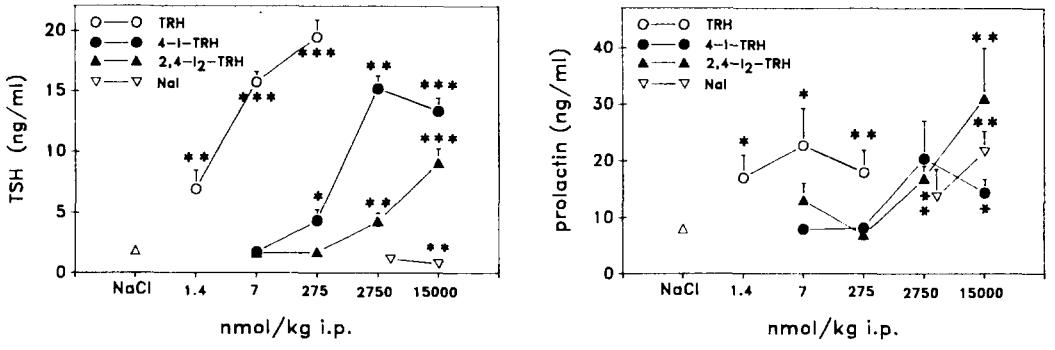

FIG. 3. Effect of TRH and its analogs on thyrotropin (TSH) and prolactin levels 15 min after intraperitoneal administration. Number of animals was between 6–14 in each group. *$p<0.05$, **$p<0.01$ vs. saline treatment.

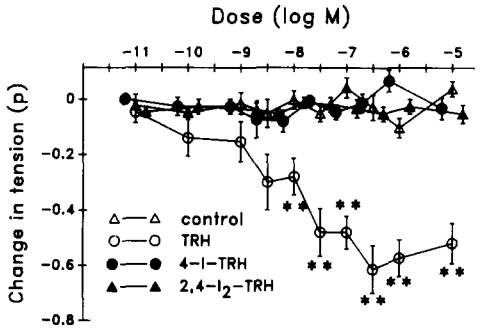

FIG. 4. Inhibition of contractions of the rat duodenal smooth muscle preparation after administration of TRH and its analogs. Number of animals was 6–8 in each group. *$p<0.05$, **$p<0.01$ vs. vehicle.

39,000 × g for 30 min in a refrigerated Sorvall RC5B centrifuge. The pellet was resuspended in buffer to a concentration of 50 to 100 mg wet weight/ml and used in the binding assay.

Fifty μl of tissue homogenate was incubated for 5 hours on ice with 3 to 5 nM [^{3}H][3-Me-His^2]TRH ([^{3}H]MeTRH) (New England Nuclear) and increasing concentrations of unlabeled TRH or TRH analogs. The final incubation volume was 100 μl. The incubation period was terminated by vacuum filtration (Brandel Harvester M-48R, Brandel) through a Whatman GF/B glass fiber filter followed by 4 rinses of 3 ml ice-cold 0.9% sodium chloride solution each. All assays were done in duplicate. The filters were placed in scintillation vials containing 5 ml Ready-Solv TM EP-scintillation fluid and counted after 24 hours in a liquid scintillation counter (Wallac LKB 1218 Rackbeta, Wallac) with an efficiency of 50–60%. Specific binding of [^{3}H]MeTRH was determined by subtracting the counts obtained from tubes containing 10^{-5} M unlabeled TRH from those of the sample. Inhibition constants were calculated according to Cheng and Prusoff (1).

Statistics

For statistical analysis in the smooth muscle experiments and basal level of the cardiovascular variables in conscious rats one-way ANOVA followed by Tukey's multiple comparison test were used (Fig. 4). In cardiovascular and endocrine studies with unequal variances Kruskal-Wallis nonparametric ANOVA and Dunn procedure were used (Figs. 2–3, Tables 1–2). The responses of those doses of the same drug that were significantly different from saline control were compared with Wilcoxon sign rank test whereby multiple comparisons were calculated with the Bonferroni correction (Fig. 2, Table 1). SAS statistical software was used for calculations of the statistics. Means of the maximal effects ± SEM are shown.

Drugs

All drugs were dissolved in 0.9% NaCl solution for the cardiovascular experiments or in Krebs solution or in sodium phosphate buffer for the smooth muscle and for the receptor binding studies, respectively. TRH was purchased from Sigma Chemical Co. (St. Louis, MO) and NaI from E. Merck (Darmstadt, BRD). The analogs 4(5)-I-Im-TRH and 2,4(5)-I_2-Im-TRH were prepared by direct iodination of TRH (10).

RESULTS

Cardiorespiratory Effects in Anesthetized Rats

TRH increased MAP significantly at the dose of 10 nmol/kg ICV while with 4(5)-I-Im-TRH a significant increase was observed first at 1000 nmol/kg ICV (Fig. 2). However, the maximum changes in MAP induced by TRH and 4(5)-I-Im-TRH were not significantly different (Table 1). 2,4(5)-I_2-Im-TRH ICV was inactive on MAP.

4(5)-I-Im-TRH and TRH ICV were equipotent in elevating HR or MV in terms of the doses needed for significant changes and the maximal responses (Fig. 2, Table 1). While having no significant effect on HR, 2,4(5)-I_2-Im-TRH increased MV at the highest dose of 1000 nmol/kg ICV. Elevation of MV induced by TRH or the analogs was due to increased ventilatory rate while only negligible changes in tidal volume were observed. TRH and 4(5)-I-Im-TRH did not differ significantly with respect to the latencies needed for the peak responses in MAP [6.2 ± 1.3 min for TRH and 4.5 ± 1.0 min for 4(5)-I-Im-TRH], in HR (4.3 ± 0.8 min and 5.7 ± 1.1 min, respectively) or in MV (5.2 ± 1.4 and 3.7 ± 1.0 min, respectively). The cardiorespiratory responses to TRH and analogs lasted for the whole observation period of 20 min. NaI at doses 1–1000 nmol/kg ICV did not exert any significant effect on MAP, HR or MV. The mean basal values of MAP ranged between 84 and 90 mmHg for all other treatments except 2,4(5)-I_2-Im-TRH (60 ± 4 mmHg) that differed significantly from the others ($p<0.05$). No significant differences were observed in the basal values of HR and MV between the treatment groups. The mean basal values of HR ranged from 441–467 bpm and that of MV from 101–127 ml/min, respectively.

Cardiovascular Effects in Conscious Rats

TRH significantly increased MAP in conscious rats at a dose of 15 μmol/kg IA and 4(5)-I-Im-TRH at 30 μmol/kg IA while 2,4(5)-I_2-Im-TRH IA was inactive (Table 2). MAP responses of

TABLE 3

APPARENT INHIBITION CONSTANTS (K_i) OF TRH AND IODINATED ANALOGS ACCORDING TO CHENG AND PRUSOFF (1)

	Pituitary	Hypothalamus	Brainstem	Cortex
TRH (nM) (n = 4)	23.9 ± 3.3	29.9 ± 1.9	19.7 ± 2.2	56.3 ± 9.7
4(5)-I-Im-TRH (μM) (n = 5)	9.4 ± 4.6	15.4 ± 5.3	6.8 ± 2.5	31.1 ± 1.0
2,4(5)-I_2-Im-TRH (μM) (n = 3)	71.3 ± 15.4	51.6 ± 8.1	50.9 ± 6.7	57.5 ± 5.9

The assays were done in duplicate using fresh tissue homogenate and [^{3}H]MeTRH as a radioligand in a final concentration of 3–5 nM. Mean ± SEM.

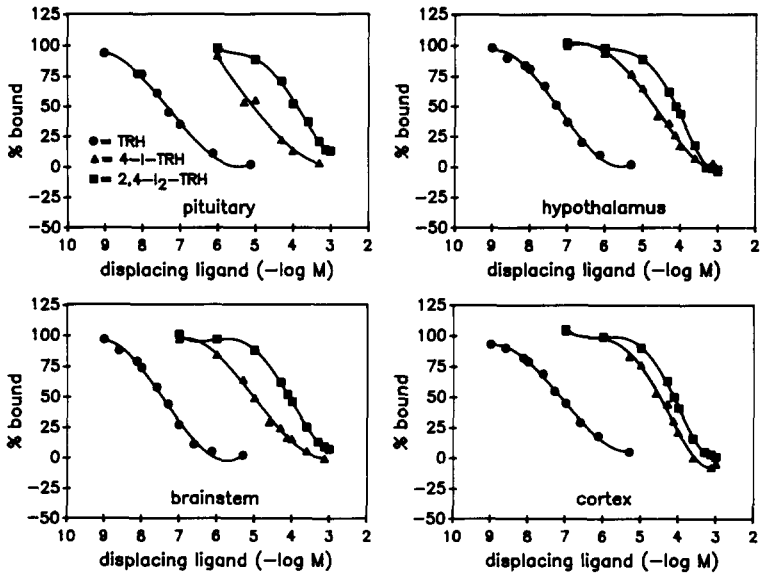

FIG. 5. Displacement of [^{3}H]MeTRH from its binding sites in the central nervous system by various concentrations of TRH and its analogs. Each point represents duplicate measurements of 3–5 separate experiments.

TRH and 4(5)-I-Im-TRH peaked within 10 min after the drug administration and lasted for less than 30 min. Of the 3 compounds tested only TRH 30 μmol/kg IA significantly increased heart rate for less than 30 min (Table 2). No significant differences were found in basal values for MAP and HR between the groups which ranged between 112 and 123 mmHg for MAP and 371–421 bpm for HR, respectively.

Release of TSH and PRL

Injections of 1.0 ml of 0.9% NaCl, IP, resulted in TSH and PRL levels of 1.9 ± 0.4 ng/ml and 10.4 ± 1.5 ng/ml, respectively (n = 13). TRH IP increased TSH and PRL levels at doses of 1.4 to 275 nmol/kg (Fig. 3). 4(5)-I-Im-TRH elevated TSH first at 275 nmol/kg IP and 2,4(5)-I_2-Im-TRH at 2750 nmol/kg IP. Both 4(5)-I-Im-TRH and 2,4(5)-I_2-Im-TRH elevated serum PRL at a dose of 2750 nmol/kg IP or higher. An equal PRL response was seen after the same dose of NaI IP, which, however, did not stimulate TSH release (Fig. 3).

Effects on the Duodenal Smooth Muscle In Vitro

TRH dose-dependently inhibited the electrically evoked contractions of the duodenal smooth muscle at doses of 10^{-8}–10^{-5} M for about 10 seconds. Peak effect of TRH was achieved at $5 \cdot 10^{-7}$ M. Both analogs were totally ineffective up to doses of 10^{-5} M (Fig. 4).

Receptor Binding

TRH displaced [^{3}H]MeTRH from its binding sites in pituitary, hypothalamus, brainstem and cortex at the concentrations of 10^{-9} to 10^{-5} M. The competition curves for 4(5)-I-Im-TRH and 2,4(5)-I_2-Im-TRH were parallel to that of TRH but TRH was about 1000 times more potent than 4(5)-I-Im-TRH, and over 1000 times more potent than 2,4(5)-I_2-Im-TRH (Fig. 5). The potency to compete with [^{3}H]MeTRH for high affinity binding sites was similar in all brain regions with the exception of a slightly lower activity of 4(5)-I-Im-TRH in cortex than in other parts. The apparent inhibition constants (K_i) in the regions studied are summarized in Table 3.

DISCUSSION

In accordance with previous studies TRH elevated MAP, HR and MV both in the anesthetized and the conscious rats, released TSH and PRL, inhibited the contractions of the duodenal smooth muscle and displaced [^{3}H]MeTRH from its binding sites (6, 19, 23, 24). Neither of the analogs showed greater activity than TRH. In male rats TSH responses to TRH are more specific and consistent than PRL responses. Unspecific stimulation of PRL secretion was also shown by the PRL increases after high doses of NaI. Equally high doses of both iodoanalogs were needed for PRL response, suggesting unspecific mode of action. The proper experimental model using castrated female rats should be used to fully clarify the effects of the iodoanalogs on PRL release.

TRH was about two hundred times more potent than 4(5)-I-Im-TRH as a TSH releaser and exhibited an almost 1000-fold higher affinity to TRH binding sites. 2,4(5)-I_2-Im-TRH was even weaker than 4(5)-I-Im-TRH in releasing TSH and in affinity to TRH binding sites. The present observation on the weak TSH-releasing potency of these analogs is in accordance with the previous finding on the nonexistent TSH-releasing potency of the lower doses of the same analogs (10).

In the previous studies TRH elevated MAP and HR at doses of 1–1000 nmol/kg ICV in anesthetized rats (11, 13, 25) or even at lower doses of 1–100 nmol/kg both in conscious and anesthetized rats (7,22). In this study the responses in MAP or HR of 10 nmol/kg and in MV of 0.1 nmol/kg ICV of TRH in anesthetized rats presented already the higher end of the dose-response curve whereafter no significant additional response to higher doses of TRH was observed.

4(5)-I-Im-TRH elevated MAP and MV to a similar degree as TRH, but in comparison to TRH, higher doses of 4(5)-I-Im-TRH were needed to achieve equal responses in MAP. While 4(5)-I-Im-TRH proved to be equipotent with TRH in producing

tachycardia via the central route it had no effect on HR when administered peripherally. On the other hand, the tachycardiac response to TRH IA was relatively weak in conscious rats as well. This difference could be due to the reflectory counterregulation in conscious rats (peripheral administration) while in anesthetized rats (ICV injections) the lack of proper cardiovascular reflexes would allow the increase in HR. Another possibility could be the weaker penetration of the drugs from the periphery into the central sites regulating heart rate than into those regulating blood pressure. In a similar fashion, the higher doses of 4(5)-I-Im-TRH needed for equal MAP responses with TRH might reflect the better penetration of TRH into the central sites involved.

In conclusion, of the two analogs studied, 4(5)-I-Im-TRH had cardiovascular activity that closely resembled that of TRH while the other, 2,4(5)-I_2-Im-TRH, was devoid of cardiovascular activity and only slightly stimulated ventilation. Both of the analogs were clearly less potent than TRH or inactive in releasing TSH or PRL, in inhibiting the contractions of the smooth muscle and in binding to TRH receptors. These results suggest that the intact imidazole ring may be essential for the endocrine and the smooth muscle effects but not for the cardiovascular activity of TRH. This is supported by the finding that modifications of the imidazole ring in TRH with an electron-withdrawing substituent [4(5)-NO_2, 4(5)-CF_3 or 2-CF_3] did not affect the cardiovascular activity but modified the PRL-releasing property (4,20). The lack of cardiovascular activity of 2,4(5)-I_2-Im-TRH, in contrast to 4(5)-I-Im-TRH, may result from the two bulky iodines causing a steric hindrance for interaction of this analog with the central receptors responsible for the cardiovascular activity of TRH.

In spite of its cardiovascular activity, 4(5)-I-Im-TRH was only weakly bound to the central TRH receptors in homogenates from fairly large brain areas studied. However, specific nuclei appear to mediate the cardiovascular (e.g., medial preoptic nucleus, preoptic suprachiasmatic nucleus, and locus coeruleus) (2, 3, 16) and the respiratory effects of TRH (e.g., mesencephalic regions) (5,12). Therefore, future studies could perhaps reveal some selectivity for 4(5)-I-Im-TRH to the receptors in these discrete areas.

ACKNOWLEDGEMENTS

This work has been supported in part by the Research Council for Medicine of The Academy of Finland, Paulo Foundation, Pharmacal Research Foundation and by the USUHS protocol R09211 to Dr. Feuerstein. The skillful technical assistance of Mrs. Beate Sarell is greatly acknowledged.

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