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# Proenkephalin A gene expression in bovine adrenal chromaffin cells is regulated by changes in electrical activity

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**Concentrations of mRNA coding for the opioid peptide precursor proenkephalin A (mRNA$^{ENK}$) were measured in primary cultures of bovine adrenal chromaffin cells maintained in serum-free medium. Using a sensitive solution hybridization assay, an increase in mRNA$^{ENK}$ levels from 45 to 300% above control with $K^+$ (10–20 mM), $Ba^{2+}$ (1 mM) and veratridine (5 μM) was found. The highest increase (300% above control) was obtained with the $Na^+$ channel agonist veratridine. This effect was nearly abolished in the presence of the $Na^+$ channel antagonist tetrodotoxin (TTX) (1 μM). Moreover, TTX partially inhibited the increase in mRNA$^{ENK}$ levels caused by $K^+$ (20 mM) depolarization (from 185 to 130% of control), but had no effect on the stimulation by $Ba^{2+}$ (1 mM). The $Ca^{2+}$ channel antagonists $D_{600}$ (50 μM) verapamil (50 μM) and $Co^{2+}$ (1 mM) inhibited the responses to either $K^+$, $Ba^{2+}$ or veratridine, whereas the $Ca^{2+}$ channel agonist Bay K 8644 (0.1 μM) potentiated the effect of 20 mM $K^+$ from 185 to 230% of control. The $K^+$- induced increase in the mRNA$^{ENK}$ levels was associated with an increase of immunoreactive proenkephalin A-derived peptides in both tissue and medium, indicating an enhanced production of opioid peptides. These results suggest that membrane depolarization may play an important role in the regulation of proenkephalin A gene expression in bovine adrenal chromaffin cells. It may represent a mode by which substances acting directly on $Na^+$ or $Ca^{2+}$ channels may modulate the regulation of proenkephalin A mRNA biosynthesis and opioid peptide production.**

*Key words:* proenkephalin A/gene expression/membrane depolarization/ion channels/solution hydridization

## Introduction

A number of studies have shown that secretion of catecholamines and proenkephalin A-derived peptides from adrenal medullary cells can be increased by nicotinic receptor stimulation with acetylcholine (ACh) and depolarizing agents such as potassium ($K^+$), barium ($Ba^{2+}$) and veratridine (VT) (Livett *et al.*, 1981; Kilpatrick *et al.*, 1981; Viveros *et al.*, 1979; Ito *et al.*, 1979). Electrophysiological and biochemical studies have provided evidence that an increase in cytoplasmic calcium is a necessary step in the secretory process (Douglas *et al.*, 1967; Douglas, 1968). The entry of $Ca^{2+}$ appears mainly to occur through $Ca^{2+}$ channels that can be activated either by a steady-state depolarization or spike activity (Kidokoro and Ritchie, 1980; review of Hagiwara and Byerly, 1981; Fenwick *et al.*, 1982). In fact, an involvement of action potentials in stimulus secretion coupling has been suggested (Biales *et al.*, 1976; Brandt *et al.*, 1976).

Nicotinic stimulation has recently been reported to increase proenkephalin A gene expression in cultured bovine adrenal chromaffin cells (Eiden *et al.*, 1984). Nicotinic receptor activation, however, also leads to an increase in sodium influx through the receptor, thereby depolarizing the cells. In this study, we investigated the effects of depolarization on proenkephalin A mRNA (mRNA$^{ENK}$) levels.

We show that proenkephalin A gene expression in cultured bovine adrenal medullary cells is increased by non-specific membrane depolarizing agents such as $K^+$, $Ba^{2+}$ and VT. The possible role of both $Na^+$ and $Ca^{2+}$ channels in stimulus biosynthesis coupling is analyzed using specific $Na^+$ and $Ca^{2+}$ channel modulators.

## Results

Figures 1 and 2 show the effects of different secretagogues on mRNA$^{ENK}$ levels in cultured bovine adrenal chromaffin cells after treatment for 48 h.

The levels of mRNA$^{ENK}$ were measured by solution hybridization (Figure 1) or by filter hybridization using the Northern blot technique (Figure 2). Depolarization with 5–20 mM $K^+$ caused a dose-related increase in mRNA$^{ENK}$ up to 85% above control (Figure 1, inset). The increase in mRNA$^{ENK}$ upon $K^+$ treatment was time dependent. Only a small increase (20–30%) was observed after 24 h and mRNA$^{ENK}$ levels slowly increased further after 48 h (not shown). After 48 h of incubation, stimulated levels reached were high enough to allow accurate determination of negative and positive modulations. A particularly large increase (4-fold) in the mRNA$^{ENK}$ levels was found after treatment with VT (5 μM). Analysis of the mRNA$^{ENK}$ levels by Northern blotting revealed a single RNA species of ~1400 bases which corresponds to that previously observed with purified poly(A) mRNA (Pittius *et al.*, 1985). The increases in the mRNA$^{ENK}$ levels in response to $K^+$ and VT are of a similar magnitude to that measured by the solution hybridization technique.

The effect of VT appears to be due to its ability to activate voltage-sensitive $Na^+$ channels, as the increase in mRNA$^{ENK}$ levels was markedly reduced in the presence of the $Na^+$ channel blocker tetrodotoxin (TTX 1 μM) (Figure 1). Moreover, TTX significantly reduced the ability of $K^+$ to increase the mRNA$^{ENK}$ levels, indicating that part of the $K^+$ effect may be mediated by the activation of $Na^+$ channels. In contrast, TTX (1 μM) did not inhibit the stimulatory effect of $Ba^{2+}$ (1 mM) on the mRNA$^{ENK}$ levels.

The involvement of $Ca^{2+}$ channels in the effects of $K^+$ (20 mM), $Ba^{2+}$ (1 mM) and VT (5 μM) is shown in Figure 3. The $Ca^{2+}$ channel antagonists $D_{600}$ (50 μM), verapamil (50 μM) and $Co^{2+}$ (1 mM) markedly reduced the stimulatory effect of these agents on the mRNA$^{ENK}$ levels. The incubation time with $Co^{2+}$ was kept to 24 h because of the potential toxicity of this ion on long-term exposure. Conversely, Bay K 8644 (100 nM), a $Ca^{2+}$ channel agonist, which appears to act by prolonging the mean

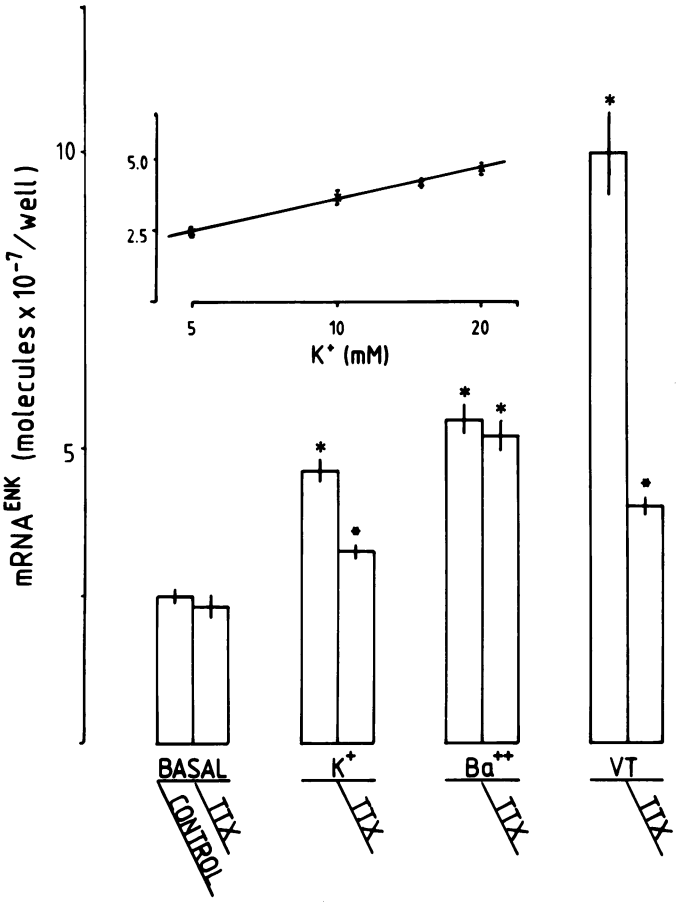


**Fig. 1.** Effects of secretagogue stimulation and its inhibition by TTX ($10^{-6}$ M) on mRNA$^{ENK}$ in primary cultures of bovine adrenal chromaffin cells, as measured by specific solution hybridization assay. Treatments were for 48 h with potassium ($K^+$, 20 mM), barium ($Ba^{2+}$, 1 mM) and veratridine (VT, $5 \times 10^{-6}$ M). The inset shows the dose-response curve to $K^+$ (5–20 mM) depolarization. Values represent means ± SEM from six individual cultures. (*) Significant value $P < 0.01$ compared with control. (★) Significant value $P < 0.01$ compared with respective stimulated condition alone (one way analysis of variance, Newman Keul's test).

opening time of voltage-sensitive $Ca^{2+}$ channels, potentiated the $K^+$-induced increase in cellular mRNA$^{ENK}$ levels.

The increase in mRNA$^{ENK}$ levels in response to 20 mM $K^+$ was associated with increased levels of immunoreactive proenkephalin A-derived peptides, both in tissue (control 8.8 ± 0.8 versus $K^+$ 25 ± 2.4 pmol/well, means ± SEM,n=8) and medium (control 0.5 ± 0.02 versus $K^+$ 2.3 ± 0.2 pmol/well, means ± SEM, $n$=8). The immunoreactive peptides were measured using a monoclonal antibody which recognizes the amino acid sequence Tyr-Gly-Gly-Phe common to all opioid active cleavage products of proenkephalin A.

Total DNA and RNA remained constant in either control or treated cells over the 2 days of incubation. mRNA$^{ENK}$ and enkephalin-like peptide content in control cells remained unchanged during 3 days of culture. Enkephalin-like peptides, however, increased in the medium indicating that a low but constant release of opioid peptides occurred (Table I). These results are in agreement with those reported by Quach *et al.* (1984) and Eiden and Hotchkiss (1983).

Assuming that the number of cells in which mRNA$^{ENK}$ was measured corresponds approximately to the number of cells plated, and that mRNA$^{ENK}$ is only present in chromaffin cells, a minimum estimate of ~50 molecules/cell is obtained.

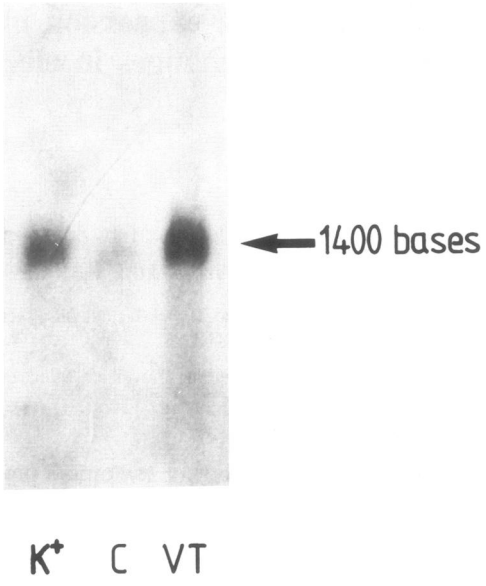


**Fig. 2.** Northern blot analysis of mRNA$^{ENK}$ in primary culture of bovine adrenal chromaffin cells. Total nucleic acids obtained from $10^6$cells were separated by electrophoresis on a 1.2% agarose gel, blotted onto nitrocellulose and hybridized with $^{32}$P-labelled sscDNA encoding proenkephalin A. VT: veratridine ($5 \times 10^{-6}$ M), C: control, $K^+$; potassium (20 mM).

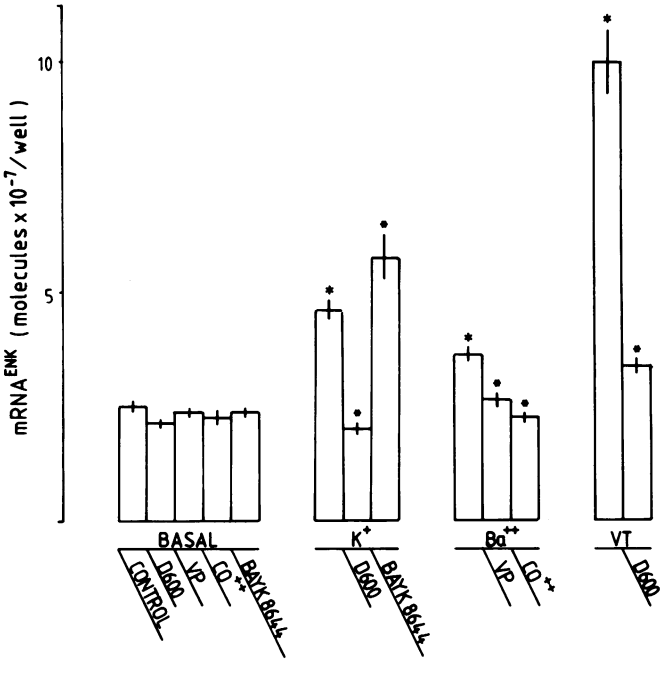


**Fig. 3.** Effects of $Ca^{2+}$ channel antagonists ($D_{600}$ $5 \times 10^{-5}$; verapamil, VP $5 \times 10^{-5}$ M and cobalt, $Co^{2+}$ 1 mM) and agonist (Bay K 8644, $10^{-7}$ M) on $K^+$, $Ba^{2+}$ and VT stimulations. Treatments with $Ba^{2+}$, verapamil and $Co^{2+}$ were for 24 h. All others for 48 h. Values represent means ± SEM from six individual cultures. (*) Significant value $P < 0.01$ compared with control. (★) Significant value $P < 0.05$ compared with respective stimulated condition alone (one way analysis of variance, Newman Keul's test).

Studies on [$^3$H]uridine incorporation into newly synthesized RNA indicate that the increase in mRNA$^{ENK}$ levels measured upon depolarization are probably not due to a general activation of gene transcription in cultured chromaffin cells. Total radioactivity incorporated was not essentially different in VT-treated cells (1694 ± 117 c.p.m. $\times 10^{-2}$) from that in control cells

**Table I.** Time course of cellular $mRNA^{ENK}$ levels and opioid immunoreactive material in tissue and medium

| Time (h) | $mRNA^{ENK}$ (molecules × $10^{-6}$/well) | ir-proenkephalin derived peptides (pmol/well) | |
|---|---|---|---|
| | | Cell content | Medium content |
| 8 | 27 | N.D. | N.D. |
| 24 | 24 | 8.4 ± 1.6 | 0.2 ± 0.02 |
| 48 | 25 | 8.8 ± 2.2 | 0.5 ± 0.06 |
| 72 | 23 | 10.0 ± 2.0 | 0.7 ± 0.05 |

Values are the mean ± SD from eight individual cultures.

(1418 ± 155 c.p.m. × $10^{-2}$). The values represent means ± SD from 4–6 individual cultures.

## Discussion

In this report, we studied proenkephalin A gene expression in cultured bovine adrenal chromaffin cells maintained in defined serum-free medium. The $mRNA^{ENK}$ cell content was quantitated using a sensitive solution hybridization assay. Basal cellular $mRNA^{ENK}$ and enkephalin-like peptide levels remained virtually constant over 3 days. Incubation of the chromaffin cells with 5–20 mM $K^+$, which produces a dose-dependent voltage change (Friedman *et al.*, 1985), caused a dose-related increase in $mRNA^{ENK}$ levels.

The $K^+$ (20 mM)-induced increase in the levels of $mRNA^{ENK}$ was accompanied by a 3-fold increase in the tissue levels and a 4- to 5-fold increase in the medium levels of proenkephalin A-derived immunoreactive peptides. For the determination of the immunoreactive opioid peptides, a monoclonal antibody was used which recognizes met- and leu-enkephalin and all larger forms of proenkephalin A-derived peptides which contain the sequence of met- or leu-enkephalin at the N terminus (Gramsch *et al.*, 1983) (e.g. peptide E, peptide F, BAM-22P, etc., see review of Höllt, 1983). Higher mol. wt forms generated during the processing of proenkephalin A occur in high amounts in the adrenal medulla (see review of Lewis and Stern, 1983). Although the antibody also recognizes opioid peptides from the proenkephalin B precursor (Gramsch *et al.*, 1983), these are present in much lower amounts in the adrenal medulla as compared with the proenkephalin A peptides. The large increase in the levels of opioid peptides in the tissue (3-fold) and in the medium (4- to 5-fold) as compared with the $mRNA^{ENK}$ levels (<2-fold) indicates that possibly depolarizing stimuli do not only result in changes in $mRNA^{ENK}$ levels but may also affect the processing of the proenkephalin A precursor.

A particularly pronounced increase in $mRNA^{ENK}$ concentrations (4-fold) was observed after treatment with VT (5 μM), a drug that acts by activating and opening of voltage-sensitive $Na^+$ channels, thereby depolarizing the cells. Although we cannot completely exclude that VT may also modulate the activity of other ion channels (Romey and Lazdunski, 1982), the low concentrations used and the observation that the specific $Na^+$ channel blocker TTX (1 μM) almost completely prevented its stimulatory effect, indicates that VT enhanced $mRNA^{ENK}$ levels by activation of voltage-sensitive $Na^+$ channels. The increase in $mRNA^{ENK}$ levels induced by $K^+$ (20 mM) was also inhibited by TTX, indicating that at least part of the effect of $K^+$ is due to activation of voltage-dependent $Na^+$ channels.

The increase in $mRNA^{ENK}$ observed with $K^+$ or VT treatment was markedly reduced in the presence of the $Ca^{2+}$ channel antagonist $D_{600}$ and potentiated by the $Ca^{2+}$ channel agonist Bay K 8644. Similarly, the stimulatory effect of $Ba^{2+}$ was completely abolished by the $Ca^{2+}$ channel blocker $Co^{2+}$ and almost abolished by the $Ca^{2+}$ channel antagonist verapamil. This suggests that stimulation of proenkephalin A gene expression by Ba ions and other depolarizing agents is mediated by $Ca^{2+}$ channels. It is currently thought that $Ba^{2+}$ may act by mimicking an increase in $Ca^{2+}$ permeability through $Ca^{2+}$ channels and/or by selectively depressing a voltage-dependent $K^+$ current, termed the 'M' current, which may lead to an enhanced electrical activity and action potential generation (for discussion see Brown *et al.*, 1981; Douglas *et al.*, 1983). However, the $Ba^{2+}$-induced increase in $mRNA^{ENK}$ levels was TTX resistant and it appears that $Ca^{2+}$ entry alone could mediate the effect.

These observations suggest that $Ca^{2+}$ is involved in the induction of proenkephalin A gene expression in adrenal chromaffin cells upon depolarization. This is in agreement with Eiden *et al.* (1984) who reported that cAMP and $Ca^{2+}$ may interact in the regulation of $mRNA^{ENK}$ levels upon nicotinic stimulation. It is not yet clear whether $Ca^{2+}$ modulates the proenkephalin gene expression by altering the cAMP levels or whether these two messenger systems interact at another level.

In conclusion, we have shown that proenkephalin A gene expression is not only modulated by nicotinic receptor activation (Eiden *et al.*, 1984), but is also regulated by membrane depolarization.

In rats, however, adrenal medullary enkephalins appear to be regulated differently at the mRNA and peptide level and evidence for possible species difference has already been proposed by Fleminger *et al.* (1984). Unilateral surgical dissection of the splanchnic nerve and chlorisondamine treatment result in an elevated enkephalin content (Fleminger *et al.*, 1984; LaGamma *et al.*, 1984) as well as $mRNA^{ENK}$ content (Kilpatrick *et al.*, 1984). Similarly, a 50-fold increase in enkephalin levels was observed in medullae grown as explant cultures, and KCl (55 mM) and VT ($10^{-5}$ M) reduced enkephalin content and $mRNA^{ENK}$ after 2–3 days in culture (LaGamma *et al.*, 1984, 1985). A similar regulation of substance P in sympathetic neurones by impulse activity and post-synaptic sodium influx has been reported (Kessler *et al.*, 1981; Kessler and Black, 1982). Thus, it remains to be investigated whether these differences reside in the different action of nicotine in rat and cows or whether other factors are present in the adrenal medulla which *in vivo* inhibit enkephalins to a larger extent in rats than in cows. One such candidate could be γ-aminobutyric acid (Kataoka *et al.*, 1984), but its function in rat adrenals has not yet been investigated.

## Materials and methods

### *Cell culture preparation*

Cells were prepared according to the general method of Livett (1984) as follows. Adrenal glands were obtained at a local slaughterhouse, retrogradely injected with phosphate-buffered saline (PBS) containing 10 mM Hepes, pH 7.4, penicillin (100 U/ml), heparin (7 U/ml), amphotericin B (25 μg/ml), streptomycin (10 μg/ml) and delivered on ice to the laboratory within 30 min. To remove red blood cells, the glands were retrogradely perfused with the above solution for 30 min at 0.5 ml/min. Then they were retrogradely perfused for 60 min with a 0.05% collagenase solution dissolved in the above medium lacking heparin (solution A) and kept at 37°C. The medullae were dissected from the surrounding cortex and minced with forceps and scalpel. The minced tissue was washed twice in 1 × PBS and incubated for 60 min in dispersion medium [DNase 125 μg/ml, Dispase 50 μg/ml, bovine serum albumin (BSA) 10 mg/ml, dissolved in solution A] at 37°C in a shaking water bath. The supernatant was recovered and the remaining tissue subjected once more to digestion for 20–30 min. The two supernatants were centrifuged, cells resuspended in 1 × PBS and passed through a 80 μm nylon mesh followed by a 40 μm nylon mesh. After centrifugation the

cells were resuspended in 1% BSA dissolved in 1 × PBS and run over a 5% BSA gradient. The pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) 7.5 mM Hepes pH 7.4 containing 10% fetal calf serum, glutamine (300 μg/ml), fluorodeoxyuridine (10 μM), antibiotics at the same concentration as above, and passed once more through a 40 μm nylon mesh. Cells were counted, viability checked by trypan-blue exclusion (90% viability) and plated on collagen-coated 24-Costar multi-well dishes at a density of $5 \times 10^5$ cells/well. Cells were allowed to attach for 48 h at 37°C under 95% $O_2$/5% $CO_2$, when medium was changed to DMEM supplemented with glutamine (0.2 mg/ml), kanamycin (50 μg/ml) and fluorodeoxyuridine (10 μM). In the experiments where $Ba^{2+}$ was used, DMEM with 7.5 mM Hepes pH 7.4 lacking phosphate and sulfate salts and bicarbonate was used (NaCl 150 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, glucose 1 g/l). Drugs were added and cells cultured for a further 24–48 h.

*Isolation of total nucleic acids (TNA)*

Cells were lysed in 10 mM Hepes (pH 7.4), 1 mM EDTA, 0.5% SDS containing proteinase K (100 μg/ml) at 37°C for 60 min followed by extraction with one volume of phenol/chloroform:isoamylalcohol (1/24:1) and re-extraction with one volume of chloroform at 4°C. Total nucleic acids were precipitated by 2.2 volumes of ethanol, 1/10 volume 3 M sodium acetate (pH 5.2) and recovered by centrifugation at 12 000 r.p.m. 4°C, for 20 min. The pellet was washed once in 70% ethanol, dried in a speed-vac rotor and dissolved in sterile distilled water. Total nucleic acids were quantified by spotting an aliquot of the sample (2 μl) onto a 2% agarose plate containing ethidium bromide (0.5 μg/ml). The quantification was performed by densitometric scanning of a photographic negative of the plate and sonicated salmon sperm DNA served as a standard (Chen *et al.*, 1983). Optical absorbance measurements at $OD_{260}$ (1 $OD_{260}$ = 40 μg TNA) were also performed.

*Solution hybridization*

Preparation of the single-stranded complementary DNA (sscDNA) coding for proenkephalin A used in the hybridization experiments was as previously described (Pittius *et al.*, 1985). Solution hybridization of the sscDNA to mRNA in excess was performed in 40% formamide, 0.6 M NaCl, 20 mM Tris (pH 7.5), 4 mM EDTA, according to Durnam and Palmiter (1983). The reactions were carried out at 48–50°C. This is in the mid-range of the temperature-dependent hybridization kinetic curve for $mRNA^{ENK}$ (N.Kley, unpublished observation). The extent of hybridization was determined by S1 nuclease digestion as previously described (Pittius *et al.*, 1985).

*Blot analysis*

Total nucleic acids were denatured with glyoxal (Thomas, 1980) run on a 1.2% agarose gel and transferrred to nitrocellulose sheets. After baking the nitrocellulose sheets for 2 h at 80°C, they were pre-hybridized overnight, according to Wahl *et al.* (1979). After hybridization to the $^{32}P$-labeled sscDNA in the same buffer containing 10% dextran sulfate at 45°C, the sheets were washed four times in 2 × SSC (1 × SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 5 min, and twice in 0.1% SSC containing 0.1% SDS at 50°C for 30 min. Dried filters were autoradiographed at −70°C with intensifying screens.

*Total RNA synthesis*

Following incubation of the cells with [$^3$H]uridine at 20 μCi/ml for the final 15 h of incubation (total incubation time was 48 h), cells were washed twice in PBS and lysed in 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 10 mM Tris pH 7.5. Nucleic acids were precipitated in 10% trichloroacetic acid on ice for 30 min and recovered by centrifugation. The precipitate was dissolved in perchloric acid and nucleic acids hydrolysed by heating to 90°C for 15 min. The sample was allowed to cool and counted by liquid scintillation counting.

The blank was obtained by adding [$^3$H]uridine a few minutes before lysing the cells.

*Mixed opioid peptide radioimmunoassay (RIA)*

Solid phase RIA using the 3-E7 monoclonal antibody to β-endrophin (kind gift of Dr Gramsch), and β-endorphin for the construction of a standard curve, was as described by Schulz and Gramsch (1985). Characteristics of the 3-E7 monoclonal antibody to β-endorphin, recognizing antigens that contain the amino acid sequence of either met- or leu-enkephalin at their N terminus, have been described by Gramsch *et al.* (1983).

*Materials used*

Enzymes, ultra-pure phenol, SDS, DMEM and fetal calf serum were purchased from Gibco-BRL (Karlsruhe, FRG), suprapure agarose from Pharmacia (Freiburg, FRG), nitrocellulose papers from Schleicher and Shuell (Dassel, FRG), [α-$^{32}P$] dATP from Amersham (Braunschweig, FRG). Collagenase, BSA and DNase were from Sigma (Taufkirchen, FRG), Dispase, Grade I, was from Boehringer (Mannheim, FRG). All other chemicals were from Sigma (Taufkirchen, FRG) or Merck (Darmstadt, FRG). All solutions and glassware were autoclaved before use when working with RNA.

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