Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5351–5355, June 1995
Neurobiology

Purification and identification of pituitary cytotropic factor

(adenosine/dopamine)

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Communicated by S. M. McCann, University of Texas Southwestern Medical Center, Dallas, TX, February 22, 1995 (received for review December 15, 1994)

ABSTRACT **It has been shown that the pituitary contains a cytotropic factor (CTF) that stimulates the secretion of catecholamines by dopaminergic neurons of the hypothalamus. In the present study, CTF was purified from rat pituitaries and found by means of mass spectrometric analysis to be adenosine. This finding was corroborated by the observations that CTF behaves identically to adenosine when subjected to liquid chromatography, is inactivated and converted to inosine by adenosine deaminase, and is qualitatively and quantitatively indistinguishable from adenosine in its biological activity. It is concluded that pituitary adenosine is a trophic factor for hypothalamic dopaminergic neurons.**

A reduction in the capacity of hypothalamic neurons to secrete dopamine is associated with aging. However, this aging-dependent decrease in function is not irrevocable because treatment with progesterone and estradiol increases dopamine synthesis and release (1, 2). The mechanism by which this treatment stimulates dopamine secretion was believed first to be the result of direct action of these hormones on the dopaminergic neurons because, relative to other brain cells, both steroids are preferentially concentrated in the nuclei of these cells (3, 4). Progesterone and estradiol were later found to be ineffective in hypophysectomized animals, leading to the hypothesis that an agent of pituitary origin was involved in the activation of these neurons (5). This latter hypothesis was supported by the finding that pituitary grafts within the brain stimulated dopamine secretion (5).

Other evidence for this view was provided by the observation that fetal hypothalamic cells increased their secretion of dopamine when cultured in the presence of an extract of the pituitary (6). The extent of the stimulation was dependent on the concentration of extract in the culture medium and to the duration of the incubation (7). The active agent in the extract was called cytotropic factor (CTF). In the extended presence of CTF, the quantity of tyrosine hydroxylase and its mRNA in the cultures also increased. These findings led to the present study, which had as its aim the isolation and identification of pituitary CTF.

MATERIALS AND METHODS

Bioassay of CTF. The biological activity of CTF was assayed by using PC12 cells, where catecholamine secretion served as the responsive variable. PC12 cells, a catecholamine-secreting cell line derived from a rat adrenal pheochromocytoma (8), were incubated at 10^5 cells per well in 48-well culture plates precoated with poly(D-lysine). The cells were cultured in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum and 10% heat-activated horse serum. Incubations with CTF were conducted at 37°C for 3 hr. At the end of the incubation, the medium was acidified, and dihydroxyphenylalanine (dopa), dopamine, and norepinephrine were quantified by liquid chromatography with electrochemical detection (9).

Purification of CTF. Frozen pituitaries of young rats from Pel-Freez Biologicals served as the source of CTF. The glands were homogenized in cold 0.1 M acetic acid (20 ml per 100 glands) with a Brinkmann Polytron. The homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was harvested and evaporated to dryness at room temperature by centrifugation under reduced pressure. The residue, equivalent to 100 pituitaries, was dissolved in 0.1 M acetic acid and subjected to gel chromatography on a Sephadex G-10 (Pharmacia) column (2.6 cm × 48 cm).

The fractions of the eluate containing partially purified CTF were dried by centrifugation under reduced pressure. The residue was dissolved in 1 μM acetic acid and diluted with 49 volumes of acetonitrile. The solution, equivalent to 50 pituitaries, was chromatographed on a Polyhydroxyethyl aspartamide column (4.6 mm × 200 mm) from Poly LC (Columbia, MD) equilibrated with a mixture of acetonitrile and 1 μM acetic acid (98:2, vol/vol). A curvilinear gradient of acetonitrile and 50 μM acetic acid (98:2, vol/vol) was used for elution from the aspartamide column. The effluent was monitored by UV absorption at 254 nm.

The chromatographic fraction containing CTF was dried. The residue, equivalent to 100 pituitaries, was dissolved in water and chromatographed on an IB-SIL 5C1 column (4.6 mm × 250 mm) from Phenomenex (Torrance, CA) that had been equilibrated with an acetonitrile/0.1 M acetic acid mixture (98:2, vol/vol). The column was developed with a linear gradient of water.

The fractions containing CTF were dried. The residue, equivalent to 300 pituitaries, was dissolved in 0.1 M acetic acid and diluted with 49 volumes of acetonitrile. Finally, CTF was chromatographed on a C_{18} μBondapak column (3.9 mm × 300 mm) from Waters that had been equilibrated with acetonitrile/0.1 M acetic acid (98:2, vol/vol). Fractions were eluted from the μBondapak column with a linear 0.1 M acetic acid/acetonitrile gradient, collected, and dried.

Electrospray Mass Spectrometry. Fifty microliters of 0.01 M acetic acid was added to selected, chromatographically separated fraction residues and vortexed. For electrospray ionization/Fourier-transform mass spectrometry (ESI/FTMS) analyses, sample solution aliquots (≈10 μM) were diluted 1:1 with methanol and infused at a flow rate of 0.75 μl/min. Analyses were performed using an ESI source (Analytica, Branford, CT) interfaced to a 3-T FTMS-2000 (Extrel-FTMS, Madison, WI) with electrostatic ion transfer optics (10). An isolation-gate valve was operated under computer control to allow ion accumulation in the trapped ion cell at 1.0×10^{-7} torr (1 torr = 133 Pa), after which the valve was closed and pressure was reduced to 0.4×10^{-8} torr in 5 sec for ion

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Abbreviations: CTF, cytotropic factor; ESI/FTMS, electrospray ionization/Fourier-transform mass spectrometry; TMS, trimethylsilyl.

excitation/detection. For accurate mass determinations, arginine and gramicidin S were added as internal calibrants, and 10 replicate spectra were recorded. Spectra were acquired in direct mode with a mass resolution of ≈30,000 at m/z 290 and represent the coaddition of four accumulations of 30 sec each. Data were baseline corrected, augmented with an equal number of zeros, and Fourier transformed without apodization.

Gas Chromatography–Mass Spectrometry. For GC–MS analysis, 20 μl of each acetic acid solution was transferred to a glass injection vial, freeze dried, and incubated with 15 μl of bistrimethylsilylfluoroacetamide (Pierce)/acetonitrile (1:1, vol/vol) at 65°C for 20 min to form trimethylsilyl (TMS) derivatives. Mass spectrometric analyses were performed on a gas chromatograph–quadrupole mass spectrometer (models 5890 and 5989; Hewlett–Packard). A 15-m DB-5 capillary column (J & W Scientific, Folsom, CA) was temperature programmed from 100°C to 320°C in 11 min after the initial temperature was held for 1 min. Mass spectra were obtained from m/z 70 to m/z 560.

RESULTS

When an acetic acid extract of rat pituitaries was subjected to gel chromatography on a Sephadex G-10 column, CTF was recovered near the bed volume (Fig. 1). When CTF-containing material from the Sephadex column was further purified by liquid chromatography on an aspartamide column, CTF was recovered in a compound peak having a retention time of 20 min (Fig. 2*A*). When partially purified CTF from the aspartamide column was subjected to liquid chromatography on an IB-SIL column, CTF was recovered in a symmetrical peak having a retention time of ≈14 min (Fig. 2*B*). When CTF from the IB-SIL column was chromatographed on a C_{18} μBondapak column, CTF was recovered in a symmetrical peak having a retention time of 12.5 min (Fig. 2*C*). Approximately 1.5 μg of highly purified CTF was obtained from 1000 pituitaries.

Dried aliquots of chromatographic fractions from the IB-SIL column were analyzed first by ESI/FTMS. Based upon accurate mass measurements, initial preliminary data permitted a tentative assignment of adenosine to the CTF activity. Subsequently, more highly purified samples from the μBondapak column were processed to confirm this assignment. Samples included (*i*) CTF active material (equivalent to 1 μg), (*ii*) an inactive procedural blank collected at the same elution time as CTF, and (*iii*) authentic adenosine collected when 1 μg of adenosine was eluted from the μBondapak column. Fig. 3 compares portions of spectra obtained from an inactive fraction (*Upper*) and a CTF active fraction (*Lower*). Accurately measured ions characteristic of active CTF are given in Table 1.

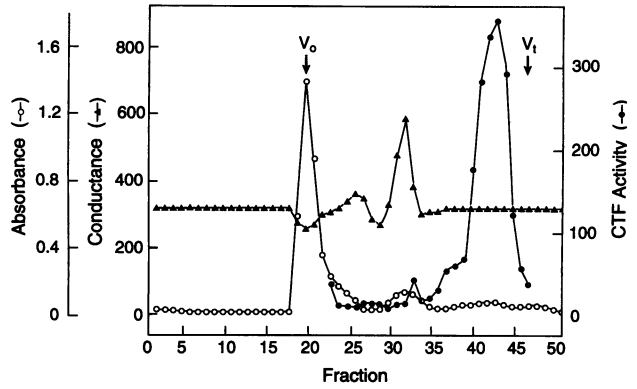

FIG. 1. Gel chromatography of acetic acid extract of rat pituitaries. The extract, equivalent to 100 glands, was chromatographed on a Sephadex G-10 column developed with 0.1 M acetic acid. The effluent was collected in 5-ml fractions. Absorbance was measured at 280 nm; conductivity is expressed in μS. CTF activity is expressed as pmol of catecholamines secreted by PC12 cells during a 3-hr incubation at 37°C. V_o and V_t denote void volume and bed volume, respectively.

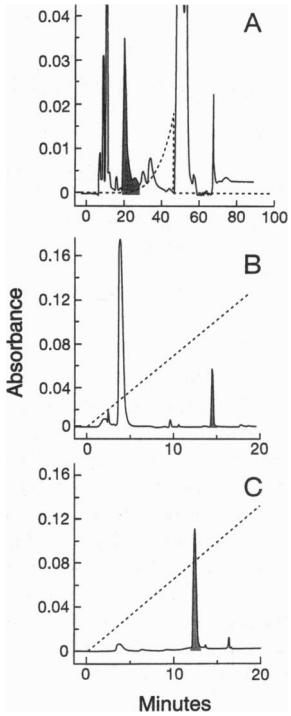

FIG. 2. Liquid chromatography of CTF. (*A*) Polyhydroxyethyl aspartamide column chromatography of CTF partially purified on a Sephadex G-10 column. The aspartamide column (particle diameter, 5 μm; pore size, 300 Å) was developed with a curvilinear gradient (broken line) using a flow of 0.6 ml/min. CTF equivalent to 50 pituitaries was injected in 500 μl. The abscissa denotes time of development. The eluate was monitored by UV spectroscopy at 254 nm; absorbance units full scale (AUFS) = 0.05. CTF was recovered in the shaded fraction. (*B*) IB-SIL 5C1 column chromatography (particle diameter, 5 μm; pore size, 125 Å) of CTF purified on an aspartamide column was developed with a linear gradient (broken line) at a flow of 1 ml/min. CTF equivalent to 100 pituitaries was injected in 500 μl; AUFS = 0.2. CTF was recovered in the shaded fraction. (*C*) C_{18} μBondapak column chromatography (particle diameter, 10 μm; pore size, 125 Å) of CTF purified on an IB-SIL column was developed with a linear gradient (broken line) at a flow of 1 ml/min. CTF equivalent to 300 pituitaries was injected in 500 μl; AUFS = 0.2. CTF was recovered in the shaded fraction.

After verification of the accurate mass measurements of CTF and adenosine, samples of CTF and adenosine were compared by GC–MS of their TMS derivatives. Partial total ion current chromatograms are shown in Fig. 4, indicating the coelution of trimethysilylated CTF and adenosine, and the absence of a similar peak in the inactive fraction. Mass spectra corresponding to adenosine-tetraTMS and CTF-TMS are shown in Fig. 5 and indicate identical fragmentation patterns.

Further corroboration of the identity of CTF and adenosine was provided by digestion with adenosine deaminase. When highly purified CTF or adenosine was digested with the enzyme for 20 min and then subjected to liquid chromatography on an IB-SIL column, the peak having a retention time of 13.8 min corresponding to CTF or adenosine was abolished, and a new peak having a retention time of 3.9 min corresponding to inosine was generated (Fig. 6). When CTF or adenosine was incubated with heat-inactivated adenosine deaminase, no effect on either was seen as judged by its elution pattern on an IB-SIL column (data not shown). When highly purified CTF and adenosine were cochromatographed on an IB-SIL column, a single symmetrical peak was recovered that had an elution time corresponding to that of purified CTF or adenosine.

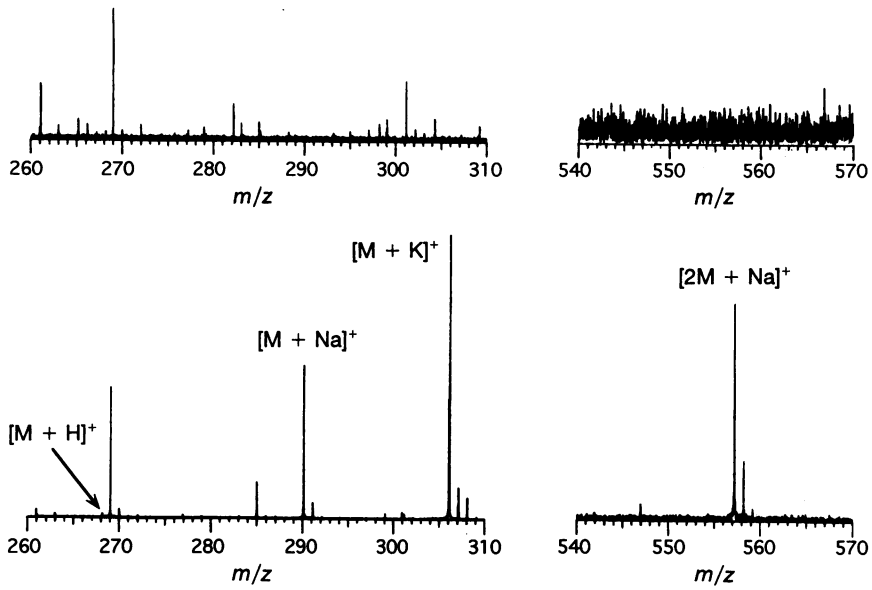

FIG. 3. ESI/FTMS spectra of infused solutions from procedural blank (*Upper*), collected at the same elution time as active CTF, and active CTF (*Lower*). Spectral regions are plotted on the same absolute abundance scale and display regions where there are significant differences between CTF and the procedural blank.

When crude pituitary extract was digested with adenosine deaminase, most of the CTF activity was abolished as judged by the secretion of catecholamines (Table 2). Heat-inactivated adenosine deaminase had no effect on the CTF activity. Similarly, treatment of adenosine with adenosine deaminase, but not heat-inactivated adenosine deaminase, greatly reduced its stimulatory effect on catecholamine secretion. When the biological activities of purified CTF and adenosine were compared, no difference in their qualitative and quantitative activities was discernible (Fig. 7).

DISCUSSION

CTF mass spectrometric analyses proceeded from the observation that CTF was a nonpeptidic, polar, acetic acid-stable product of endogenous metabolic processes. An indication of the identity of CTF as adenosine came first from the high-resolution mass measurement of ions in the ESI spectra that were consistent with CTF being a compound with a molecular weight of 267 and producing ions measured at $[267.096529 + H]^+$, $[267.095949 + Na]^+$, $[267.096083 + K]^+$, and $[2(267.096726) + Na]^+$. Based on these measured masses, the most likely elemental composition was determined to be $C_{10}H_{13}N_5O_4$. This composition matches that of adenosine with a molecular weight of 267.096754, suggesting it as likely among those candidate compounds with roles in mammalian biochemistry. Adenosine was confirmed as being present in active CTF by comparing total ion chromatograms and mass spectra from GC–MS analyses of trimethylsilylated derivatives of authentic adenosine and CTF. Peaks were coeluted at a retention time of 9.54 min for active CTF-TMS and adenosine-tetraTMS, but there was no corresponding signal in an inactive fraction. Electron ionization mass spectra of CTF-TMS and adenosine-tetraTMS eluted at 9.54 min were identical, providing definitive evidence that adenosine was present in the active CTF extract.

When highly purified CTF was incubated for 20 min with adenosine deaminase and then subjected to liquid chromatog-

Table 1. Mass of ions characteristic of CTF

Proposed composition	Measured mass, Da (mean ± SD)	Error (based on adenosine), Da
$[M + H]^+$	268.104354 ± 0.000448	−0.000322
$[M + Na]^+$	290.085717 ± 0.000177	0.000258
$[M + K]^+$	306.059790 ± 0.000162	0.000125
$[2M + Na]^+$	557.183220 ± 0.001307	−0.000490

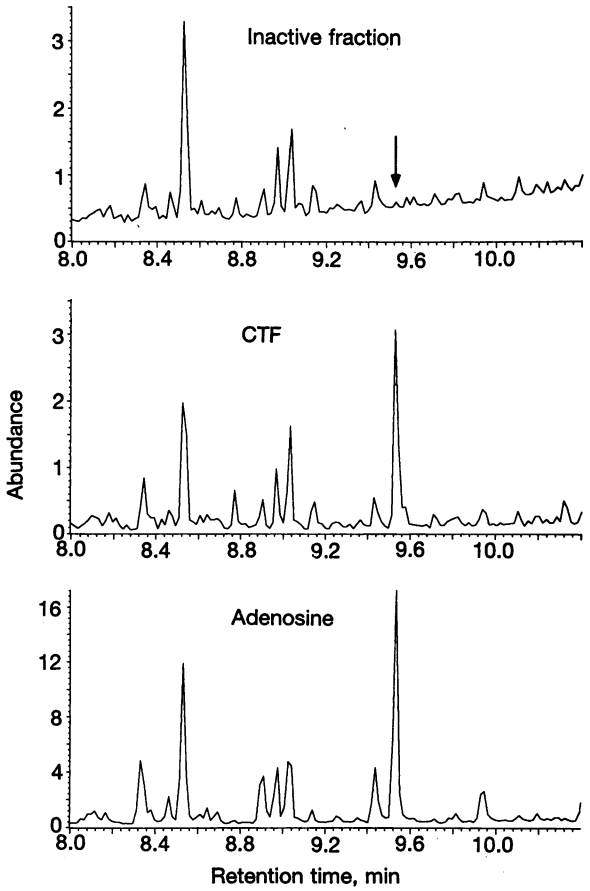

FIG. 4. Total ion current chromatograms of trimethylsilylated samples analyzed by GC–MS. The peak eluted at 9.54 min is characteristic of adenosine-tetraTMS (*Bottom*), and a coeluted peak is observed in CTF-TMS (*Middle*), but not in the procedural blank (*Top*). Abundance denotes arbitrary units of ion current × 10^{-4}.

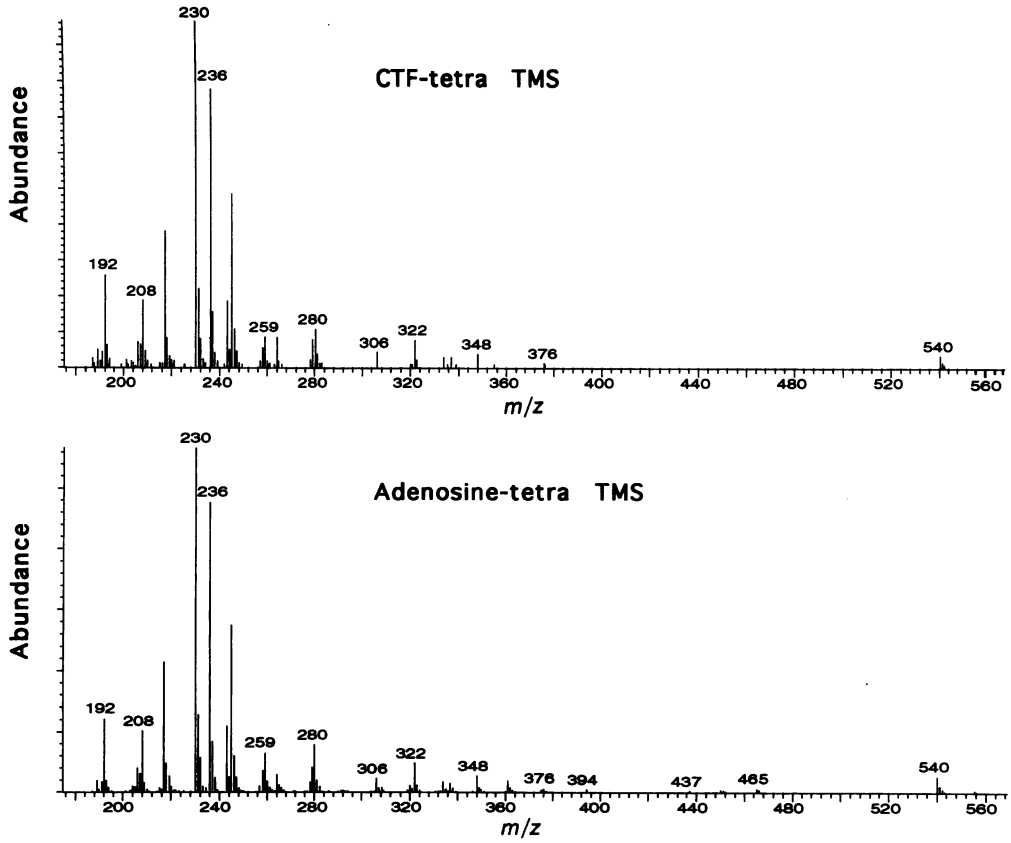

FIG. 5. Electron ionization mass spectra from the peak eluted at 9.54 min for adenosine-tetraTMS (*Lower*) and CTF-TMS (*Upper*), confirming their identity.

raphy on an IB-SIL column, no substance was recovered having a retention time corresponding to CTF. However, a substance having a retention time corresponding to inosine was recovered. When CTF was incubated with heat-inactivated adenosine deaminase and then subjected to liquid chromatography on an IB-SIL column, there was no effect on CTF. When adenosine was treated with adenosine deaminase or heat-inactivated adenosine deaminase, the results were identical to those seen with CTF.

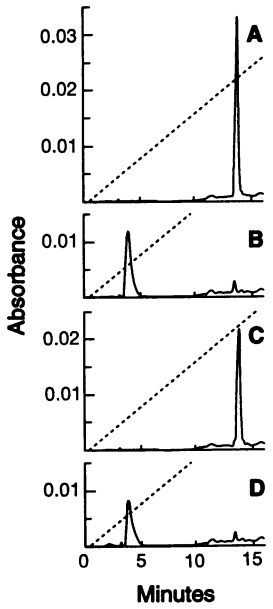

FIG. 6. Inosine formation from adenosine or CTF treated with adenosine deaminase. Liquid chromatography was performed with an IB-SIL LC column (see Fig. 2 for details). (*A* and *C*) Chromatograms of adenosine (*A*) and CTF (*C*) prior to adenosine deaminase digestion. The peak retention times of adenosine and CTF were 13.8 min. (*B* and *D*) Chromatograms showing the disappearance of adenosine and CTF, respectively, and the appearance of inosine after digestion with adenosine deaminase. The peak retention time of inosine is 3.9 min.

In addition to the identity of their physical properties, the biological activities of purified CTF and adenosine were indistinguishable. When evaluated by their stimulation of catecholamine secretion by PC12 cells, CTF and adenosine induced qualitatively and quantitatively similar responses.

The extent to which CTF activity in pituitary extract was attributable to adenosine is deserving of consideration. When crude pituitary extract was digested with adenosine deaminase,

Table 2. Effect of adenosine deaminase digestion on the activity of crude pituitary CTF or adenosine

	Secretion, pmol/3 hr		
Stimulatory agent	Dopa	DA	NE
Solvent control	14 ± 0.3	47 ± 2	23 ± 0.3
CTF			
Untreated	52 ± 4	242 ± 20	90 ± 5
ADase-treated	27 ± 1	77 ± 4	40 ± 1
HIADase-treated	54 ± 1	236 ± 4	90 ± 1
Adenosine			
Untreated	38 ± 0.7	161 ± 4	54 ± 1
ADase-treated	11 ± 0.6	28 ± 3	14 ± 1
HIADase-treated	33 ± 2	138 ± 8	46 ± 2

PC12 cells were stimulated with CTF (equivalent to 1 pituitary gland per ml) or adenosine (1 μM) that had not been treated or had been digested with adenosine deaminase (ADase) or heat-inactivated ADase (HIADase). Secreted dopa, dopamine (DA), and norepinephrine (NE) were measured. Values are means ± SEM, $n = 4$.

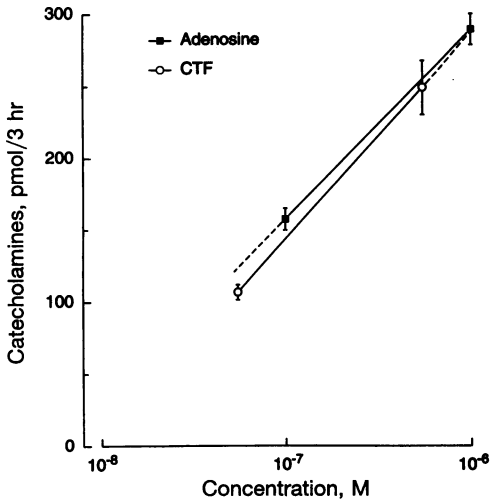

FIG. 7. Comparison of the qualitative and quantitative biological activities of CTF (○) and adenosine (■). Authentic adenosine served as the reference; CTF was quantified by its absorbance at 254 nm, with adenosine as the standard. The data are presented as means ± SEM, $n = 3$.

the CTF activity of the extract was markedly reduced. This finding supports the conclusion that the CTF activity of pituitary extract is attributable to adenosine.

Coincubation of hypothalamic cells with anterior pituitary cells or of PC12 cells with cloned rat pituitary tumor cells that contain CTF results in an increase in the secretion of catecholamines (unpublished observations). Such findings support the view that adenosine secreted by anterior pituitary cells has a role as a trophic agent in the modification of function of brain neurons. It has long been recognized that hypothalamic hormones regulate pituitary function; however, there is less evidence that a pituitary hormone regulates brain neurons. The present findings demonstrate that adenosine from pituitary cells has such a role.

It is interesting to speculate that reduction in dopamine secretion by the aged brain may be a consequence of insufficient adenosine stimulation of dopaminergic neurons. The observation that anterior pituitary grafts in the brain of old rats significantly prolong their lifespan supports a role for trophic stimulation by a pituitary hormone of brain cells (11). We suggest that adenosine may have such a function. The pathway by which pituitary CTF reaches the hypothalamic dopaminergic neurons is not established. However, rapid transport by retrograde blood flow in the pituitary stalk seems a likely route (12).

We thank Jodie Roberts and Sharyn Monroe for excellent technical assistance. The editorial assistance of Helen Wirkler is gratefully acknowledged. This work was supported by Grant 5 R37 AGO4344 from the National Institute on Aging (Bethesda, MD).

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