Somatostatin inhibits release of thyrotropin releasing factor from organ cultures of rat hypothalamus

(norepinephrine/bacitracin/cerebrospinal fluid/hypothalamic organ culture/hypothalamic releasing factors)

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ABSTRACT   Somatostatin in concentrations of 10^{-6} to 10^{-8} M inhibited basal release of thyrotropin releasing factor in organ culture of rat hypothalamus. Norepinephrine in doses of 10^{-4}–10^{-6} M induced release of thyrotropin releasing factor which increased progressively with time and was temperature and dose dependent. This enhanced thyrotropin-releasing-factor release was inhibited by somatostatin at 10^{-6}–10^{-8} M.

The tetradecapeptide somatostatin, which is extensively distributed throughout the central nervous system, gastrointestinal tract, and pancreas (1–3), inhibits release of various hormones including growth hormone (4), thyrotropin (5), glucagon (6) and insulin (4). Somatostatin infusion lowers circulating thyrotropin in man (7), and passive immunization of rats with specific somatostatin antiserum increases basal and thyrotropin releasing factor (TRF)-stimulated thyrotropin secretion (8). These experiments imply an interrelationship between TRF and somatostatin and pituitary thyrotropin secretion. Potential local effects of somatostatin within the hypothalamus itself, either directly on peptide hormone secretion or via an action upon neurotransmitters, are equally likely and have not been critically examined.

Studies of the regulation of TRF synthesis and release are complicated by the fact that the hormone is widely distributed throughout the central nervous system so that measurements of the hormone in the peripheral blood, regardless of their accuracy, may not faithfully mirror concentrations of TRF actually delivered to the anterior pituitary (9). Moreover, measurements of hormone levels in the hypophyseal portal circulation require surgical cannulation under anesthesia which entails the administration of anesthetics, many of which affect hypothalamic hormone release (10), and in most instances measurements require prior hypophysectomy as well, to permit sampling of the entire hypothalamic outflow (11). Our own approach has been to utilize short-term organ culture of rat hypothalamus as a model for delineating factors modulating *in vitro* release of TRF. We have found that somatostatin at 10^{-6} M–10^{-8} M inhibits basal release of TRF. Moreover, norepinephrine (NE) in doses of 10^{-4}–10^{-6} M increases release of TRF, an effect which is not seen in the presence of somatostatin.*

MATERIALS AND METHODS

Materials were purchased commercially as follows: organ culture materials from Falcon Plastics, Cockeysville, MD; nutrient mixture F-12 with L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution, and fetal calf serum from Gibco, Grand Island, NY; bacitracin, L-norepinephrine·HCl, and dithioerythritol (2,3-dihydroxy-1,4-dithiolbutane) from Sigma Chemical Company, St. Louis, MO; TRF and somatostatin from Calbiochem, La Jolla, CA. TRF analogues were gifts from Parke–Davis and Abbott Laboratories.

Male Sprague–Dawley rats (250 g body weight) were killed by decapitation at 10–11 a.m. After the brain was exposed by removal of the skull with sterile instruments, it was carefully placed in a sterile plastic petri dish which contained Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution, bacitracin (2.1×10^{-5} M), and glucose (6 mg/ml). Prior to use the dishes had been "gassed" with 5% CO_2/95% air. Dissection was performed in a laminar flow hood with sterile instruments at a plane 1 mm lateral to the midline and at a depth of 1 mm. The fragments were obtained from the area between the posterior margin of the optic chiasm and the anterior portion of the mammillary body. Microscopic examination (Fig. 2) demonstrated that the fragments included parts of the paraventricular, dorsomedial, ventromedial, suprachiasmatic, and arcuate nuclei, as well as the median eminence. Care was taken to ensure that the entire procedure from the time of death to initiation of the primary culture was performed within 1 min. From decapitation to placement into the petri dish took <40 sec. An additional 15–20 sec was required for hypothalamic dissection and transfer to the primary incubation medium for explanation. The explant was placed on a small piece of lens paper on a triangular wire mesh grid in a center well of an organ culture dish, and 0.5 ml of culture medium was added. The culture medium contained 88 vol of nutrient mixture F-12 with L-glutamine, 10 vol of heat-inactivated fetal calf serum, 1 vol of Hepes (10 mM in final concentration), 1 vol of Tricine (10 mM in final concentration), glucose (6 mg/ml in final concentration), and bacitracin (2.1×10^{-5} M in final concentration). The final pH was 7.3–7.5. The hypothalami were cultured in groups of three and maintained in a humidified incubator overnight (24 hr) at 37° in an atmosphere of 5% CO_2/95% air. The use of heat-inactivated fetal calf serum and bacitracin served to prevent enzymatic degradation of TRF (12, 13) and represented a major change from published techniques (14, 15). At the conclusion of the primary culture a preincubation was carried out for 10 min, followed by experimental incubation for 30 min with 0.5 ml of incubation medium with and without putative modulators of TRF release. Both incubations were performed in incubation medium that was similar to cerebrospinal fluid in terms of its electrolyte composition—i.e., 126 mM NaCl/1 mM Na_2HPO_4/0.877 mM $MgSO_4$·$7H_2O$/22 mM $NaHCO_3$/1.45 mM $CaCl_2$. Modifications were maintenance of glucose at 6

Abbreviations: TRF, thyrotropin releasing factor; NE, norepinephrine.

* Hirooka, Y., Hollander, C. S., Suzuki, S., Ferdinand, P. & Juan, S-I. (1977) *Program of the American Thyroid Association, 53rd Meeting*, T-23 (abstr.).

mg/ml (approximately 3-fold greater than the normal concentration in order to facilitate longer cell viability), the addition of bacitracin (2.1×10^{-5} M, pH 7.4) to prevent enzymatic breakdown of TRF, and the use of potassium at twice the normal level in an attempt to maintain the intracellular potassium concentration. Both preincubations and experimental incubations were done at 37° in an atmosphere of 5% CO_2/95% air. Measurements of glucose concentrations in the organ culture medium were performed by utilizing the glucose oxidase method (16). TRF release was quantitated by assay of the medium with a highly specific, sensitive radioimmunoassay for TRF (17–20). Statistical significance for all experiments in this study was determined by the Student *t* test. TRF content was determined by a modification of published methods (21, 22). Our procedure has been described in detail (17–20). In essence three hypothalami, the contents of one sample dish, were homogenized in 1 ml of ice cold methanol utilizing a Potter–Elvehjem apparatus by 10 up-and-down strokes of the pestle rotating at 2.5 rpm, were suspended in ice cold (4°) 90% methanol (0.5 ml of homogenate per 2 ml), and were centrifuged at $1000 \times g$ for 30 min. The resultant supernatant was dried with an air stream while immersed in a water bath at 60°, stored at −20°, and dissolved in 0.5 ml of phosphate/sodium chloride buffer (0.25% bovine serum albumin/0.15 M NaCl/0.01 M phosphate, pH 7.5) prior to assay. With this procedure the mean yield of exogenously added cold TRF in six experiments was 99.7%.

RESULTS

Authenticity of radioimmunoassayable TRF was indicated by immunologic, chromatographic, and enzymatic criteria. Immunologically, there was virtual absence of crossreaction with closely related TRF analogs (Table 1). We assessed the extent of crossreaction of a given analog with TRF antibody by comparing the relative concentration of the compound with that

Table 1.   Specificity of TRH antibody

Compound*	Relative reactivity
TRH	1.0
Pro-His-Pro-NH$_2$, Glu-His-Ser-NH$_2$, LDL-TRH	0.001
Glu-His-Pyrrolidinamide, Glu-His-Tyr-NH$_2$	0.00033
Heat inactivated hypothalamic extract†	0.0001
Substance P	0.00004
DLL-TRH, <Glu-His-Ser-NH$_2$	0.000025
Glu-His-Pro-Tyr-NH$_2$, α-endorphin, β-endorphin, δ-endorphin	0.000008
<Glu-His-Tyr-NH$_2$	0.000005
LLD-TRF	0.000004
Glu-His-Morpholinamide	0.0000016

Crossreaction of anti-TRH antibody with TRH analogues and various compounds. The relative reactivity of TRH has been assigned an arbitrary value of 1.0.
* Relative reactivity = 0.000001: <Glu-His-Leu-NH$_2$; TRF free acid; <Glu-His-Pro-OH; <Glu-His-Gly-NH$_2$; <Glu-His-OCH$_3$; DDD-TRF; <Glu-His; His-Pro; D-histidine-HCl; <glutamic acid; L-histidine-HCl; L-proline; gonadotropin releasing hormone or luteinizing hormone releasing hormone. Relative reactivity = <0.0000001: melanocyte stimulating hormone, adrenocorticotropic hormone 1–39 (Li); dopamine; dithioerythritol; NE; thyroxine (T4); triiodothyronine (T3); KCl; serotonin; Met-enkephalin; human thyrotropin (NIH); rat thyrotropin (NIH); somatostatin, prostaglandins E$_1$, E$_2$, and F$_2$α; cyclic AMP; dibutyryl cyclic AMP; serotonin; melatonin.
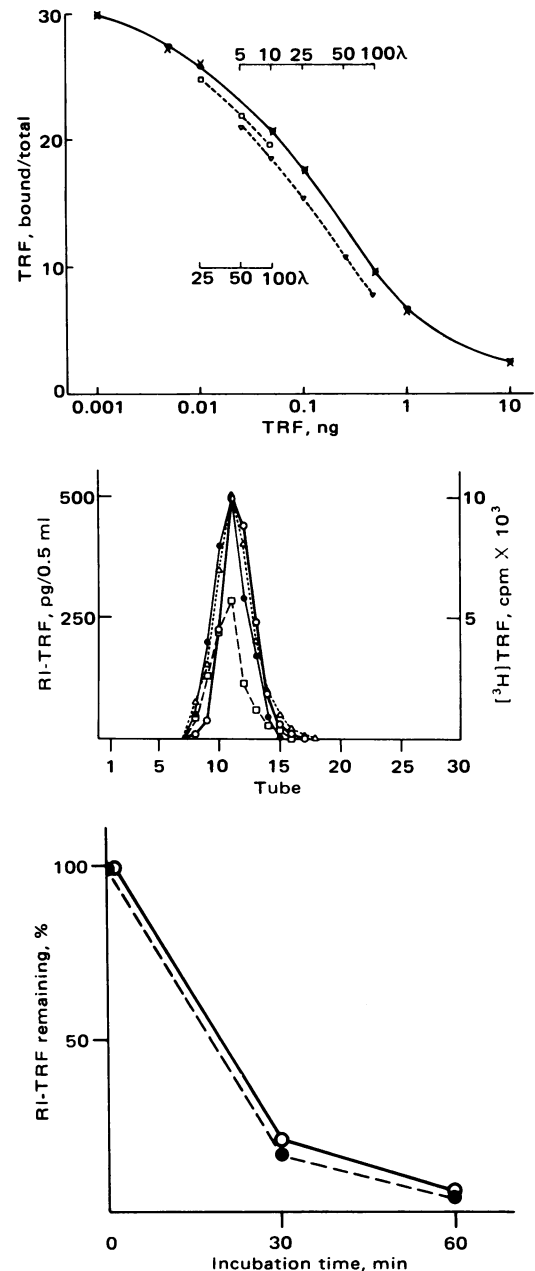† Hypothalami were incubated at 37° for 2 hr and were heated at 60° for 1 hr.

FIG. 1.   Radioimmunoassay of TRF in hypothalamic organ culture. (*Top*) The ordinate is bound TRF over total. The abscissa is TRF in ng for the standard curves for synthetic TRF in buffer (O) or in cerebrospinal fluid-like medium (×). The abscissas for dilutions of hypothalamic extracts (∇) and for immunoassayable TRF released into the cerebrospinal fluid-like medium in the course of experimental incubation (□) have been drawn as *Insets* in the upper and lower portions of the figure, respectively. In this assay the minimum TRF detected was 2 pg. Sensitivity of the assay was generally of the order of 1–2 picograms. (*Middle*) Chromatography was performed in a Sephadex G-10 column (1×15 cm bed volume). The ordinate reflects radioimmunoassayable TRF or [^{3}H]TRF (O). Δ, Synthetic TRF; □, hypothalamic extract; ●, medium. (*Bottom*) Enzymatic degradation of TRF in plasma. This figure illustrates the results of incubation of synthetic TRF (●) and rat hypothalamic extract (O) with rat plasma for 60 min.

of TRF needed to produce 50% inhibition of binding to antibody. The highest degree of crossreactivity was 1/1000, which was seen only with these peptides: Pro-His-Pro-NH$_2$, Glu-His-Ser-NH$_2$, and LDL-TRF. Moreover, there was no interference by the experimental medium with the antigen–anti-
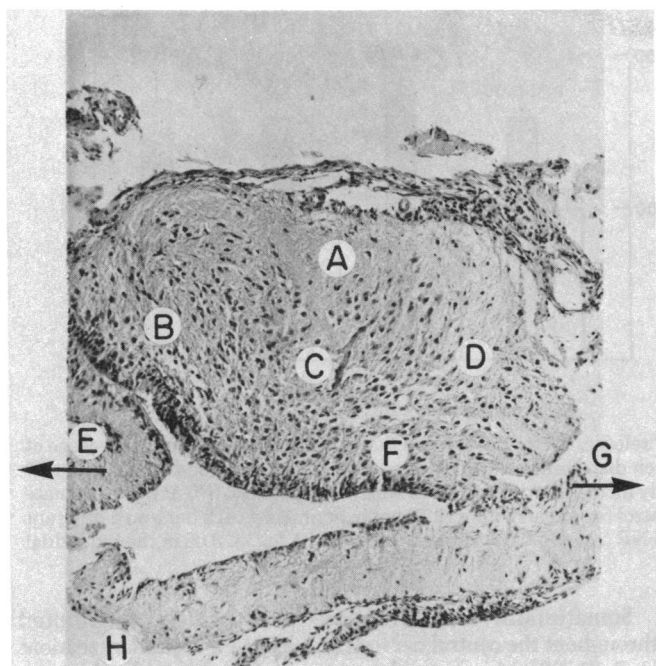
FIG. 2.   Light microscopy of the hypothalamus. The hypothalamic tissue shown was cultured for 1 day and incubated *in vitro* for 40 min (10 min preincubation, 30 min experimental incubation). The tissue was fixed in 10% formalin, stained with hematoxylin and eosin, and photographed at a magnification of 115 times actual size. A, Paraventricular nucleus; B, dorsomedial nucleus; C, ventromedial nucleus; D, suprachiasmatic nucleus; E, mammillary body; F, arcuate nucleus; G, optic chiasm; H, median eminence.

body reaction (Fig. 1 *top*). In addition, there was parallelism of dilutions of putative TRF present in hypothalamic extract and TRF added to the experimental media. The appearance of parallelism was confirmed by logit log transformation of the data and comparison of the resultant regression lines by analysis by the F test (23, 24). Chromatographically (Fig. 1 *middle*), there was identical mobility of synthetic and [^{3}H]TRF with that of putative TRF in the hypothalamic extract or media on a Sephadex G-10 column. Finally, enzymatic evidence for the authenticity of measured TRF was adduced by demonstrating similar inactivation by rat plasma of synthetic TRF and TRF present in a hypothalamic extract (Fig. 1 *bottom*).

The general validity of the organ culture system was indicated by (*a*) good preservation of the fine structure of the incubated hypothalamus (Fig. 2) and satisfactory replication of dissection of hypothalami as assessed by histological examination; (*b*) reproducibility of TRF content in dissected hypothalami to within 9.9% for 11 experiments involving 38 hypothalamic cultures (coefficient of variation; see Table 2). Content was expressed as pg/dish (consisting of three hypothalami) or as pg/mg of protein (method of Lowry *et al.*; ref. 25); (*c*) stability of TRF content in hypothalamic tissue before and after

primary culture and after incubation in cerebrospinal fluid-like media (2667 ± 88.2, 2700 ± 100, 2700 ± 100 pg/dish containing three hypothalami); and (*d*) linear metabolism of glucose (31.3 ± 1.6 ng/min for 30 min).

In pilot studies it was established that when the hypothalami are incubated in groups of three per dish, basal release of TRF is reproducible within individual experiments at ±5% (SEM). However, interassay variability averaged ±10% for six experiments and was occasionally as high as ±20%. We have therefore expressed the results of these studies as percentage of control to facilitate comparisons between experiments. At a dose of 10^{-4} M, NE reproducibly (four experiments) increased release of TRF by 58–75% which was in each case significant when compared with the control. These responses were dose dependent between 10^{-6} M and 10^{-4} M, occurred at 37° but not at 0°, and increased progressively with time (see Fig. 3). Addition of NE, alone or in combination with dithioerythritol to inhibit oxidation of NE, failed to alter enzymatic degradation of TRF at 37° in the presence of rat plasma, suggesting that the effect of NE does not involve merely stabilization of TRF. Addition of somatostatin alone at concentrations of 10^{-6} M, 10^{-7} M, and 10^{-8} M resulted in significant inhibition of TRF release. There was inhibition to 47.1% of control at 10^{-6} M somatostatin ($P < 0.02$), to 53.7% of control at 10^{-7} M ($P < 0.001$), and to 37.3% of control at 10^{-8} M ($P < 0.02$) (Fig. 4). To ascertain if somatostatin was also capable of inhibiting neurotransmitter-mediated release of TRF, we utilized the model of NE-induced TRF release. For these studies 10^{-4} M NE was selected to achieve a near maximal response. When incubated with 10^{-7} M and 10^{-8} M somatostatin, the TRF response to NE appeared to be completely abolished ($P < 0.05$) (Fig. 5).

DISCUSSION

Our application of the organ culture technique to study TRF release was stimulated by prior reports that fragments of hypothalamus and median eminence from mink (26), guinea pig (15, 27), and rat (26, 28) appeared viable for up to 7–13 days *in vitro*. Because McKelvy *et al.* noted degeneration of neural processes and loss of bioassayable TRF after 3 days in organ culture (15), we selected short-term primary incubation for 24 hr to lessen the possibility of neuronal dedifferentiation *in vitro*, and we demonstrated good preservation of the fine structure of the incubated hypothalami. Our finding of enhanced release of TRF with NE is in good agreement with prior observations (29). The level of TRF released in the combined presence of NE and somatostatin was similar to the basal TRF release in this system (control in Fig. 5). There are several potential explanations for this finding. Conceivably, responsiveness to NE is actually retained in the presence of somatostatin, and the apparent absence of a response to NE reflects a depression in the basal response. Alternatively one could postulate the converse—i.e., NE blocks the inhibitory effects of somatostatin on basal secretion which is itself unchanged. However, further studies on the interaction of somatostatin and NE and on the

Table 2.   Reproducibility of hypothalamic dissection

Experiment number	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Mean
Mean*	3.35	2.97	2.98	2.70	3.23	2.80	3.39	3.45	3.33	3.50	2.67	3.12
SD	0.13	0.21	0.21	0.17	0.25	0.31	0.25	0.50	0.38	0.34	0.15	0.31
SEM	0.07	0.12	0.10	0.10	0.15	0.18	0.14	0.25	0.19	0.17	0.09	0.09
N†	3	3	4	3	3	3	4	4	4	4	3	38

* TRF content, ng/dish.
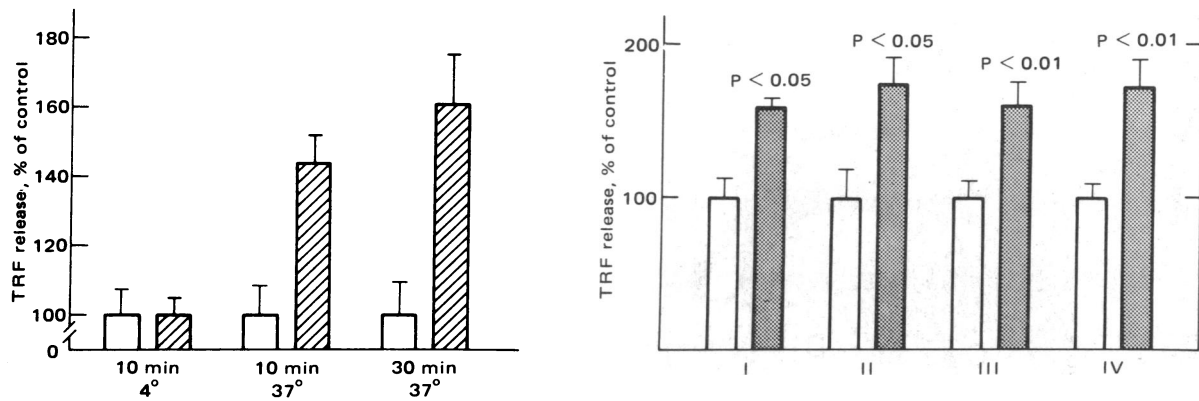† Number of dishes containing three hypothalamic fragments each.

FIG. 3.   Time and temperature dependence of the effects of NE on TRF release. (*Left*) Responses (shaded bars) are shown as percentage of control (open bars). (The height of the bar represents the mean ±SEM; each dish contained three hypothalami.) No response was seen at 4° in three experiments. In a dozen experiments at 37°, the response significantly increased with increased time of incubation. (*Right*) The ordinate shows the TRF response during 30 min of incubation as a percentage of the control (open bars). Four different experiments, each done on a different day, are labeled I, II, III, and IV. Although the significance of the response to NE (shaded bars) varies from $P < 0.01$ to $P < 0.05$ in the individual experiments, the variations in the response were small.

hypothalamic content and turnover of TRF are required to select between these and other possible explanations for this phenomenon. Our studies to date do not completely exclude the possibility that the observed basal secretion could simply reflect leakage of TRF from the organ culture. However, our findings with NE make it more likely that it represents a more specific secretory process from the TRF terminals. In addition, the contribution of synthesis of new TRF to the basal release also requires further clarification. Whatever its precise mechanism its seems possible that this action of somatostatin, like its postulated effects in the pancreas, is mediated by virtue of its release into a microenvironment, thereby influencing processes of adjacent cells rather than acting as a hormonal agent via release into the general circulation.

Besides the hormones mentioned at the outset, somatostatin has been shown in a detailed study to be effective in suppressing the spontaneous release of many other hormones in a surprisingly wide variety of clonal cell lines in tissue culture (30). These include growth hormone, thyrotropin, NE, adrenocorticotropin, prolactin, luteinizing hormone, IgG, follicle stimulating hormone, thyroxine, corticosterone, and testosterone (30). Thus, it is perhaps not altogether surprising that somatostatin present in high concentrations in various nuclei in the hypothalamus and median eminence (3) inhibits the release of TRF, another hypothalamic hormone.

Somatostatin and TRF are both widely distributed throughout the central nervous system, often in similar locations (1, 3, 31–33). Moreover, there is a considerable body of neurophysiological (34) and behavioral (35) evidence that suggests that both hormones may function as neurotransmitters in these locations. It is therefore intriguing to speculate that somatostatin might also inhibit TRF release in these extra hypothalamic areas in a manner analogous to its effects upon TRF in the hypothalamus.

In these studies considerable care was taken to minimize potential *in vitro* artifacts. Precautions taken include documentation of authenticity of the nature of the radioimmunoassayable TRF, the use of bacitracin and heat-inactivated fetal calf serum to stabilize TRF, and the demonstration that the apparent effect of NE does not simply reflect *in vitro* stabilization of TRF. Moreover, we have clear evidence of the functional ability of the system with regard to TRF release and
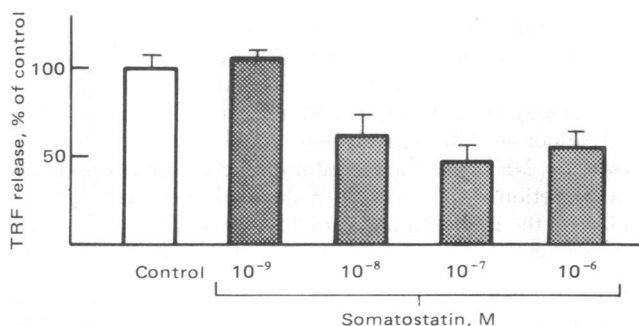

FIG. 4.   Effect of somatostatin on basal release of TRF from hypothalamus *in vitro*. The ordinate shows TRF release as percentage of control. The height of the bars represents mean ±SEM. The SEM of the control was ±1.9%. Each dish contained three hypothalami. Fourteen dishes were measured for the control (open bar). Additions of somatostatin are depicted by the stippled bars. Three dishes were employed for 10^{-9} M somatostatin and six dishes each at 10^{-8} M and 10^{-7} M. No inhibition was seen at 10^{-9} M but significant inhibition was discernible at 10^{-8} M ($P < 0.02$), 10^{-7} M ($P < 0.001$) and 10^{-6} M ($P < 0.02$).
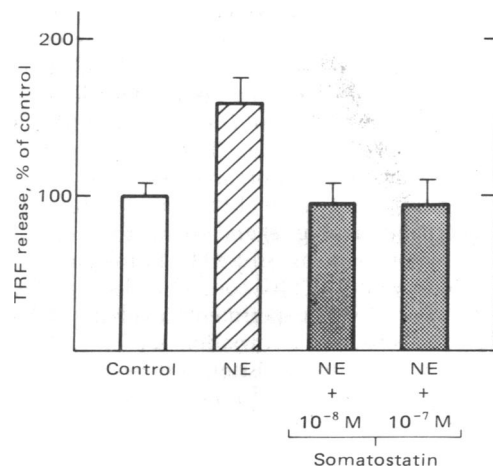

FIG. 5.   Effect of NE alone and in combination with somatostatin on the release of TRF from hypothalamus *in vitro*. Fourteen different dishes were employed as the control (open bar). NE alone at 10^{-4} M, added to six dishes (crosshatched bar), produced a statistically significant increase in TRF release as compared to control ($P < 0.02$). Additions of NE and somatostatin at either 10^{-7} M or 10^{-8} M (stippled bars, three dishes for each somatostatin concentration) appear to suggest complete inhibition of the TRF response to NE by somatostatin ($P < 0.05$) at either concentration as compared to NE alone. However, a comparison with Fig. 4 reveals that the apparent blunting of the TRF response to NE in the presence of somatostatin largely reflects a depressed basal response (see text).

its responsiveness to levels of somatostatin of the order of 10^{-8} M—i.e., similar to or possibly somewhat less than those that we and others have found in the hypothalamus and median eminence (3). Nevertheless our *in vitro* observations clearly cannot be extrapolated to the *in vivo* situation without further studies.

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