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# Differential Potencies of Cocaine and Its Metabolites, Cocaethylene and Benzoylecgonine, in Suppressing the Functional Expression of Somatostatin and Neuropeptide Y Producing Neurons in Cultures of Fetal Cortical Cells

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**ABSTRACT.** Using aggregate cultures derived from 17-day-old fetal rat cortex, we addressed the question: Does cocaine alter the functional expression of neuropeptide Y (NPY) and somatostatin (SRIF) neurons and, if so, are cocaethylene (CE) and benzoylecgonine (BZE) as active as cocaine? NPY/SRIF production in response to brain-derived neurotrophic factor (BDNF) or phorbol-12-myristate-13-acetate (PMA) was used as a functional criterion. A 5-day exposure to cocaine did not affect basal or stimulated (BDNF or PMA) production of NPY but it markedly suppressed BDNF- or PMA-stimulated production of SRIF. Exposure to CE led to a drastic suppression of basal as well as stimulated (BDNF or PMA) production of both NPY and SRIF. These effects of cocaine and CE were concentration dependent (1–100 μM). BZE did not alter any of these functional parameters. Next, we evaluated the fate of cocaine, CE, and BZE in the culture medium. Cocaine was converted to BZE, whereas BZE was not converted to cocaine. CE was converted to cocaine and BZE, with substantial amounts of cocaine and CE remaining in the medium after 72 hr (≈20% each). In summary, cocaine, CE, and BZE exhibited differential potencies in suppressing the expression of cultured NPY and SRIF neurons: CE was more potent than cocaine and BZE was inactive. SRIF neurons were more susceptible than NPY neurons to the effects of cocaine. The higher potency of CE may be due to a property of the compound and/or to CE serving as a source for a slow, continuous formation of cocaine by the brain cells themselves. BIOCHEM PHARMACOL **54**;4: 491–500, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** cocaine; cocaethylene; neuropeptide Y; somatostatin; brain culture; fetus

NPY‡ is one of the most abundant peptides in the brain [1–3]. The high abundance of NPY in the perinatal and adult brain, the co-expression of NPY and biogenic amines [4–6] and peptides [7, 8], and the close apposition of NPY axons to catecholaminergic [9, 10] and peptidergic [11–13] neurons are suggestive of a wide range of NPY actions in the brain. The observation that changes in the expression of NPY in the brain correlate with several behavioral manifestations of cocaine implicates NPY in the neuropathology of cocaine abuse. Two examples are provided: (1) *Major depression*. The end of a cocaine binge is associated with an episode of major depression [14]. NPY levels in the cerebrospinal fluid and in the cortex (but not in other brain regions) are reduced markedly in brains of people expressing major depression [15, 16] and in an experimental rat model for major depression [17]. Long-term cocaine induces a reduction in NPY gene expression in several regions of the rat cortex [18]; and (2) *Seizures*. Cocaine induces seizures in first-time and in chronic cocaine abusers (for references, see [19]). Experimental regimens mimicking epileptic seizures in the human lead to a dramatic increase in NPY and its mRNA in hippocampal/cortical neurons in the rat [20–22]. Cocaine-induced seizures in the rat are associated with changes in the expression of hippocampal NPY neurons [19].

In several brain regions of the adult rat, particularly the cortex and hippocampus, most of the NPY neurons co-express SRIF, whereas not all SRIF neurons co-express NPY [7, 8, 23]. NPY [24] and SRIF [25] neurons are generated in the rat cortex on days 14–20 of fetal life. It is quite possible that cocaine exerts differential effects on the developing and mature peptidergic neurons. Supporting this possibility are the findings that the binding sites for cocaine in the fetal and adult brain differ [26]. Cocaine administration to

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‡ *Abbreviations*: BDNF, brain-derived neurotrophic factor; BZE, benzoylecgonine; CE, cocaethylene; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; NPY, neuropeptide Y; PMA, phorbol-12-myristate-13-acetate; and SRIF, somatostatin.

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pregnant and lactating rats leads to an increase in SRIF content in the cortex and a decrease in the hippocampus of newborn and 15-day-old neonatal rats [27]. On the other hand, cocaine administration to adult rats does not affect SRIF content in the brain [28], but it does lead to a reduction in NPY and, to a lesser extent, SRIF content in several cortical regions [18].

It is well documented that cocaine abuse is associated with a wide range of physiological abnormalities. Moreover, cocaine is metabolized rapidly in the periphery to several compounds (for references, see [29]), some of which may be as active as or even more active than cocaine within the brain. Thus, it is still questionable if the reported effects of *in vivo* cocaine administration on NPY/SRIF neurons are due to central and/or peripheral effects of cocaine and if cocaine as such is the active component within the brain. In this study, we addressed these questions: (i) Does cocaine, acting directly on brain cells in culture, alter (suppress/enhance) the functional expression of the developing NPY neuron? (ii) If so, does cocaine exert differential effects on the cultured NPY and SRIF neurons? (iii) What is the potency of cocaine and two major naturally occurring metabolites, i.e. CE and BZE?

An aggregate culture system that can serve as a model to study the developmental expression of the fetal NPY neuron has been established in our laboratory. In this system, the NPY neurons behave similarly to perinatal *in situ* NPY neurons in that progressively with time, the NPY neurons undergo morphological [30] and functional differentiation, i.e. increase in NPY production and processing of proNPY to NPY [31, 32]. Moreover, NPY production is a regulated process: it is induced by BDNF [33] and by agents activating the cyclicAMP and protein kinase C signalling pathways (forskolin and PMA, respectively) [34, 35]. Using this culture system, we compared the effects of cocaine on the functional expression of NPY and SRIF neurons, using NPY/SRIF production in response to BDNF or PMA as functional criteria. We evaluated the kinetic parameters of cocaine action and the role of cocaine metabolites, specifically, BZE, the product of cocaine metabolism in our culture system, and CE, a unique potent metabolite of cocaine produced in people consuming cocaine and alcohol simultaneously [36].

## MATERIALS AND METHODS

### *Cell Culture*

REAGENTS. DMEM (Cat. No. 380-2430) and other tissue culture reagents were from Life Technologies (Grand Island, NY). DNase I was from Boehringer (Indianapolis, IN); ITS+ (content/L: 6.25 mg insulin, 6.25 mg transferrin, 6.25 μg selenous acid, 1.25 g BSA, and 5.35 mg linoleic acid) was from Collaborative Research (Bedford, MA), $^{125}$I porcine NPY and $^{125}$I-SRIF were from Amersham (Arlington Heights, IL), synthetic rat NPY and synthetic SRIF were from Bachem (Torrance, CA), and recombinant BDNF was provided by Amgen Inc. (Thousand Oaks, CA). Cocaine hydrochloride (Cat. No. C-5776), BZE free base (Cat. No. B-4147), CE free base (Cat. No. C-0932) and all other reagents were from Sigma (St. Louis, MO).

PREPARATION AND MAINTENANCE OF CULTURES. DMEM containing 5 μL/mL antibiotic/antimycotic, 1% ITS, 0.01 nM triiodothyronine, and 1 nM cortisol was used for cell dissociation and culture (this solution is referred to as DMEM$^{sITS}$). Sprague–Dawley female rats (Harlan, Indianapolis, IN) were anesthetized with Nembutal on day 17–18 of pregnancy (day of sperm = day 0), and the fetuses were removed. The cortex was dissected and cells were dissociated using a procedure described previously in detail [31, 37]. Briefly, diced tissue was incubated at 37° for 20 min in $Ca^{2+}$/$Mg^{2+}$-free Hanks' solution followed by 30 min of incubation in 0.125% trypsin; DMEM containing 20 μg/mL DNase and 10% fetal bovine serum was added and the cells were dissociated mechanically. Cells were pelleted (150 g, 7 min) and resuspended in DMEM$^{sITS}$, and $6 \times 10^6$ cells were distributed into 25-mL Erlenmeyer flasks (final culture volume: 3.5 mL). Exclusion of trypan blue was taken as an index of viability, and it was >95%. Flasks were gassed with a mixture of 90% air/10% $CO_2$, capped tightly, and then incubated at 37° under constant rotation (77 rpm) using a gyratory shaker.

### *Experimental Protocol*

Aliquots of stock solution of BDNF (2 μg/10 μL 0.1% BSA in saline) and of cocaine ($10^{-2}$ M in $H_2O$) were kept frozen at −70°. Stock solutions of BZE and CE (0.1 M each) and PMA (20 μM) were made in ethanol and kept at −20°. On the day of the experiment, stock solutions were diluted to the desired concentration with DMEM$^{sITS}$. Unless otherwise stated, cultures were maintained for 9 days using the following protocol. On day 3, 2 out of 3.5 mL medium was replaced with a solution of cocaine or metabolite giving a final concentration in the incubation culture medium of the designated dose; on day 6, 2 mL was again replaced with a solution containing the designated dose of cocaine or metabolite. Media for the control cultures contained the appropriate amount of ethanol (0.1% ethanol for 100 μM CE/BZE). To assess NPY/SRIF production, on day 8 the entire medium was replaced with DMEM$^{sITS}$ containing BDNF (50 ng/mL) or PMA (20 nM); controls were replaced with DMEM$^{sITS}$ alone or DMEM$^{sITS}$ containing 0.1% ethanol, respectively. Twenty-four hours later, cultures were terminated and processed for peptide, DNA, and LDH assays. In one experiment, aggregates were cultured for a total period of 14 days with exposure to cocaine on days 3–13; media changes on days 8 and 10 were as described above for day 6. In each experiment, each treatment group consisted of 3–4 replicates.

### *Assays*

Media and aggregates were processed as described [31], and NPY and SRIF contents were quantified by radioimmuno-

assay. NPY was assayed, using synthetic rNPY as the standard, $^{125}$I-NPY as the tracer, and an NPY-antiserum raised in our laboratory [37]. SRIF was assayed as described [32], using synthetic SRIF as the standard, $^{125}$I-SRIF as the tracer, and an SRIF antiserum generated in our laboratory [38]. LDH was assayed in the medium (0.1-mL aliquots) using Sigma LDH assay Kit TOX-7. There was no difference in LDH activity between BDNF- and control-treated cultures; therefore, the values were pooled, and the mean value for each experiment was taken as a single determination. Aggregate DNA content was assayed fluorometrically [39].

### *Analysis of Cocaine Metabolites*

Aggregates were incubated with cocaine/metabolite at the designated dose as described above; media were harvested at various time intervals thereafter and kept frozen at −70°. Cocaine and its metabolites were extracted from media using a previously described method [40, 41], and each mobile phase extract was subjected to gas chromatography, using a Hewlett Packard 5890A Gas Chromatograph equipped with a nitrogen-phosphorus detector (NPD). The area under the peak of cocaine/metabolite was calculated and the amount computed from standard curves of the respective standards, using a linear regression analysis ($r^2$ = 0.96 to 0.99 for cocaine/metabolites). Intra- and interassay variability was <5% as determined through use of a standard sample every third sample injection. Limits of quantitation (LOQ) of the NPD detector were: 19 ng/mL for cocaine; 100 ng/mL for BZE; and 35 ng/mL for CE. Positive identification of cocaine and its metabolites was done by GC/MS on a Finnigan SSQ 700 GC/MS system.

### *Statistical Analyses*

Statistical significance of the differences between multiple mean-groups was evaluated by one-way analysis of variance followed by Newman–Keuls (equal N) or Tukey HSD (unequal N) Post-Hoc test for multiple comparison of probabilities (Systat computer program).

## RESULTS

### *Effect of Cocaine on NPY and SRIF Production*

In all the experiments, aggregate and medium contents of NPY and SRIF were assayed. In our previous studies, and re-confirmed in this study, we consistently observed that the amounts of NPY/SRIF secreted into the medium over a period of 24 hr exceeded by far those contained in the aggregates, and this was true for control cultured aggregates as well as for stimulated (BDNF, PMA, forskolin, or PMA + forskolin) aggregates. Moreover, in stimulated aggregate cultures, most of the increase in peptide content was noted in the medium. A typical example of the distribution of NPY/SRIF between the aggregates and the medium is presented in Fig. 1. Since the entire culture

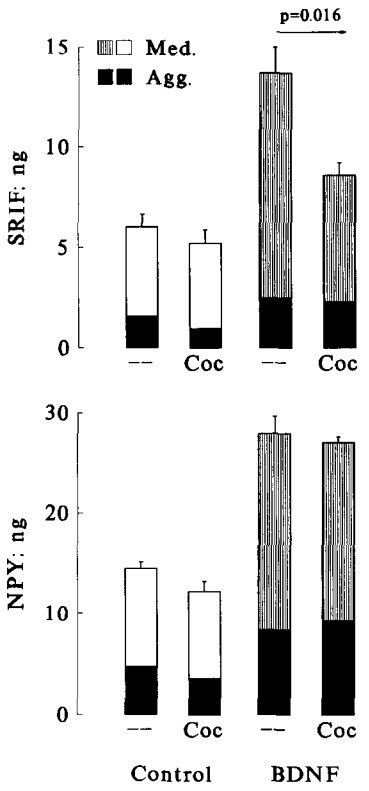


**FIG. 1. Effect of cocaine on BDNF-stimulated production of SRIF and NPY. Aggregates were incubated with cocaine (100 μM) on days 3 and 6 as described in Materials and Methods. On day 8, the entire medium was replaced with DMEM$^{sITS}$ with or without BDNF (50 ng/mL), and 24 hr later cultures were terminated. In this and subsequent figures, peptide content is ng/incubation flask. Data are means of 3–4 determinations; the SEM of the total peptide content (aggregate + medium) is indicated. Results are representatives of five different experiments. Statistical significance of the difference in total culture content of SRIF between cocaine- and control-treated cultures is indicated.**

medium was replaced 24 hr before termination of the experiment, the medium content of NPY/SRIF can be taken as a reliable criterion for peptide production, and it is used as such in the following text. To simplify presentation of the data in subsequent figures, only medium peptide content is presented.

As can be seen in Fig. 1, exposure of aggregates to 100 μM cocaine on days 3–8 did not affect basal (non-stimulated) SRIF production, but it markedly suppressed BDNF stimulation of SRIF production. When the net BDNF-stimulated SRIF production was estimated (peptide medium content with BDNF minus the respective control), SRIF production was inhibited by 70% (Table 1). A shorter exposure of the aggregates to cocaine on days 6–8 was essentially ineffective. In contrast to SRIF, cocaine did not attenuate BDNF stimulation of NPY production.

To assess whether this reduction in BDNF stimulation of SRIF production reflects general toxicity of cocaine, we measured the DNA contents of the aggregates (total cell number) and LDH activity in the same medium processed

**TABLE 1. Effect of cocaine on medium content of NPY and SRIF and aggregate content of DNA**

| | DNA | BDNF | | Control (C) | | Net = BDNF minus C | |
|---|---|---|---|---|---|---|---|
| | (μg) | NPY (ng) | SRIF (ng) | NPY (ng) | SRIF (ng) | NPY | SRIF |
| Control | 35.8 ± 2.0 | 19.6 ± 1.5 | 11.2 ± 1.5 | 9.7 ± 0.4 | 4.4 ± 0.5 | 9.9 | 6.8 |
| Cocaine | 38.2 ± 0.9 | 17.8 ± 0.3 | 6.3 ± 0.5 | 8.5 ± 0.8 | 4.2 ± 0.3 | 9.3 | 2.1 |

Data are from the experiment outlined in Fig. 1. Data are means ± SEM, N = 3–4 for peptide content and 7–8 for DNA.

for peptide assay (index for cell integrity). Cocaine treatment did not lead to a significant decrease in DNA content of the aggregates (Table 1) nor to a significant increase in LDH activity released into the medium. Taking the mean value of LDH activity in the medium of control-treated aggregates as 100%, LDH activity in the medium of cocaine-treated aggregates was 130 ± 10.5% (N = 4 separate experiments). These results indicate that exposure of the aggregates for 5 days to a concentration of 100 μM cocaine does not lead to major cell loss or deterioration of cell integrity.

Previous studies indicated that BDNF action on the NPY neuron is mediated by the TrkB receptor [33, 42], which is a protein tyrosine kinase. The question is whether the effect of cocaine is limited to the signalling pathway initiated by activation of the protein tyrosine kinase or whether other major signalling pathways are affected, e.g. the protein kinase C pathway. To address this question, we examined the effect of cocaine exposure on PMA stimulation of SRIF and NPY production. Cocaine exposure on days 3–8 (Fig. 2, left panel) or days 3–13 (Fig. 2, right panel) led to a concentration-dependent suppression of SRIF but not NPY production in response to PMA.

### *Is Cocaine or Its Metabolite(s) the Active Component in Our Culture System?*

To ascertain the fate of cocaine in our culture system, aggregates were incubated with cocaine (10 μM) on day 6 and media were collected for chemical analysis at various time intervals. Cocaine was converted into BZE progressively with time such that ≈50% was converted by 8 hr and ≈90% by 48 hr (Fig. 3). When media were incubated at 37° in the absence of aggregates, only 13% of the cocaine was converted to BZE by 48 hr (the purity of cocaine, 0 time no incubation, was ≥95%). These results indicate that cocaine is metabolized by the cultured aggregates to BZE, which is recovered extracellularly in the culture medium, and that there is minimal spontaneous conversion of cocaine to BZE in our culture medium at 37°. The question then arises: is cocaine itself or BZE the active compound in our culture system?

Aggregates were exposed to either cocaine or BZE (100 μM each) on days 3–8, and basal and BDNF- or PMA-stimulated peptide production was assessed 24 hr later. As shown in Fig. 4, cocaine suppressed BDNF stimulation of SRIF production (the net BDNF-stimulated production was suppressed by 77%), whereas BZE did not do so. Moreover, neither cocaine nor BZE suppressed BDNF stimulation of NPY production (Fig. 5). The same cocaine specificity was noted for PMA stimulation of SRIF and NPY production. These results indicate that BZE is inactive in our culture system and that cocaine as such or a very short-lived intermediate metabolite of cocaine is the active compound.

### *Effect of CE on NPY and SRIF Production*

CE has been reported to be as potent or even more potent than cocaine in some biological systems [43, 44]. Therefore, it was of interest to assess whether the effect of CE on SRIF/NPY production is similar to that of cocaine. Aggregates were exposed to 100 μM CE in parallel with those exposed to cocaine or BZE. The effect of CE differed from that of cocaine in three respects: (1) CE markedly sup-

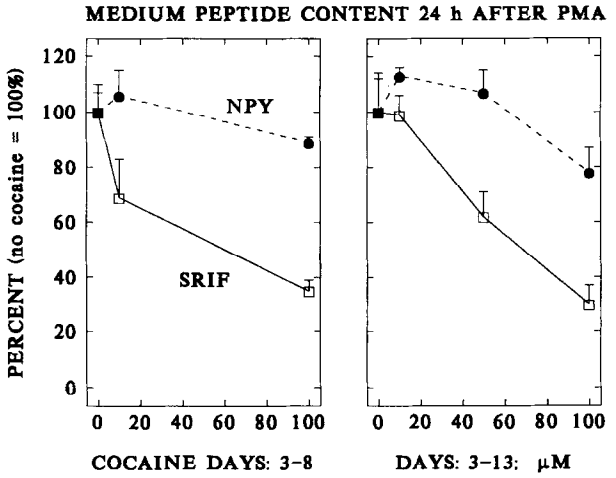


**FIG. 2. Suppression of PMA-induced SRIF and NPY production as a function of the concentration of cocaine. Aggregates were incubated for a total period of 9 days or 14 days in two separate experiments. Cocaine was included in the medium at the designated concentration (0, 10, 50, or 100 μM) on days 3 and 6 (*left panel*) or days 3, 6, 8, and 10 (*right panel*). The concentration of cocaine (μM) is indicated on the X-axis. Twenty-four hours before termination of culture, the entire medium was replaced with DMEM$^{sITS}$ with or without PMA (20 nM). The medium content of SRIF or NPY of the control incubated cultures was taken as 100%, respectively; the values of the controls were: *left panel*, 29.4 and 32.0 ng, respectively; *right panel*, 9.5 and 38.3 ng, respectively. Data are means ± SEM of 3–4 determinations; SRIF is presented in a solid line and NPY in a broken line.**

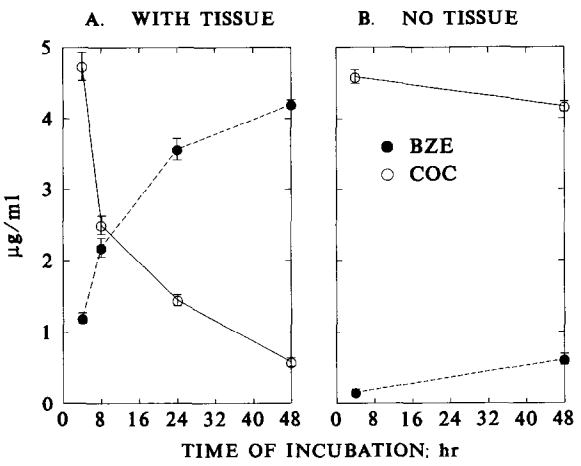


**FIG. 3. Time–course of cocaine metabolism to BZE. On day 6 of culture, cocaine was added to the culture medium to a final concentration of 10 μM, and cultures were terminated 4, 8, 24, or 48 hr thereafter (*left panel*). In parallel, culture medium without aggregates was incubated with cocaine at 37° (*right panel*, scale is the same as the left panel). Media were processed for analysis of cocaine/metabolites as described in Materials and Methods. Data are means ± SEM, N = 6.**

pressed ($P \leq 0.002$) basal production of both SRIF (Fig. 4) and NPY (Fig. 5); (2) CE suppressed ($P \leq 0.001$) BDNF or PMA stimulation of both peptides (data for PMA are not shown; results were similar to those for BDNF); and (3) CE led to a significant reduction in DNA content of the aggregates (Fig. 6). Next, we assessed the concentration-dependence of CE action and found that a concentration of 1 μM CE did not alter any of the parameters assayed. However, a concentration of 10 μM CE suppressed BDNF-stimulated production of SRIF and NPY, but it did not suppress basal peptide production or DNA content of the aggregates (Fig. 7). In addition, we found that only the 100 μM concentration of CE led to an increase (≈2-fold) in LDH activity released into the culture medium. These results are consistent with CE (at concentrations >10 μM) leading to loss of cell integrity and to cell death. Importantly, such neurotoxicity was not demonstrable for cocaine, BZE, or the ethanol added to the control-treated cultures.

A possible explanation for our results is that CE itself is more potent than cocaine in our culture system and that it is metabolized very slowly by the aggregates or not at all. To address this possibility, aggregates were incubated on day 3 with either cocaine, BZE, or CE (100 μM each), and the profile of metabolites present in the medium was evaluated 72 hr later. When cocaine was used as the substrate (Fig. 8A, left panel), 7% remained as unmetabolized cocaine and ≈93% was converted to BZE. When BZE was used as the substrate (Fig. 8B, left panel), none was converted to cocaine and 70% remained as unmetabolized BZE; the remaining 30% was converted to an unidentified material (possibly ecgonine). When CE was used as the substrate (Fig. 8C, left panel), 20% remained unmetabolized CE, 18% was converted to cocaine, and 62% was converted to BZE. None of these substrates was metabolized at 37° in the absence of aggregates (Fig. 8, right panels). Taken together with the kinetic profile of cocaine metabolism shown in Fig. 3, these results suggest that CE is slowly metabolized to cocaine, which in turn is metabolized to BZE; whether ethanol is formed during this process has yet to be established. Even if ethanol is formed, it is most unlikely that the effects of CE seen in our study are the result of ethanol toxicity. The highest concentration of ethanol, attained in cultures incubated with 100 μM CE over a period of 72 hr, would be 0.04% (80% conversion), which is less than the amount of ethanol included in the control cultures (0.1%).

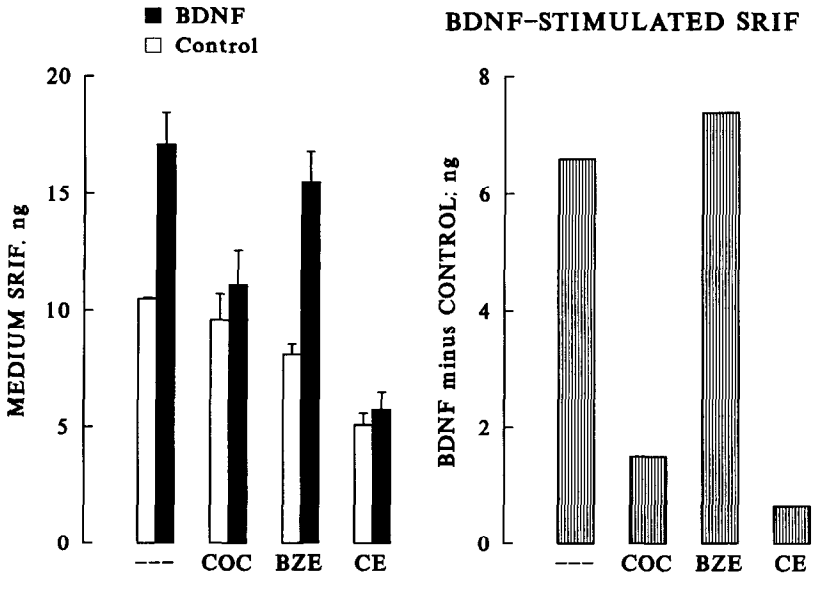


**FIG. 4. Comparison of the effects of cocaine, BZE, and CE on BDNF-induced SRIF production. Aggregates were incubated for a total period of 9 days. Cocaine, BZE, or CE (100 μM each) was added to the media on days 3 and 6; ethanol was added to the media of cocaine- and $DMEM^{sITS}$ control-treated cultures to a final concentration of 0.1%. On day 8, the entire medium was replaced with $DMEM^{sITS}$ with or without BDNF (50 ng/mL), and 24 hr later cultures were analyzed for the content of SRIF, NPY (data shown in Fig. 5), and DNA (data shown in Fig. 6). *Left panel*: medium content of SRIF; *right panel*: net BDNF-stimulated SRIF production (medium content with BDNF minus medium content of the respective control). Data are means ± SEM, N = 4. Basal and BDNF-stimulated production of SRIF by CE-treated cultures was significantly ($P < 0.002$ and $P < 0.001$, respectively) lower than that of control-treated cultures. Changes in SRIF production by BZE-treated cultures were not significant. Results are representative of three separate experiments. Similar results were obtained when aggregates were cultured with cocaine, CE, or BZE and then challenged with PMA.**

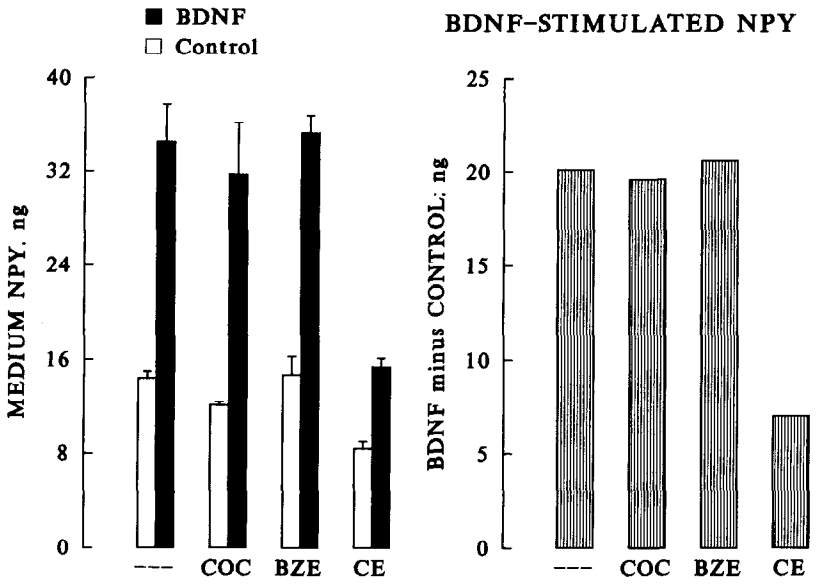


FIG. 5. Comparison of the effects of cocaine, CE, and BZE on BDNF-induced production of NPY. Data obtained from the experiment described in the legend of Fig. 4. *Left panel*: medium content of NPY; *right panel*: net BDNF-stimulated NPY production (medium content with BDNF minus medium content of the respective control). Basal and BDNF-stimulated production of NPY by CE-treated cultures was significantly ($P < 0.002$ and $P < 0.001$, respectively) lower than that of control-, cocaine- or BZE-treated cultures.

## DISCUSSION

Although cocaine abuse affects a wide range of brain functions, most of the investigations on the mechanisms of cocaine action conducted in the past two decades dealt with monoaminergic neurons, specifically dopaminergic, noradrenergic, and serotonergic neurons. However, results from recent studies indicate that cocaine may affect a much broader spectrum of neurons, i.e. peptidergic neurons such as NPY and SRIF. Systemic administration of cocaine to adult rats leads to a decrease in NPY but not in SRIF in several cortical regions [18, 19]. On the other hand, administration of cocaine to pregnant and lactating rats results in an increase in SRIF content in the cortex of the newborn and neonatal rats [27]. In this study, using NPY/SRIF production in response to BDNF as a functional criterion, we demonstrated that cocaine and two of its

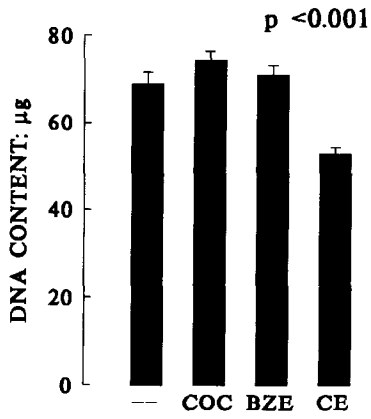


FIG. 6. Comparison of the effects of cocaine, CE, and BZE on the DNA content of the aggregates. Data were obtained from the experiment described in the legend of Fig. 4. There was no difference in DNA content between BDNF- and control-treated aggregates; therefore, data were pooled (N = 8). DNA content of CE-treated aggregates was significantly ($P < 0.001$) lower than that of cocaine-, BZE-, or control-treated aggregates.

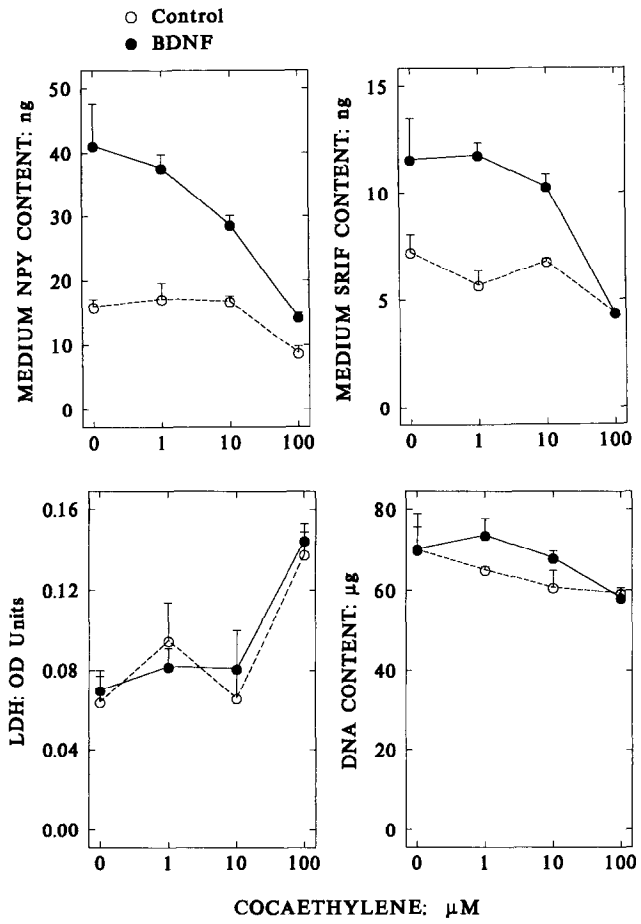


FIG. 7. NPY and SRIF production, LDH activity released into the medium, and DNA content of the aggregates as a function of the concentration of CE. Aggregates were incubated with either 0, 1, 10, or 100 µM CE on days 3 and 6 (media of control cultures contained the appropriate amounts of ethanol). On day 8, aggregates were challenged with BDNF (50 ng/mL), and cultures were terminated 24 hr later. Data for NPY (*upper left*), SRIF (*upper right*), LDH (*lower left*), and DNA (*lower right*) are expressed per culture. Data are means ± SEM, N = 3–4.

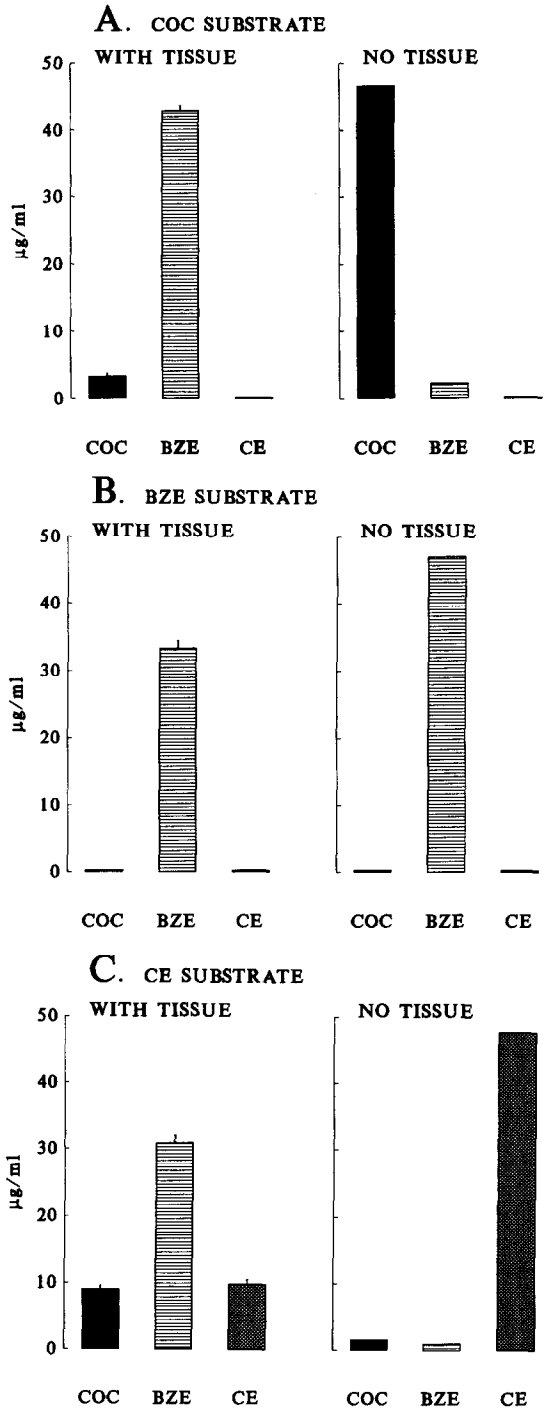


FIG. 8. Profile of metabolites formed from cocaine, BZE, or CE in the presence and absence of aggregates. *Left panels*: aggregates were exposed to either cocaine, BZE, or CE (100 μM each) for 3 days (the same experiment presented in Figs. 4–6). Ecgonine was detected (14.1 μg/mL) only after incubation with BZE but not with cocaine or CE. *Right panels* (scale is the same as for the left panels): culture media, without aggregates, were incubated in parallel with the aggregate cultures. Media were collected and processed for analysis of metabolites as described in Materials and Methods. Note, there was no spontaneous non-enzymatic metabolism of either cocaine, CE, or BZE. By comparison to the molar concentration of cocaine, BZE, or CE included in the culture medium, the measured values of these substances were higher by 35–60%. Data are means ± SEM, N = 4.

derivatives, i.e. CE and BZE, exhibited differential potencies in suppressing the functional expression of NPY and SRIF neurons in cultures of fetal cortical cells. In this respect, CE was much more potent than cocaine and BZE was ineffective (Figs. 4 and 5). The characteristics of this effect of CE/cocaine are such that CE suppressed BDNF-induced production of both NPY and SRIF, whereas cocaine suppressed only the production of SRIF. The finding of a similar order of potency for suppression of PMA-induced production of NPY and SRIF indicates that cocaine/CE affects the expression of multiple intracellular signalling pathways, in this case the protein tyrosine kinase (BDNF) and protein kinase C (PMA) pathways.

Our finding of suppression of SRIF production is in contradistinction to those of Rodriguez-Sanchez *et al.* [27]. This discrepancy may reflect the fundamental differences between our experimental systems: *in vitro* vs *in vivo*. In the *in vivo* system, longer exposure periods to cocaine have been utilized, e.g. 12 days during pregnancy or 12 days during pregnancy followed by 15 days during lactation. In our *in vitro* culture system, cocaine suppression of PMA stimulation of SRIF production was observed regardless of whether cocaine exposure was for 5 or 10 days (Fig. 2). Thus, it is quite possible that some of the effects seen in the *in vivo* study are due to peripheral effects of cocaine/metabolite manifested in the pregnant/lactating mother and/or her fetuses. It is also possible that the effect of cocaine on the fetal/neonatal cortical SRIF neuron requires input from other brain regions, as is the case for the NPY neuron. Whalestedt *et al.* [18] reported that cocaine suppression of NPY production in the cerebral cortex requires input from mesocortical/mesolimbic intact fibers. Such input most likely does not operate in the culture system, which may explain the lack of effect of cocaine on the NPY neuron. The novel finding in this study is that both SRIF and NPY neurons are highly susceptible to CE. Whether other neuronal cell types express a similar susceptibility has yet to be established.

Use of high doses of cocaine is known to be lethal. The concentration range of cocaine found in post-mortem adult brain and blood varies, reaching levels as high as 10–100 μM [29, 45, 46]; levels of cocaine in the meconium of infants born to drug-dependent mothers can be as high as 65 μM [47], and CE levels in post-mortem brain and blood can reach levels of 10–50 μM [36]. Our finding that the effective concentrations of cocaine and CE are in the range of 10–100 μM and the report by Hearn *et al.* [43] that CE is more potent than cocaine in mediating lethality in the rat raise the question whether CE and/or cocaine are neurotoxic in our culture system. Three lines of evidence are consistent with CE causing loss of cell integrity and death in a concentration-dependent manner. A concentration of 100 μM CE led to a doubling in the activity of LDH released into the culture medium, to a significant reduction in the DNA contents of the aggregates, and to a marked reduction in basal (non-stimulated) production of both SRIF and NPY (Fig. 7). By these functional criteria, lower

concentrations of CE or 100 μM cocaine did not lead to substantial cell loss. The latter is in accord with previous results, indicating that prolonged exposure to 100 μM cocaine is not neurotoxic to fetal mesencephalic neurons in culture [48]. These results, however, do not exclude the possibility of a selective, concentration-dependent, lethal effect of CE on NPY and SRIF neurons and of cocaine on SRIF neurons. Whether suppression of NPY/SRIF production is one of the early steps in the cascade of events leading eventually to cell death or whether it reflects another aspect of CE/cocaine actions on these neurons is a question for future investigation.

Why is CE more potent than cocaine in our culture system? Cocaine is converted to BZE by the cultured brain cells, the rate of which appears to be dependent on the type of cell culture (≈50% conversion by 8 hr in our study (Fig. 3) and by 18 hr in another study [48]). As BZE is inactive in our culture system, we deduce that cocaine as such or a very short-lived intermediate metabolite of cocaine is the active substance leading to suppression of the SRIF neuron. CE is metabolized slowly to cocaine and BZE, and substantial amounts of the former two remain in the culture for up to 72 hr (Fig. 8C). Since BZE is not metabolized by the cultured cells, this profile of CE metabolites suggests the following sequence of CE metabolism: CE → cocaine → BZE. To our knowledge, this is the first demonstration of metabolism of CE to cocaine by brain cells in culture. Thus, one possibility is that CE serves as a source for slow and continuous formation of cocaine, which, in turn, suppresses expression of the SRIF neuron. The prolonged continuous exposure to cocaine may also suppress the functional expression of the NPY neuron and may affect other brain cells to such an extent that they lose cell integrity.

Another possibility is that CE as such is as active as or even more active than cocaine in our culture system and, if so, the effects of CE could be synergistic/additive to those of cocaine. Supporting this possibility are the following lines of investigation. It is by now well established that the transporters for dopamine, norepinephrine, and serotonin serve as major sites of cocaine action in the brain (for references, see [49]). In addition to these transporters, cocaine can bind to muscarinic receptors and the sodium channel, albeit with a lower affinity than that to the monoamine transporters [50–53]. By comparison with cocaine, CE binds to the muscarinic receptor and the sodium channel with a higher affinity [44, 53], to the dopamine transporter with a similar affinity, and to the serotonin transporter with a lower affinity [36, 54]. It is also possible that additional receptors/transporters are affected differentially by CE and cocaine. Interestingly, BZE does not bind to either of these cocaine/CE binding molecules [44, 51, 55, 56].

It is tempting to speculate on the transporters/receptors that may mediate the actions of CE and cocaine on the cultured NPY/SRIF neurons. Dopamine regulates NPY gene expression in the rat brain in a positive or negative manner depending on the brain region [57–60]. However, it is unlikely that the dopamine transporter mediates cocaine/CE actions in our culture system, since dopamine neurons are very sparse or absent in fetal and adult rat cortex [61, 62] and in cortical cultures [63, 64]. On the other hand, two other potential binding sites for cocaine/CE are abundant in the cortex, i.e. muscarinic receptors and sodium channels. Muscarinic receptors are co-expressed with SRIF and γ-aminobutyric acid in neurons in several brain regions of the fetal and adult rat and in brain cultures [65–67], many of which may co-express NPY. Sodium channels are abundant on neurons throughout the brain, and cocaine is known for its anesthetic effects (for references, see [52, 68]). The profile of cocaine metabolites active in our culture system and the concentration range for cocaine/CE action are consistent with the involvement of muscarinic receptors and/or the sodium channels present on the NPY/SRIF neurons themselves and/or on other cells regulating the functional expression of these peptidergic neurons.

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