Reserpine-induced Alterations in the Processing of Proenkephalin in Cultured Chromaffin Cells

INCREASED AMIDATION*

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We have used antisera directed towards eight different portions of the proenkephalin molecule to examine the processing rates and patterns of proenkephalin-derived peptides in chromaffin cell cultures in the presence and absence of reserpine. Reserpine treatment produced profound effects on the molecular weight profile of nearly all enkephalin-containing peptides. Increased production of low molecular weight immunoreactive [Met^2]enkephalin, [Leu^5]enkephalin, [Met^2]enkephalin-[Arg^4]Gly^7-Leu^8, and [Met^2]enkephalin-Arg^6-Phe^7 was observed in reserpine-treated cultures; immunoreactivity corresponding to several intermediate-sized enkephalin-containing peptides such as Peptide B and the high molecular weight [Met^2]enkephalin-Arg^4-Gly^7-Leu^8 immunoreactive peptide was decreased. The production of two amidated opioid peptides, amidorphin and metorphamide, was greatly accelerated in the presence of reserpine. The increased levels of low molecular weight enkephalins could not be accounted for by assuming decreased basal release. These results indicate that reserpine treatment is able to increase the extent of post-translational processing of proenkephalin within chromaffin cells.

Opioid peptides of the enkephalin family are widely distributed within the central and peripheral nervous system and are thought to participate as neurotransmitters/neuromodulators in many neuronal pathways. The high concentrations of enkephalins found in the adrenal medulla of several species (1) and the presence of circulating enkephalin-immunoreactive peptides (2) are suggestive of a hormonal role for adenomedullary enkephalins. This role is as yet undefined but may relate to the known cardiovascular effects of synthetic opiates and endogenous opioid peptides (reviewed in Ref. 3).

Wilson et al. (4, 5) first demonstrated that reserpine treatment of cultured adrenal chromaffin cells is able to increase levels of radioreceptor-assyayable enkephalins, including [Met^2]enkephalin and [Leu^5]enkephalin. This effect was initially attributed to an increased biosynthesis of proenkephalin (5). However, Eiden et al. (6) and Naranjo et al. (7) have shown that reserpine treatment of chromaffin cell cultures effectively decreases levels of proenkephalin mRNA. Based on their finding of increased amounts of low molecular weight immunoreactive [Met^2]enkephalin, Eiden et al. (6) have suggested that reserpine is able to increase the production of [Met^2]enkephalin from proenkephalin. Whether the production of other low molecular weight enkephalins is similarly increased by reserpine was not investigated; a generalization of this effect to include all of the known processing products of proenkephalin would strongly suggest that reserpine exerts a direct effect on the post-translational processing of proenkephalin. An alternative possibility which must be considered is that reserpine acts to decrease the spontaneous release of low molecular weight opioid peptides from chromaffin cells, thus allowing them to accumulate intracelullarly.

In the present study, I have addressed the mechanism by which reserpine can induce changes in the levels of low molecular weight opioid peptides by investigating the post-translational processing of enkephalin-immunoreactive peptides in chromaffin cell cultures in the presence and absence of reserpine. Eight different antisera, directed toward different portions of the proenkephalin molecule, were used to characterize the molecular weight profile of enkephalins present in the adrenal medulla, in cultured adrenal chromaffin cells, and in cultures treated with reserpine. I have also investigated the influence of reserpine treatment on the release of enkephalin-containing peptides. My results demonstrate that reserpine treatment of chromaffin cell cultures rapidly increases the levels of a variety of smaller proenkephalin-derived peptides, some of which require further post-translational processing such as amidation.

MATERIALS AND METHODS

Preparation of Chromaffin Cell Cultures—Chromaffin cell cultures were prepared from bovine adrenal glands (obtained from a local slaughterhouse) following the method of Wilson et al. (8) with the following modifications. The initial collagenase digestion of the glands was performed using repeated manual retrograde perfusion with warm 0.5% collagenase (Sigma, Type 1A) in place of mechanical perfusion; fresh collagenase was used each time. DNase ('6 mg/ml, Sigma) was included in the collagenase medium and in the Percoll gradient in order to reduce aggregation. The collagenase digestion was terminated by washing the cells three times with Locke's solution containing 2% bovine serum albumin (Fraction V, Miles Laboratories). Following the Percoll gradient step, the dissociated cells were washed three times with Locke's solution and were finally resuspended in a minimal volume of Dulbecco's modified Eagle's medium (Gibco) containing 10% heated fetal calf serum (Gibco), 10 mM HEPES, 40 mg/liter gentamycin, 10 μM cytosine arabinoside, 106 units/ml penicillin, and 100 μg/ml streptomycin. An aliquot of the cell suspension was counted using Trypan Blue to determine cell yield and viability. Cells were resuspended in Dulbecco's modified Eagle's medium and plated in collagen-coated multiple 13 or 24 well plates.

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1 The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
was raised in this laboratory by immunization of New Zealand White rabbits with synthetic Peptide B. The antiserum has been described previously (10, 11). Briefly, this antiserum was obtained through the courtesy of Drs. J. Schwartz and M. Mocchetti (National Institutes of Mental Health); this antiserum is predominantly carboxyl-directed; [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{6}Gly\textsuperscript{7}Leu\textsuperscript{8} antiserum was raised in this laboratory by immunization of New Zealand White rabbits with synthetic Peptide B coupled to thyroglobulin. The characteristics of the synenkephalin antiserum, provided by D. Liston and J. Rossier, were reported by Liston et al. (17).

Radioimmunoassays were carried out using an antiserum generously donated by S. Sabol (National Institutes of Health); the specificity of this antiserum has been described previously (10, 11). Briefly, this antiserum is predominantly carboxyl-directed; [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{6}Phe\textsuperscript{7} antiserum was raised in this laboratory by immunization of New Zealand White rabbits with [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{6}Gly\textsuperscript{7}Leu\textsuperscript{8} conjugated to succinylated hemocyanin; details of the antiserum production and specificity have been reported (14). Metorphamide antiserum was obtained from E. Weber (Oregon Health Sciences University); the characteristics of this antiserum were previously reported (15). Amidorphin antiserum was provided by B. Seizinger, D. Liebisch, and A. Herz (Max-Planck Institut) and has been described by Seizinger et al. (16), while the characteristics of the synenkephalin antiserum, provided by D. Liston and J. Rossier, were reported by Liston et al. (17). Antiserum to Peptide B was raised in this laboratory in New Zealand White rabbits with synthetic Peptide B coupled to thyroglobulin. The production of this antiserum will be described in a separate paper.\textsuperscript{3} This antiserum shows no detectable cross-reaction with [Met\textsuperscript{5}]enkephalin, [Leu\textsuperscript{5}]enkephalin, or [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}Gly\textsuperscript{5}Leu\textsuperscript{6} [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}Phe\textsuperscript{5} cross-reacts by 0.37%.

Radioimmunoassays were carried out in duplicate in a total volume of 0.3 ml radioimmunoassay buffer (0.1 M sodium phosphate buffer, with 50 mM sodium chloride, 0.1% bovine serum albumin, 0.1% bovine \(\beta\)-mercaptoethanol, and 0.1% sodium azide, pH 7.4) containing approximately 10,000 cpm of iodinated peptide and appropriate final dilution (ranging from 1:14,000 to 1:50,000). Following overnight incubation at 4°C, antibody-bound labeled peptide was separated from free labeled peptide using polyethylene glycol with bovine \(\gamma\)-globulin as a carrier. More specific details of the radioimmunoassay procedure used have been described recently (18).

RESULTS

Fig. 1 depicts the molecular weight profile of several immunoreactive enkephalins present in extracts prepared from fresh bovine adrenal medulla. Most of the immunoreactive [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}Phe\textsuperscript{5} as well as immunoreactive [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}Gly\textsuperscript{5}Leu\textsuperscript{6} is contained in species with apparent molecular weights between 4 and 8 kDa. In contrast, immunoreactivity corresponding to [Leu\textsuperscript{5}]enkephalin and [Met\textsuperscript{5}]enkephalin was eluted at the position expected for the authentic pentapeptides. This result is most likely due to the fact that these two antiseras cross-react poorly with higher molecular weight immunoreactive substances. Fig. 1 also shows the molecular weight profile of amidated opioid peptides derived from proenkephalin. Immunoreactivity corresponding to amidorphin and metorphamide was eluted at positions appropriate to the molecular weight of these peptides. The molecular weight profile of immunoreactive Peptide B, which also elutes at the expected molecular weight position, is also shown in this figure. These results indicate that the major enkephalin-containing peptides in both the adrenal medulla as well as in chromaffin cell cultures are peptides of intermediate molecular weight (i.e. larger than the penta- to octapeptides, but more fully processed than proenkephalin). In Fig. 2 (left panels), the molecular weight profiles of immunoreactive enkephalins present in 3-day-old chromaffin tissue culture dishes (Costar) at a density of 2.5 \(\times\) 10\textsuperscript{5} cells/cm\textsuperscript{2}. The medium was changed on the third day after plating; reserpine (1 \(\times\) 10\textsuperscript{-4} M, diluted in Dulbecco's modified Eagle's medium) was added at this time. Cultures were homogenized in 0.5 ml of ice-cold extraction buffer (1 n HCl containing 20 mM HCl and 0.1% \(\beta\)-mercaptoethanol) at various time points after the addition of reserpine. In order to compare the profile of immunoreactive enkephalins present in chromaffin cell cultures with the tissue from which they were derived, fresh medullary tissue was dissected free from cortical tissue on ice and homogenized with 10 volumes of ice-cold extraction buffer. A 10% extract from both tissue and culture were centrifuged at 20,000 \(\times\) g for 30 min and the supernatant removed and concentrated by lyophilization prior to gel filtration.

The effect of reserpine on the basal release of enkephalins into the medium was examined by removing medium from the cells at varying time points following exposure to reserpine (1 \(\times\) 10\textsuperscript{-4} M) or control medium. Medium samples were then centrifuged and assayed directly for [Met\textsuperscript{5}]enkephalin and [Leu\textsuperscript{5}]enkephalin; standard curves were also run in medium. The nicotine-stimulated release of enkephalins from chromaffin cell cultures was studied using cultures which had either been treated with reserpine or control medium for 3 days. Replating was also performed to varying concentrations of nicotine in balanced salts solution for a 15-min period at room temperature (9). The balanced salts solution was then removed and stored frozen prior to radioimmunoassay for [Leu\textsuperscript{5}]enkephalin. Radioimmunoassay standards were also run in balanced salts solution.

**Gel Filtration**—Separation of the various immunoreactive molecular weight forms of proenkephalin-derived peptides was achieved using a 60 \(\times\) 0.9-cm column of Sephadex G-75, equilibrated, and eluted in 1 n NaCl containing 0.1 mg/ml crystalline bovine serum albumin (Behring Diagnostics). Columns were run at 4°C; the flow rate was 2 ml/h and 0.68-ml fractions were collected. The column was standardized using blue dextran, soybean trypsin inhibitor, \(^{125}\)I-labeled Peptide B, \(^{125}\)I-[Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}Phe\textsuperscript{5}, and cobalt chloride.

**Radioimmunoassays and Antiserum Specificity**—The [Met\textsuperscript{5}]enkephalin radioimmunoassay was carried out using an antiserum generously donated by S. Sabol (National Institutes of Health); the specificity of this antiserum has been described previously (10, 11). Briefly, this antiserum is predominantly carboxyl-directed; [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{6} shows only a 0.5% cross-reaction, while [Leu\textsuperscript{5}]enkephalin cross-reacts by 10%. The details of the [Leu\textsuperscript{5}]enkephalin radioimmunoassay have been previously reported (12). The [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}Phe\textsuperscript{5} antiserum was obtained through the courtesy of Drs. J. Schwartz and I. Mocchetti (National Institutes of Mental Health); this antiserum is also primarily carboxyl-directed (13) and exhibits 50% cross-reaction with [Leu\textsuperscript{5}]enkephalin cross-reacts by 10%. The cross-reactivity corresponding to amidorphin and metorphamide was eluted at positions appropriate to the molecular weight of these peptides. The molecular weight profile of immunoreactive Peptide B, which also elutes at the expected molecular weight position, is also shown in this figure. These results indicate that the major enkephalin-containing peptides in both the adrenal medulla as well as in chromaffin cell cultures are peptides of intermediate molecular weight (i.e. larger than the penta- to octapeptides, but more fully processed than proenkephalin).

3 Lindberg, I., and White, L. (1986) Biochem. Biophys. Res. Commun., in press.
FIG. 2. Molecular weight forms of immunoreactive enkephalins (penta- to octapeptides) in control and reserpine-treated chromaffin cell cultures. An acid extract was prepared from either control chromaffin cell cultures (left panels) or cultures which had been treated with reserpine (10^-6 M) for 3 days. Following chromatography on Sephadex G-75, aliquots of the resultant fractions were dried and assayed for the enkephalins shown. Met^-ENK-RF, Met^-enkephalin-Arg^-Phe^-; Met^-ENK-RGL, Met^-enkephalin-Arg^-Gly^-Leu^-; Leu^-ENK, Leu^-enkephalin.

cell cultures are shown. In general, immunoreactive chromaffin cell enkephalins present in cultures possess similar molecular weights to those observed in adrenal medulla. Immunoreactive [Met^-] and [Leu^-]enkephalin are predominantly of one molecular weight form. Several larger immunoreactive molecular weight forms of [Met^-]enkephalin-Arg^-Gly^-Leu^- and [Met^-]enkephalin-Arg^-Phe^- peptides are observed in these cultures; however, as with extracts prepared from the whole adrenal medulla, most of the immunoreactive [Met^-] enkephalin-Arg^-Phe^- and [Met^-]enkephalin-Arg^-Gly^-Leu^- peptides exhibit masses of 4 and 8 kDa. The right half of this figure shows the profile of immunoreactive enkephalins present in parallel cultures which had been treated for 3 days with 10^-6 M reserpine. A large increase in immunoreactive [Met^-] and [Leu^-]enkephalin peptides can be observed. In addition, there is a shift in the molecular weight profile of [Met^-] enkephalin-Arg^-Gly^-Leu^- immunoreactive peptides following reserpine treatment. In the control cells, only 10% of [Met^-]enkephalin-Arg^-Gly^-Leu^- immunoreactive peptides elute in the position of the octapeptide; following reserpine, 44% of the [Met^-]enkephalin-Arg^-Gly^-Leu^- immunoreactive peptides elute in this position. A similar effect is observed when [Met^-]enkephalin-Arg^-Phe^- peptides are assayed; in control cells, approximately 33% of [Met^-]enkephalin-Arg^-Phe^- immunoreactive peptides elute in the position of the heptapeptide. Following reserpine, 75% of immunoreactive [Met^-]enkephalin-Arg^-Phe^- elutes at the position of the heptapeptide. Reserpine thus appears to promote a shift in molecular weight of immunoreactive peptides from intermediate sized to low molecular weight forms. No differences in protein concentration were observed in reserpine-treated as opposed to control cultures (0.14 ± 0.01 versus 0.12 ± 0.01 mg protein, respectively) (means ± S.D., n = 6).

The molecular weight profiles of amidated opioid peptides present in cultured chromaffin cells are shown in the left panels of Fig. 3; both immunoreactive amidorphin as well as metorphamide exhibit elution times characteristic of the authentic peptides. The levels of metorphamide are extremely low in control cultures, but rise dramatically upon treatment of the cultures with reserpine (Fig. 3, right panels). Amidorphin levels also exhibit a pronounced reserpine-induced increase, although the magnitude of this increase is not as great as that of metorphamide.

In Fig. 4, the molecular weight profile of chromaffin cell immunoreactivity corresponding to two intermediate-sized enkephalin-containing peptides, Peptide B and synenkephalin, is shown. Synenkephalin immunoreactivity consists predominantly of two forms, corresponding to apparent masses of approximately 25 and 15 kDa; the position of the lower mass immunoreactive peak corresponds to the elution position of iodinated synenkephalin (not shown). Peptides with Peptide B immunoreactivity elute as a single immunoreactive species in the position of the iodinated Peptide B marker (Fig. 4, left panels). Following treatment of cultures with reserpine, a slight decrease in the amount of the larger synenkephalin-immunoreactive peptide is observed, while no change in the amount of synenkephalin itself is seen. Peptide B-immunoreactive peptides appear to decrease following reserpine treatment (Fig. 4, right panels). In general, the profile of the immunoreactive proenkephalin-derived peptides present in chromaffin cell cultures was very similar to that ob-

FIG. 3. Molecular weight forms of amidated opioid peptides in control and reserpine-treated cultures. Aliquots of fractions taken from the chromatography described in Fig. 2 were assayed for the two amidated opioid peptides shown.
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Fig. 4. Molecular weight forms of intermediate sized enkephalin-containing peptides in control and reserpine-treated cultures. Aliquots of fractions taken from the chromatography described in the legend to Fig. 3 were assayed for synenkephalin immunoreactivity (panels A and B) and Peptide B immunoreactivity (panels C and D).

observed in the parent tissue, implying that chromaffin cells represent an appropriate model in which to study the biosynthesis of enkephalins.

The time course of reserpine-induced changes in low molecular weight peptide concentration was assessed by measuring levels of three opioid peptides at varying times following the administration of reserpine to the cultures (Fig. 5). These peptides were chosen for assay because the previous experiments showed that they consisted predominantly if not exclusively of one molecular weight form. As shown in the top panel of Fig. 5, the levels of immunoreactive metorphamide increase steadily after exposure of the cells to metorphamide (dashed line); control cells maintain constant levels of this peptide (solid line). In contrast, the time course of activation of amidorphin production (middle panel) indicates that this peptide does not appear to increase as much as metorphamide. Unlike metorphamide, increased amidorphin production exhibits a plateau at about 12–24 h. Similarly, [Leu'lenkephalin production, shown in the bottom panel, also does not respond to reserpine as extensively as does metorphamide production. As observed in the case of amidorphin, reserpine-stimulated [Leu'lenkephalin production also exhibits a plateau at about 12–24 h. Some experiment to experiment variability in the degree of stimulation by reserpine of low molecular weight enkephalins was observed. The reasons underlying this variability are not known but may have to do with the fact that reserpine effects appear to be highly dependent on drug/tissue ratios (19). It is thus possible that variations in cell plating and/or fibroblast content may have contributed to a variable effectiveness of the drug. However, it is of interest to note that metorphamide production always showed a far greater increase in response to reserpine than amidorphin or [Leu'

Fig. 5. Time course of activation of the production of amidated opioid peptides and Leu-enk in response to reserpine. Cultures were homogenized at various time points after the addition of reserpine (1 × 10^-6 M) to the cultures. The dashed lines represent cultures treated with reserpine, while the solid lines represent parallel control cultures. Leu-ENK, [Leu']enkephalin.

TABLE I
Effect of reserpine on basal release of [Met']enkephalin into the medium

| Age of culture | Cumulative release of [Met']enkephalin |
|---------------|--------------------------------------|
|               | Control | Reserpine |
| days          |         |           |
| 3             | 23.3 ± 0.9 | 23.3 ± 1.0 |
| 4             | 7.77 ± 0.53 | 6.44 ± 0.58* |
| 5             | 11.7 ± 0.60 | 8.42 ± 0.32* |
| 6             | 14.8 ± 0.86 | 10.4 ± 0.25* |

*Significantly different from corresponding control (p < 0.05, Student's t test).

The possibility that the reserpine-induced increase in enkephalin production observed after reserpine treatment was due directly to decreased release of low molecular weight peptides was tested by examining the levels of [Met']enkeph-

alin in media from cultures which had been exposed to reserpine or control media for varying amounts of time. As may be seen in Table I, exposure of cells to reserpine significantly reduced the basal release of [Met']enkephalin into the medium. However, at 3 days, these media concentrations represent only approximately 6% and 4% of the cellular levels of
[Met⁶]enkephalin in the control and reserpine-treated samples, respectively. Since cellular low molecular weight enkephalin levels were stimulated between 2- to 3-fold by reserpine treatment it appears unlikely that inhibition of basal release can be solely responsible for the increased levels of enkephalins observed during reserpine treatment. The effect of acute or chronic treatment with reserpine on the nicotine-stimulated release of enkephalins was investigated in a separate experiment (shown in Fig. 6). Chromaffin cells cultured in the presence of reserpine released greater quantities of immunoreactive [Leu⁶]enkephalin in response to nicotine than did cultures never exposed to reserpine. However, the presence of reserpine during the release experiment did not affect the nicotine-stimulated release of [Leu⁶]enkephalin (Fig. 6). Taken together with the findings presented in Table I, these results argue against the notion that the primary action of reserpine can be solely responsible for the increased levels of enkephalins.

Ascorbate has been reported to be an essential cofactor in the amidation of α-melanotropin (20, 21). It was therefore of interest to examine whether the reserpine-induced stimulation of metorphamide and amidorphin, both amidated peptides, also exhibits a requirement for added ascorbate. Treatment of cultures was carried out in the presence and absence of ascorbate (250 μM). In agreement with the results of Wilson and Kirshner (9), ascorbate supplementation was found to have no effect on the basal levels of opioid peptides; ascorbate also had no effect on the reserpine-induced increase in metorphamide or amidorphin (Table II).

**DISCUSSION**

The above data indicate that reserpine is able to increase the production of not only [Met⁶]enkephalin, but also [Leu⁶] enkephalin, [Met⁶]enkephalin-Arg⁴-Phe³, and [Met⁶]enkephalin-Arg⁴-Gly⁵-Leu⁶. Reserpine thus appears to accelerate the generation of all low molecular weight enkephalins; however, the levels of intermediate-sized enkephalin-containing peptides, such as Peptide B and the 5.3-kDa fragment of proenkephalin, are decreased in response to reserpine treatment. These results suggest that reserpine is able to increase the general activity of proteolytic processing enzymes, perhaps within the chromaffin granule. In order to ascertain whether activation of amidation, another important post-translational processing event, can also occur in the presence of reserpine, we examined the effects of reserpine treatment on the production of two amidated opioid peptides derived from proenkephalin. The levels of both amidated enkephalins, but in particular metorphamide, were observed to increase rapidly in response to reserpine treatment, suggesting that amidation is indeed activated in the presence of reserpine. This effect may be due to stimulation of amidating enzymes by reserpine; Hook et al. (22) have reported that certain kinetic parameters of the carboxypeptidase B-like processing enzyme are altered in response to reserpine treatment. Alternatively, it may be speculated that amidation is not normally a rate-limiting processing step; when reserpine acts to increase the amounts of glycine-extended precursors to amidated peptides, these precursors then become rapidly amidated. Unlike the production of α-melanotropin in intermediate pituitary cell cultures (20, 21), the production of amidated proenkephalin-derived peptides in chromaffin cell cultures was not affected by the inclusion of ascorbate in the medium. These results are surprising in view of the rapid loss of endogenous ascorbate from chromaffin cells placed in culture (23) and imply that either the existing ascorbate concentration is sufficient to maintain amidation, or that other reducing equivalents are utilized for this reaction (24).

Interestingly, the time course of reserpine-induced effects on the production of amidated peptides suggests that metorphamide production continues to increase long after the production of [Leu⁶]enkephalin has reached a plateau. These results might reflect the sequential nature of the processing steps required to generate these peptides from their presumed common precursor, Peptide E (which contains the sequence of metorphamide at its amino end and which terminates in [Leu⁶]enkephalin (25)). Pulse-chase studies of enkephalin production will be required to demonstrate the precise effects of reserpine on this step of the enkephalin biosynthetic pathway; such experiments are now in progress.

The inhibition of basal release observed in the presence of reserpine, while not in itself sufficient to explain the large increases in intracellular low molecular weight enkephalins, represents an intriguing phenomenon. The data presented above as to the effects of nicotine on the release of enkephalins from reserpine-treated cells indicate that reserpine does increase enkephalins in a pool which is stimulus secretion coupled. At the same time, reserpine apparently paradoxically lowers the basal release of enkephalins into the medium. It is interesting to note that forskolin and cyclic AMP, agents which also increase intracellular enkephalin levels (but act by increasing the transcription of proenkephalin mRNA) both

**TABLE II**

**Lack of effect of ascorbate on reserpine-induced enkephalin production**

Chromaffin cell cultures were subjected to the treatments indicated for 3 days. Data represent the mean ± S.E. of four determinations (wells).

| Condition | Amidorphin | Metorphamide |
|-----------|------------|--------------|
| Control   | 8.1 ± 0.6  | 0.094 ± 0.014 |
| + ascorbate (250 μM) | 8.5 ± 0.6 | 0.093 ± 0.009 |
| Reserpine (1 μM) | 16.3 ± 0.7 | 0.793 ± 0.049 |
| + ascorbate (250 μM) | 16.0 ± 1.2 | 0.768 ± 0.068 |

**FIG. 6. Lack of effect of reserpine on nicotine-stimulated enkephalin release.** Varying concentrations of nicotine were added to chromaffin cell cultures and the Leu-enk released into the medium was estimated by radioimmunoassay. The open triangles represent results obtained using chromaffin cell cultures which had been previously chronically treated for 3 days with reserpine (1 × 10⁻⁶ M). The open circles represent data obtained from cells acutely exposed to reserpine (concurrently with nicotine), while the closed circles represent cells exposed only to nicotine. Leu-ENK; [Leu⁶]enkephalin.
enhance rather than decrease basal release of enkephalins into the medium (26, 27).

The mechanism of action of reserpine in increasing the post-translational processing of intermediate sized enkephalin-containing peptides remains elusive. The subcellular loci of the post-translational processing events involved in the generation of low molecular weight enkephalins have not yet been determined. However, in other prohormone systems, late proteolytic processing steps as well as other processing events (such as removal of carboxyl-terminal basic residues, acetylation, and amidation) are thought to take place within the secretory granule (reviewed in Refs. 28 and 29). In addition, reserpine is known to bind to the chromaffin granule membrane, where it acts to inhibit catecholamine transport into the granule by blocking the amine translocator (reviewed in Refs. 28 and 29). In addition, reserpine-induced activation of post-translational processing. This effect may be a purely physical phenomenon, such as an improved intragranular milieu for proteolytic processing enzymes resulting from decreased catecholamine content. Alternatively, it is possible that binding of reserpine to chromaffin granules induces conformational alterations in granule membrane structure which then result in increased activity of intragranular processing enzymes. Further research will be necessary to distinguish between these possibilities.

Note Added in Proof—Similar results have recently been reported by Eiden, L. E., and Zamir, N. (1986) (J. Neurochem. 5, 1651–1654).

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