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Steady-state level and turnover rate of the tripeptide Tyr-Gly-Gly as indexes of striatal enkephalin release *in vivo* and their reduction during pentobarbital anesthesia

(opioid peptides/neuropeptide release/neuropeptide metabolism)

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ABSTRACT **Tyr-Gly-Gly (YGG) was recently shown to be an extraneuronal metabolite of opioid peptides derived from proenkephalin A, formed in brain by the action of "enkephalinase" (membrane metalloendopeptidase, EC 3.4.24.11) and degraded by aminopeptidases. The dynamic state of YGG in mouse striatum was studied by evaluating the changes in its level elicited by inhibitors of these peptidases. Inhibition of YGG synthesis by Thiorphan or acetorphan reduced YGG levels with a $t_{1/2}$ (mean ± SEM) of 12 ± 2 min, indicating an apparent turnover rate (mean ± SEM) of 18 ± 2 pmol/mg of protein per hr. An apparent turnover rate of 18 ± 2 pmol/mg of protein per hr was derived from the rate of YGG accumulation elicited by the aminopeptidase inhibitor bestatin. In addition, accumulation of Tyr-Gly-Gly-Phe-Met (YGGFM) in an extrasynaptosomal fraction after blockade of its degradation by Thiorphan and bestatin occurred at a rate of 18 ± 3 pmol/mg of protein per hr, which is likely to reflect the rate of enkephalin release *in vivo*. Hence, the three series of data suggest that striatal enkephalins rapidly turn over—e.g., with a $t_{1/2}$ in the 1-hr range. Pentobarbital anesthesia reduced by about 60% the rate of YGG accumulation elicited by bestatin and the extrasynaptosomal YGGFM accumulation elicited by Thiorphan and bestatin. This suggests that the activity of striatal enkephalin neurons is depressed during anesthesia. Pentobarbital (and chloral hydrate) did not affect the steady-state level of YGGFM but rapidly reduced that of YGG. Hence, the steady-state levels of YGG seem a reliable index of changes in enkephalin release, and measuring levels of characteristic fragments might therefore provide a general means of evaluating neuropeptide release *in vivo*.**

An understanding of the functional roles of neuronal systems in the brain greatly depends on the definition of the physiological, pharmacological, or pathological conditions under which their activity is modified *in vivo*. Thus, the development of methods to evaluate the turnover rates of monoamines or amino acids has greatly contributed to the understanding of the functions of the neurons from which they are released and of the modes of action of the major classes of neuroactive drugs (1–3).

Unfortunately, these methods are not yet available for evaluation of the turnover rates for endogenous opioid peptides (OPs) and other neuropeptides. Small changes in steady-state levels of OPs occur under a variety of conditions (4, 5) but cannot be easily interpreted as signifying changes in release; furthermore, neuronal activity can be modified without changes in the steady-state levels of neurotransmitters. Direct measurement of OPs collected in central nervous system perfusates has been used to evaluate their release (6, 7) but the method is not easily applicable without anesthesia and the resulting interference; in addition, it requires complete inhibition of degradation processes, which may interfere with the release mechanisms (8, 9). Levels of brain proenkephalin A mRNA were recently shown to be modified by chronic drug treatments (10, 11) but their measurement may not provide direct information about transient changes in OP release. Finally, the rates of radiolabeled amino acid incorporation into OPs are not easily measurable, mainly because it is difficult to achieve a reasonable degree of incorporation (4, 12, 13).

To solve this problem, we investigated the possibility of extending to OPs a strategy currently used to evaluate changes in monoamine release—i.e., the measurement of steady-state levels or turnover rates of characteristic metabolites (1–3). This approach obviously requires a knowledge of the metabolic pathways of endogenous OPs. Endogenous enkephalins [Tyr-Gly-Gly-Phe-Met (YGGFM) and Tyr-Gly-Gly-Phe-Leu (YGGFL)] are degraded through (*i*) cleavage of the Tyr-Gly bond at residues 1 and 2 by a bestatin-sensitive aminopeptidase activity (14, 15) corresponding mainly to aminopeptidase M (microsomal aminopeptidase; aminopeptidase N; EC 3.4.11.2) (16–18) and (*ii*) cleavage of the Gly-Phe bond at residues 3 and 4 by "enkephalinase" (membrane metalloendopeptidase; kidney brush border neutral proteinase; EC 3.4.24.11), which thereby releases the tripeptide Tyr-Gly-Gly (YGG) (reviewed in refs. 19–24) (Fig. 1). [^{3}H]YGG is formed when [^{3}H]enkephalins are brought into contact with cerebral tissues *in vitro* (15, 25) or *in vivo* (26, 27), and its degradation is prevented by bestatin (15).

Using a sensitive RIA, we recently characterized YGG as an endogenous constituent of mouse (28, 29) and rat brain tissues (52). Endogenous YGG appears to be formed extraneuronally by enkephalinase degradation of the OPs derived from the proenkephalin A precursor because (*i*) its regional distribution corresponds to that of YGGFM, a marker of proenkephalin A neurons, (*ii*) its levels decrease after ablation of these neurons, (*iii*) its extracellular localization has been shown by subcellular fractionation and studies with brain slices, (*iv*) its level rises considerably as a result of depolarization of proenkephalin A neuron terminals, which are abundant in slices from globus pallidus, and (*v*) inhibitors of the ectoenzyme enkephalinase inhibit YGG formation selectively (28, 29, 52). The substrate specificity of enkephalinase (30–33) indicates that YGG is formed by hydrolysis of the amide bond in which the glycine residue is engaged with an aromatic amino acid—i.e., Phe-4 in OPs; in addition, most OPs derived from proenkephalin A [YGGFM, YGGFL, and Tyr-Gly-Gly-Phe-Arg-Phe (YGGFRF)] are readily cleaved,

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Abbreviations: Y, Tyr; YG, Tyr-Gly; YGG, Tyr-Gly-Gly; YGGFM, Tyr-Gly-Gly-Phe-Met; YGGFL, Tyr-Gly-Gly-Phe-Leu; YGGFRF, Tyr-Gly-Gly-Phe-Arg-Phe; MGG, Met-Gly-Gly; FGG, Phe-Gly-Gly; GGY, Gly-Gly-Tyr; i.c.v., intracerebroventricular(ly); OP, opioid peptide.

whereas dynorphins and β-endorphin are poor substrates (18, 33).

In the present work, we attempted to evaluate YGG turnover in mouse striatum by measuring its rates of decline and rise following inhibition of enkephalinase and aminopeptidase, respectively. These rates were compared to the rate of accumulation of YGGFM in an extrasynaptosomal fraction following blockade of its two metabolic pathways. To validate these measurements under conditions of altered neuronal activity, we assessed the effects of pentobarbital anesthesia, which is known to modify the activity of a variety of neuronal systems.

MATERIALS AND METHODS

Animals. Male Swiss mice weighing 20–25 g (Iffa-Credo, France) were kept under artificial light (12-hr cycle) with food and water ad libitum. Experiments were performed between 1 and 4 p.m. Drugs were administered by the intracerebroventricular (i.c.v.) route in a volume of 10 μl per mouse according to Haley and McCormick (34). Intraperitoneal (i.p.) injections were performed in a volume of 200 μl per mouse. Body temperature was measured using a thermistor probe (Ellab TE_3, probe RM6, Copenhagen, Denmark) inserted into the colon. Mice were killed by decapitation, since this method resulted in the same levels of brain YGGFM (35) and YGG (28) as more complex procedures, including microwave irradiation.

Extraction and Purification of YGG and YGGFM. The striata were dissected out on a cold plate and 2 min after decapitation, at most, were homogenized by sonication (Ultra-sons, Annemasse, France) in 30 vol of cold 0.4 M $HClO_4$. After centrifugation (5000 × *g* for 3 min), aliquots of the clear supernatant were taken for YGG and YGGFM assays. Extracts were adjusted to pH 6 with 3 M KOH and 0.4 M phosphate buffer and loaded onto cartridges of bonded SiO_2 (Sep-Pak C_{18}, Waters Associates), which were then washed with 0.5 ml of H_2O. YGG and YGGFM were eluted from the cartridge using 1.5 ml of MeOH. The recoveries (mean ± SEM), determined with [^{3}H]YGG or [^{3}H]YGGFM as internal standard (20,000 dpm), were 80% ± 8% and 95% ± 5%, respectively. The eluates were dried under reduced pressure, and the residues were redissolved in 0.2 ml of H_2O and subjected to RIAs. All values given were corrected for recovery.

RIA of YGG. Purified tissue extracts were assayed as described (28, 29). Briefly, standards and tissue extracts were derivatized with *p*-benzoquinone, the excess of this reagent being removed by extraction into $CHCl_3$. Several dilutions of derivatized materials (0.1 ml) were mixed with the tracer, consisting of ^{125}I-labeled YGG-benzoquinone-YGG (15,000 dpm), and a 1:18,000 dilution of the antiserum was usually added in a final volume of 0.3 ml. After incubation, the bound radioactivity was precipitated by addition of polyethylene glycol and counted in a γ-spectrometer. The IC_{50} value of YGG in the RIA was 0.9 nM. Cross-reactivities of related peptides (in % of YGG immunoreactivity) were the following: <0.01 [Tyr (Y)], 0.6 [Tyr-Gly (YG)], 6 [Met-Gly-Gly (MGG)], 7 [Phe-Gly-Gly (FGG)], <0.01 [Gly-Gly-Tyr (GGY)], and 1 (YGGFM). YGG levels in tissue extracts were calculated by comparison with the standard curve, using logit-logarithmic analysis.

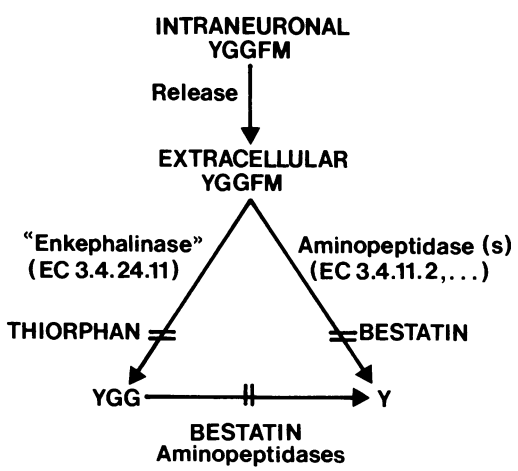

FIG. 1. Pathways for YGG synthesis and degradation.

RIA of YGGFM. The procedure of Gros *et al.* was used (35).

HPLC Analysis of YGG Immunoreactivity. Some of the extracts were prepurified on bonded SiO_2 cartridges and, after addition of [^{3}H]YGG as internal standard, injected into a C_{18} μBondapak column (Waters Associates) in a volume of 0.2 ml. Elution was performed with 0.02% AcOH at a rate of 1.5 ml/min, and 1.5-ml fractions were collected for 20 min and radioimmunoassayed for YGG. The retention times were 5 min for Y, 7 min for YG, and 8 min for YGG. YGGFM was eluted with 70% MeOH. Recovery (mean ± SEM) of the [^{3}H]YGG added to the brain extracts was 93% ± 7%.

Subcellular Fractionation. A simple fractionation procedure was used for rapid separation of the synaptosomal and nonsynaptosomal fractions in mouse striatum (28). Striata from the same animal were homogenized in 1 ml of ice-cold 0.32 M sucrose containing 150 μg of bestatin per ml and 50 μg of Thiorphan per ml using a glassTeflon Potter (0.10–0.15 mm clearance, 10 up and down strokes at 1500 rpm). A 0.5-ml aliquot of the homogenate was immediately centrifuged at 180,000 × *g* for 3 min (Airfuge, Beckman); the resulting supernatant was treated with $HClO_4$ (final concentration, 0.4 M), and the pellet was sonicated in 0.5 ml of 0.4 M $HClO_4$. Both extracts were centrifuged and the resulting supernatants were radioimmunoassayed for YGGFM. When [^{3}H]YGGFM (200,000 dpm) was added to the medium as an internal standard before tissue homogenization, polystyrene bead column chromatography of the extracts showed that 94% ± 5% (mean ± SEM) of the radioactivity recovered in the deproteinized supernatant corresponded to intact YGGFM.

Assay of Enkephalinase Activity. A membrane fraction was rapidly prepared from the striatum of mice treated with acetorphan (36), and enkephalinase activity was measured using 40 nM [D-Ala^2,Leu^5][^{3}H]enkephalin as substrate (37).

Chemicals and Drugs. Chemicals were from the following sources: synthetic peptides, Bachem, Bubendorf, Switzerland; 1,4-benzoquinone, puriss. grade, Fluka; polyethylene glycol 4000, Touzart et Matignon, Paris; organic solvents, analytical grade, Merck; other chemicals, Sigma. [^{3}H]Tyr-Gly-Gly was prepared by hydrolysis of [Met^5][^{3}H]enkephalin (30 Ci/mmol; 1 Ci = 37 GBq; Amersham) using pure kidney enkephalinase (32) and isolated by polystyrene bead column chromatography (38); its radioactive purity was checked by HPLC (32). [D-Ala^2,Leu^5][^{3}H]enkephalin (32 Ci/mmol) was from Commissariat à l'Energie Atomique (Saclay, France). Thiorphan and acetorphan were kindly provided by the Laboratoire Bioprojet (Paris); bestatin, by the Laboratoire Roger Bellon (Paris); and pentobarbital, by the Laboratoires Clin Midy (Paris).

RESULTS

Decline of YGG Levels After Inhibition of YGG Biosynthesis by Thiorphan or Acetorphan. The two enkephalinase inhibitors used were Thiorphan and its parenterally active derivative acetorphan (36). As no parenterally active derivative is

available for the aminopeptidase inhibitor bestatin, most experiments were carried out with Thiorphan, injected i.c.v. to allow direct comparison of the results with those obtained for bestatin. In control mice, striatal YGG levels were not significantly modified by i.c.v. injection of saline. Thiorphan injection elicited a rapid and apparently monoexponential decline of striatal YGG immunoreactivity with a maximal decrease of about 70%, 30–60 min after injection (Fig. 2). This result was confirmed by HPLC analysis, which showed an equivalent decrease in the peak of YGG immunoreactivity. In contrast, striatal YGGFM levels did not alter significantly (not shown). The fractional rate constant for the decline in YGG (3.5 ± 0.5 hr^{-1}), corresponding to a $t_{1/2}$ (mean ± SEM) of 12 ± 2 min, was derived from the slope of the straight line obtained by plotting the logarithm of YGG/YGG_0 (where YGG_0 = initial YGG level) versus time (1–3, 39). Multiplication of this rate constant by the initial steady-state YGG level led to an apparent turnover rate (mean ± SEM) of 18 ± 3 pmol/mg of protein per hr.

The kinetics of YGG decline after i.v. administration of acetorphan at 10 mg/kg were apparently similar to those determined with Thiorphan, as decreases of 70% ± 8% and 65% ± 10% were observed after 15 and 60 min, respectively. Striatal enkephalinase inhibition was maximal from 5 to 120 min after administration of acetorphan at 10 mg/kg, as evaluated from the *ex vivo* inhibition of enzyme activity, and corresponded to 80%. Since similar values were found following injection of acetorphan at 1 mg/kg (36); this may correspond to total enzyme inhibition *in vivo*.

Accumulation of YGG After Inhibition of its Degradation by Bestatin. After i.c.v. administration of bestatin, striatal YGG levels increased linearly for 15–20 min and then reached a plateau at about twice their steady-state levels (Fig. 3), whereas YGGFM levels were not significantly modified (not shown). The rate of YGG accumulation (mean ± SEM), calculated from the initial linear portion of the curve, was 17.9 ± 2.5 pmol/mg of protein per hr.

Accumulation of Extrasynaptosomal YGGFM After Administration of Thiorphan and Bestatin. Simultaneous inhibition of enkephalinase and aminopeptidase M, the two peptidase activities responsible for hydrolysis of endogenous YGGFM, has been reported to increase the striatal level of this peptide (14, 40), but this effect is of limited amplitude and is inconstant (unpublished observations). Because these two peptidases are ectoenzymes and hydrolysis of released YGGFM occurs extraneuronally, we assessed the effect of inhibiting the two peptidases on YGGFM levels in a crude synaptosomal fraction and in the corresponding supernatant (i.e., the extrasynaptosomal fraction); both fractions were

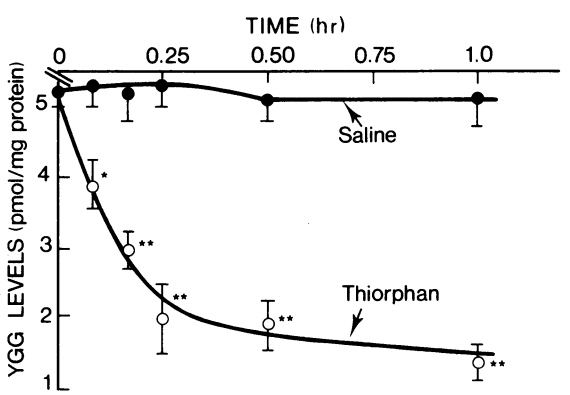

FIG. 2. Decline of YGG levels in mouse striatum after administration of the enkephalinase inhibitor Thiorphan. Mice were given saline or 100 μg of Thiorphan i.c.v. and were killed at different times after injection. Values are means ± SEM of five to eight experiments. *, $P < 0.05$; **, $P < 0.01$ versus the respective controls.

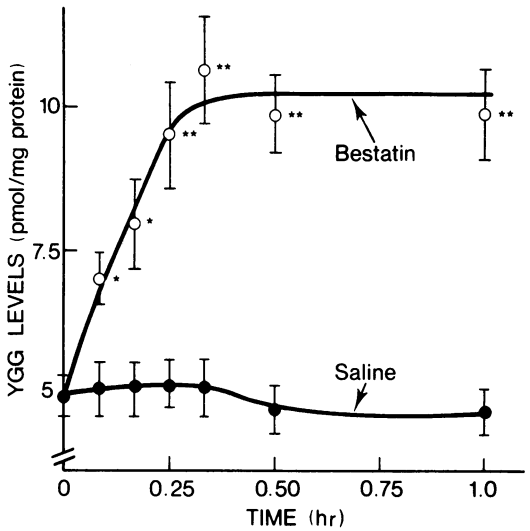

FIG. 3. YGG accumulation in mouse striatum after administration of the aminopeptidase inhibitor bestatin. Animals received saline or 150 μg of bestatin i.c.v. and were killed at different times after injection. Values are means ± SEM of 5–11 experiments. *, $P < 0.05$; **, $P < 0.01$ versus the respective controls.

obtained under conditions of negligible YGGFM hydrolysis (see *Materials and Methods*). In controls, extrasynaptosomal YGGFM constituted 21% ± 6% of the total (Table 1), whereas the corresponding value for YGG was 73% ± 4% (28). At any time after combined administration of Thiorphan and bestatin, neither synaptosomal YGGFM nor total YGGFM was significantly modified (not shown). In contrast, extrasynaptosomal YGGFM increased linearly for 15 min and then reached a plateau at about twice its steady-state level (Fig. 4). Consequently, the ratios of extrasynaptosomal to synaptosomal levels rose from 0.21 ± 0.02 in controls to 0.37 ± 0.03 ($P < 0.01$) 30 min after administration of the peptidase inhibitors (mean ± SEM of 13–15 experiments). The rate of extrasynaptosomal accumulation of YGGFM calculated from the initial portion of the curve was 18 ± 3 pmol/mg of protein per hr.

Effects of Pentobarbital-Induced Anesthesia on YGG and Extrasynaptosomal YGGFM. Mice received pentobarbital i.p. at 60 mg/kg and were immediately placed under an infrared lamp so that their internal temperature (37.8 ± 0.2°C) did not vary during the period of anesthesia (from about 3 to 60 min after the injection).

Pentobarbital did not significantly change striatal YGGFM levels (Table 1). In contrast, there was a gradual decrease in YGG levels, and a plateau was reached after 30 min at about half the initial level (Fig. 5). When this new steady-state level was reached, i.c.v. administration of bestatin elicited, for the next 15 min, linear accumulation of YGG at a rate of 7 ± 1 pmol/mg of protein per hr versus 18 ± 2 pmol/mg of protein per hr in unanesthetized mice. Because high concentrations of barbiturates inhibit enkephalinase activity *in vitro* (41), we measured this activity in rapidly prepared striatal membranes 60 min after administration of pentobarbital. This activity did not differ from that of the controls (71 ± 2 fmol/mg of protein per min versus 65 ± 5 fmol/mg of protein per min). Striatal YGG levels were similarly reduced in mice anesthetized i.p. with chloral hydrate at 400 mg/kg (−35% and −54% after 15 and 30 min, respectively). The accumulation of extrasynaptosomal YGGFM elicited by Thiorphan and bestatin was no longer significant in anesthetized mice and only constituted about 20% that observed in nonanesthetized animals (Table 1).

Table 1. Effects of pentobarbital-induced anesthesia on the accumulation of striatal YGGFM elicited by Thiorphan and bestatin

	Extrasynaptosomal YGGFM, pmol/mg of protein		Total YGGFM, pmol/mg of protein	
	Control	Pentobarbital	Control	Pentobarbital
Vehicle	5.8 ± 0.5	6.9 ± 0.5	28 ± 1	29 ± 2
Thiorphan + bestatin	9.8 ± 0.8*	7.5 ± 0.7	34 ± 2†	32 ± 2
Difference	4.0 ± 0.8	0.7 ± 0.7‡	6 ± 2	3 ± 2†

Animals were given pentobarbital i.p. at 60 mg/kg (or saline) with 50 μg of Thiorphan + 75 μg of bestatin (or vehicle) administered 30 min later i.c.v.; animals were killed 15 min after the last injection. Values are means ± SEM of six to nine experiments.
*$P < 0.01$ versus vehicle.
†NS, not significant.
‡$P < 0.01$ versus controls.

DISCUSSION

The effects of peptidase inhibitors indicate that turnover rate of the tripeptide YGG is fast in mouse striatum, and the effects of pentobarbital indicate that its level and apparent turnover rate diminish rapidly when neuronal activity is depressed during anesthesia. This is consistent with previous data indicating that YGG is formed after enkephalin release into the extracellular space, a process for which such formation may constitute a reliable index.

Following inhibition of enkephalinase, the synthesizing enzyme of YGG (28, 52), the latter declined with a $t_{1/2}$ of 12 min, whereas after inhibition of the aminopeptidase(s) responsible for its hydrolysis (15), its level doubled within 15 min. Very similar values for the YGG turnover rate—i.e., about 18 pmol/mg of protein per hr—were obtained from analysis of either the monoexponential decline or the initial linear accumulation of this tripeptide. Again, a similar value was found (18 ± 3 pmol/mg of protein per hr) for the apparent rate of YGGFM release deduced from its extrasynaptosomal accumulation after presumably complete inhibition of its metabolism by Thiorphan and bestatin (15, 17). From these three consistent estimations it may be inferred that YGGFM release and its degradation into YGG occur at a high rate, implying that the half-life of enkephalins in striatum is comparable to that of monoamines in many brain areas—i.e., about 1 hr. This conclusion is somewhat unexpected since the ribosomal synthesis, maturation, and axonal transport processes that precede neuropeptide exocytosis are often assumed to make the turnover rate of neuropeptides slower than that of monoamine or amino acid transmitters.

However, these absolute turnover rates cannot be readily accepted as accurate estimates for YGGFM release for several reasons. It is not known (and it is difficult to establish) whether or not any significant efflux of YGG or YGGFM occurs from the brain extracellular spaces during the 20 min after inhibitor injection. Peptidase inhibition might not be complete immediately after such injection, a possibility likely to introduce errors in the evaluation of turnover rates as rapid as those presently measured. Inhibition of enkephalin-metabolizing peptidases *in vivo* elicits a number of central effects (21), including modification of monoamine turnover (42–44), and might trigger control mechanisms regulating enkephalin release (8, 9). YGG does not arise from YGGFM selectively, since both the substrate specificity of enkephalinase (30–33) and release experiments from brain slices (52) indicate that YGGFL and Tyr-Gly-Gly-Phe-Met-Arg-Phe (YGGFMRF) (i.e., other fragments of the proenkephalin A precursor) contribute to YGG formation. Bestatin inhibits not only the hydrolysis of the Y-G amide bond of YGG but also that of enkephalins, so that the latter should be entirely driven into the enkephalinase pathway, which is normally responsible

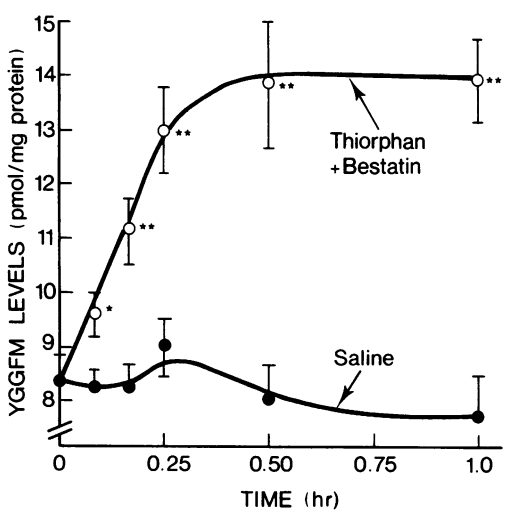

FIG. 4. Accumulation of extrasynaptosomal YGGFM in mouse striatum after its degradation inhibition by Thiorphan + bestatin. Animals received saline or 50 μg of Thiorphan + 75 μg of bestatin i.c.v. and were killed at different times after injection. Values are means ± SEM of 5–14 experiments. *, $P < 0.05$; **, $P < 0.01$ versus the respective controls.

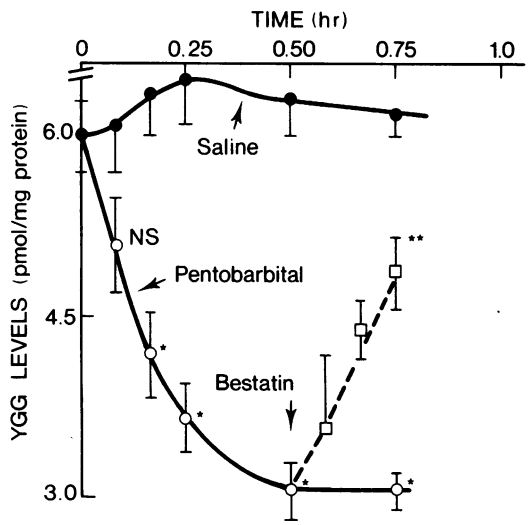

FIG. 5. Steady-state levels and bestatin-induced accumulation of YGG in mice anesthetized with pentobarbital. Mice were given pentobarbital i.p. at 60 mg/kg either alone (solid line) or with 150 μg of bestatin, administered 30 min later i.c.v. (broken line), and were killed at various intervals thereafter. Values are means ± SEM of 5–15 experiments. NS, nonsignificant; *, $P < 0.01$ versus saline; **, $P < 0.01$ versus pentobarbital alone.

for about half the total YGGFM degradation (15). Hence, the fairly close agreement between the present estimates of the YGG turnover and YGGFM accumulation rates might to a certain extent be fortuitous. However, as most of the errors tended to lead to underestimation of the rates of enkephalin release, the conclusion that this is a rapid process remains fully valid. Furthermore, estimations of neurotransmitter turnover rates are more useful in determining comparative differences under different experimental conditions than in determining absolute rates. In this respect the present methods seem to be validated by the observation that pentobarbital anesthesia similarly decreased the accumulation rates of YGG and YGGFM by 60–80% (Fig. 5 and Table 1).

In addition, steady-state YGG rapidly fell to about half its preanesthesia level in the absence of treatment with peptidase inhibitors (Fig. 5), whereas steady-state YGGFM did not significantly alter. A similar effect was observed when mice were anesthetized with chloral hydrate. This decrease in steady-state YGG level during anesthesia seems attributable to impairment of enkephalin release and of YGG formation shown with peptidase inhibitors. Barbiturates reduce the activity of several classes of aminergic neurons (45–49). As far as we know, their effects on peptidergic neuron activity have not been reported previously. The drastic reduction in the activity of enkephalin neurons, shown by the present data, might be relevant to the hyperalgesic and anticonvulsive effects of barbiturates (50) since enkephalins induce opposite effects (51).

Hence, the steady-state level of YGG appears to constitute a useful index of changes in enkephalin neuron activity in the brain, just as the levels of certain extraneuronal metabolites reflect changes in monoamine neuron activity. Measurement of this simple index offers the great advantage of avoiding the administration of any metabolic inhibitor. A similar experimental approach could conceivably be applied to other classes of cerebral neuropeptides as soon as their metabolic pathways are identified.

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