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Buprenorphine hydrochloride induces apoptosis in NG108-15 nerve cells

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Abstract

A morphine alkaloid derivative, buprenorphine hydrochloride, induces apoptosis in NG108-15 cells. Apoptosis was detected mainly by apoptosis-specific DNA fragmentation and morphological changes. This apoptosis was dose-dependent and the time-course experiment indicated that DNA fragmentation occurred within 4 h after administration of buprenorphine hydrochloride. Specific inhibitors of the previously characterized apoptotic signal cascade as well as antagonists for opioid receptors were tested. Zn^{2+}, herbimycin A, caspase inhibitors YVAD (Ac-Tyr-Val-Ala-Asp-CHO) and DEVD (Ac-Asp-Glu-Val-Asp-CHO), naloxone and naltrindole had no effect on apoptosis-specific DNA fragmentation. The serine protease inhibitor TPCK (*N*-tosyl-L-phenylalanyl chloromethyl ketone) specifically inhibited apoptosis-specific DNA fragmentation induced by buprenorphine hydrochloride; however, cell viability measurements revealed that cell death still occurred in NG108-15 cells. Thus TPCK pretreatment before buprenorphine hydrochloride administration induced apoptosis-independent cell death, presumably necrosis, in NG108-15 cells. This suggests that an unidentified serine protease, presumably functioning in the buprenorphine hydrochloride-specific death-signal cascade, could be pivotal for the rapid apoptosis observed in NG108-15 cells upon treatment with buprenorphine hydrochloride. © 1998 Elsevier Science B.V.

Keywords: Buprenorphine hydrochloride; NG108-15; Apoptosis; TPCK (*N*-tosyl-L-phenylalanyl chloromethyl ketone); Serine protease

1. Introduction

For nerve cells, it is widely acknowledged that certain kinds of physiological stress such as interruption of the blood supply can cause serious changes in the physiological cell function or even death (Wieloch, 1985). To study the effects of xenobiotics on nerve cells, we have focused on analgesics. Analgesics are widely used clinically, as they are indispensable drugs for patients receiving trauma treatment or suffering from cancer pain. However, the detailed pharmacological mechanisms of analgesics, such as the molecular pathways of pain transduction, still remain unsolved (Naranjo et al., 1991). In addition, the effects of analgesics on nerve cells have also yet to be investigated (Cubells et al., 1994).

Buprenorphine hydrochloride is the most commonly used analgesic following morphine in the clinical environment (Heel et al., 1979). When considering ligand-receptor theory, there are some reports of the pharmacological actions of buprenorphine hydrochloride in nerve cells of the brain of experimental animals (Martin, 1979; Tyers, 1980). However, the stresses on nerve cells caused by buprenorphine hydrochloride are poorly understood. In this report, we used buprenorphine hydrochloride as a model analgesic and cells of the NG108-15 cell line (Nelson et al., 1976) as model nerve cells. Study of the mechanism of the physiological changes in nerve cells which are induced by stressors such as buprenorphine hydrochloride may provide knowledge helpful for understanding several neural disorders such as Alzheimer's disease, and amyotrophic lateral sclerosis.

We found that a 100-μM concentration of buprenorphine hydrochloride caused apoptosis in NG108-15 cells as early as 4 h after its administration. Inhibition of DNA fragmentation by TPCK (*N*-tosyl-L-phenylalanyl chloromethyl ketone) suggested that this apoptosis could be mediated by an unidentified serine protease(s) in the apoptosis signal transduction cascade.

This is the first finding that the analgesic buprenorphine hydrochloride induces apoptosis in cultured cells of the nerve cell line, NG108-15.

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2. Materials and methods

2.1. Cell culture and morphology observation

NG108-15 cells were routinely cultured at 37°C in Dulbecco's Modified Eagle's Medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, 1×10^{-4} M hypoxanthine, 1×10^{-6} M aminopterin and 1×10^{-5} M thymidine. One hundred micrograms per milliliter of streptomycin was used as an antibiotic. Cultivation was conducted under 100% humidity and 5% CO_2 conditions. An Olympus model IMT-2 inverted phase-contrast microscope was used to observe morphological changes in NG108-15 cells. Hoechst 33258 dye (Molecular Probes, OR, USA) was used to identify shrunken nuclei under a Nicon Microphoto FX fluorescence microscope.

2.2. Detection of apoptotic cells

Cell viability was assayed by the trypan blue dye exclusion method. The DNA fragmentation assay was performed as described previously (Wyllie, 1980; Cohen and Duke, 1984; Tepper and Studzinski, 1992) with a slight modification; namely after harvested cells (ca. 2×10^4/ml) were washed with phosphate-buffered saline without $CaCl_2$ and $MgSO_4$ (PBS(−)), they were suspended in lysis solution (0.1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA) for 30 min. The supernatant of the cell lysate was collected by centrifugation at $10\,000 \times g$ at 4°C and was followed by an RNase (Boehringer Mannheim, Germany) digestion (20 mg/ml) at 37°C for at least 2 h. It was then processed by a 20 mg/ml Proteinase K (Boehringer Mannheim) digestion at 37°C overnight. Cellular DNA was ethanol precipitated and electrophoresed on a 1.8% agarose gel and stained with ethidium bromide.

2.3. Inhibitory experiments

General inhibitors of several apoptotic pathways, $ZnCl_2$, herbimycin A (Sigma, MO, USA), TPCK (*N*-tosyl-L-phenylalanyl chloromethyl ketone, Sigma, MO, USA), YVAD (Ac-Tyr-Val-Ala-Asp-CHO, Peptide Institute, Osaka, Japan), DEVD (Ac-Asp-Glu-Val-Asp-CHO, Peptide Institute) and opioid receptor antagonists, naloxone (Naloxone HCL, Sigma), and naltrindole (Naltrindole HCL, Research Biochemicals International, MA, USA), which is a δ-opioid receptor-specific antagonist, were added to NG108-15 cells 30 min or 1 h before administration of 100 μM buprenorphine hydrochloride. Then, NG108-15 cells were grown in a CO_2 incubator at 37°C until harvesting time. At 6 h after addition of buprenorphine hydrochloride, cells were harvested and washed once in PBS(−) and cellular DNA was extracted from the cell pellet as described in Section 2.2.

3. Results

In our preliminary experiment, we observed that treatment with 100 μM buprenorphine hydrochloride caused significant cell death in NG108-15 nerve cells 24 h after administration. Next we conducted a detailed viability experiment by observing cells microscopically. Fig. 1A shows the viability of NG108-15 cells at 0 h (T0), 2 h (T2), 4 h (T4), 6 h (T6), 8 h (T8), 12 h (T12) and 24 h (T24) after administration of buprenorphine hydrochloride. NG108-15 cells started detaching from culture dishes and floating in the medium as early as 2 h after buprenorphine hydrochloride treatment, and after 24 h only cell debris was observed. To investigate the nature of buprenorphine hydrochloride-induced cell death, we stained cells at T6 with Hoechst 33258 dye. Hoechst 33258 specifically stains DNA and is widely used to detect shrinkage of the nuclei, which is indicative of apoptosis. Typical fluorescence photographs of shrunken nuclei 6 h after administration of 100 μM buprenorphine hydrochloride are shown in Fig. 1B. These data strongly suggest that apoptosis was induced in NG108-15 cells by addition of 100 μM buprenorphine hydrochloride added to the cultures. To confirm this finding, we next conducted a DNA-fragmentation assay.

The DNA fragmentation reported in apoptosis studies is not a result of simple 'DNA damage' caused by exogenously added substances but occurs specifically as an end result of activation of the apoptotic-death signal cascade (Wyllie, 1980). When apoptosis occurs, DNA endonucleases are activated by ill-defined apoptosis-specific mechanisms and start to cleave DNA. Consequently, DNA fragments in multiplies of 180 bp in length can be observed in a typical apoptosis-specific 'DNA ladder' on a 1.8% agarose gel (Wyllie, 1980). Fig. 2 shows the time course of the DNA fragmentation assay after addition of 100 μM buprenorphine hydrochloride to NG108-15 cells. At intervals of 4 h, cellular DNA was extracted as described in Section 2 and run on a 1.8% agarose gel. The highest degree of DNA fragmentation was observed at T4 and gradually disappeared with time. Since we adjusted the total cell number (about 2×10^4 cells per lane) instead of the total DNA amount, the disappearance of the DNA ladder from T4 through T24 simply indicates the progression of apoptosis with an increasing population of dead cells.

Fig. 3 shows the dose dependence of buprenorphine hydrochloride in inducing DNA fragmentation of NG108-15 cells. Cellular DNA was extracted 6 h after administration of buprenorphine hydrochloride at each concentration. Thirty micromolar buprenorphine hydrochloride was the lowest concentration which induced apoptosis in NG108-15 cells. However, we chose a 100-μM concentration of buprenorphine hydrochloride to induce apoptosis in NG108-15 cells because DNA fragmentation could be observed more clearly at this concentration. After observing this buprenorphine hydrochloride-inducible apoptosis,

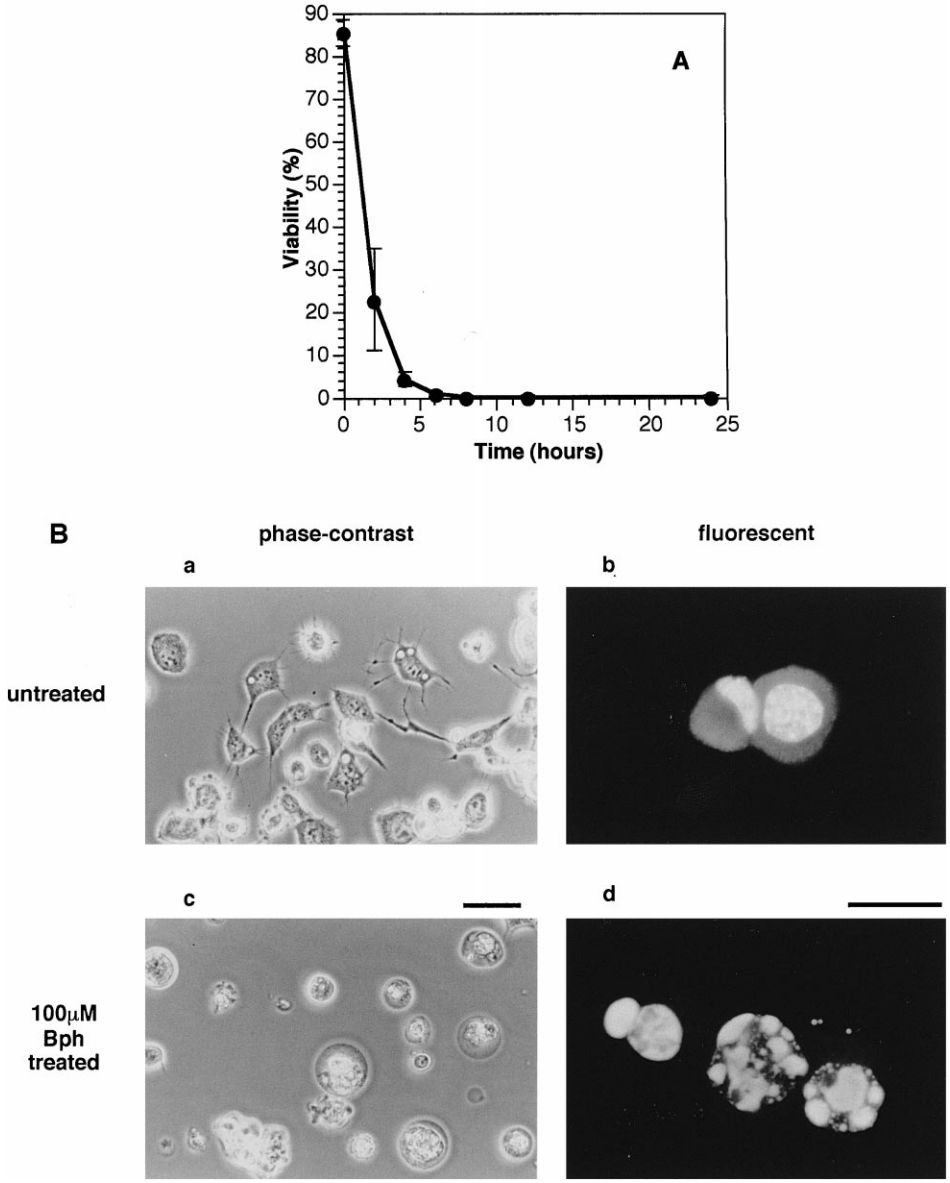

Fig. 1. Panel (A) shows the viability of NG108-15 cells after administration of 100 μM buprenorphine hydrochloride. Cell viability, expressed as mean ± s.d. of values obtained from 3 independent experiments, was evaluated by the trypan blue dye exclusion method. Panel (B) shows typical examples of phase-contrast and fluorescence microscopy pictures of both untreated as well as buprenorphine hydrochloride-treated NG108-15 cells in which apoptosis was induced with 100 μM buprenorphine hydrochloride for 6 h. Pictures a and c are phase-contrast pictures. Pictures b and d are fluorescence pictures. Pictures a and b show untreated NG108-15 cells. Pictures c and d show typical features of 100 μM buprenorphine hydrochloride-treated NG108-15 cells. Phase-contrast pictures were taken using an Olympus IMT-2 phase-contrast microscope. Hoechst 33258 dye was used to stain the DNA of the shrunken nuclei in the fluorescence pictures. Bars, indicate 30 μm.

we next wanted to determine the nature of the signal resulting from buprenorphine hydrochloride as well as how it causes apoptosis in NG108-15 cells.

Serum starvation (Kugawa, unpublished data for NG108-15 cells), removal of cytokines (Williams et al., 1990), X-ray radiation (Yamada and Ohyama, 1988), and stimulation of Fas (Yonehara et al., 1989) or oncogene pathways (Shi et al., 1992), are well known to induce apoptosis in several cell lines (reviewed by Fraser and Evan, 1996; White, 1996). To identify the signal transduction pathway responsible for inducing apoptosis in NG108-15 cells after treatment with buprenorphine hydrochloride, we used five specific inhibitors and two opiopid receptor antagonists. They are $ZnCl_2$, herbimycin A, TPCK, YVAD, DEVD, naloxone and naltrindole.

The results of DNA fragmentation with the highest concentration of inhibitors or antagonists are shown in Fig. 4. DNA fragmentation of apoptosis is reported to be inhibited by 1 mM $ZnCl_2$ because DNA endonuclease is suppressed by Zn^{2+} in HL-60 or rat thymocyte cells (Shiokawa et al., 1994; Tanuma and Shiokawa, 1994). Neither the highest (1 mM) nor the lowest (0.1 mM)

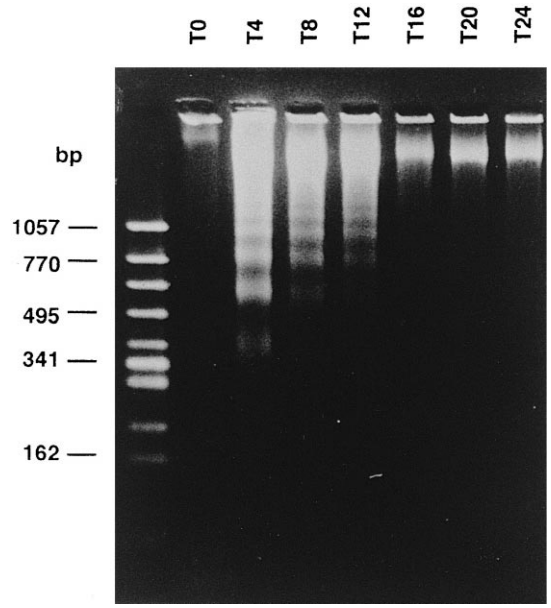

Fig. 2. The time course of DNA fragmentation in NG108-15 cells caused by 100 μM buprenorphine hydrochloride. Cellular DNA was extracted as described in Section 2, run on a 1.8% agarose gel, and stained with ethidium bromide. Total cellular DNA loaded on each lane came from ca. 2×10^4 cells. Leftmost lane is the ϕX174/HincII digested molecular weight marker. The symbols above each lane indicate hours after administration of 100 μM buprenorphine hydrochloride.

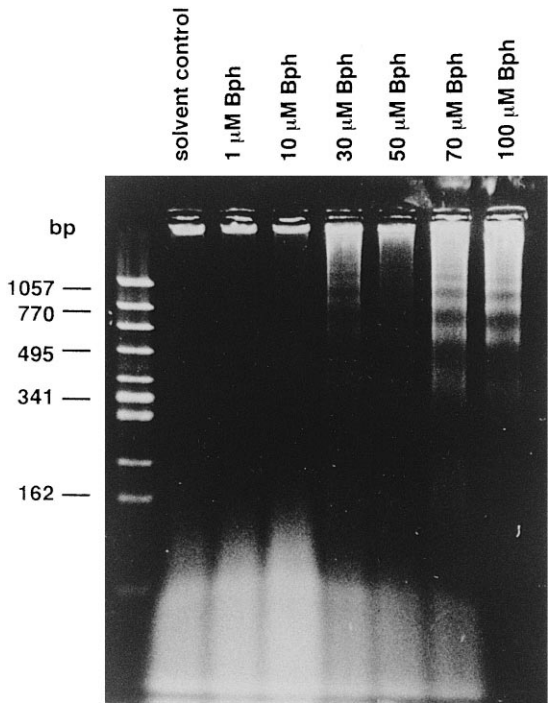

Fig. 3. The dose dependency of buprenorphine hydrochloride in inducing DNA fragmentation in NG108-15 cells. NG108-15 cells were collected 6 h after administration of each concentration of buprenorphine hydrochloride. Cellular DNA extraction and agarose gel electrophoresis were conducted as described in Section 2. The leftmost lane is a molecular weight marker. Forty percent methanol was used as a vehicle and its effect on DNA fragmentation is shown in the 'solvent control' lane.

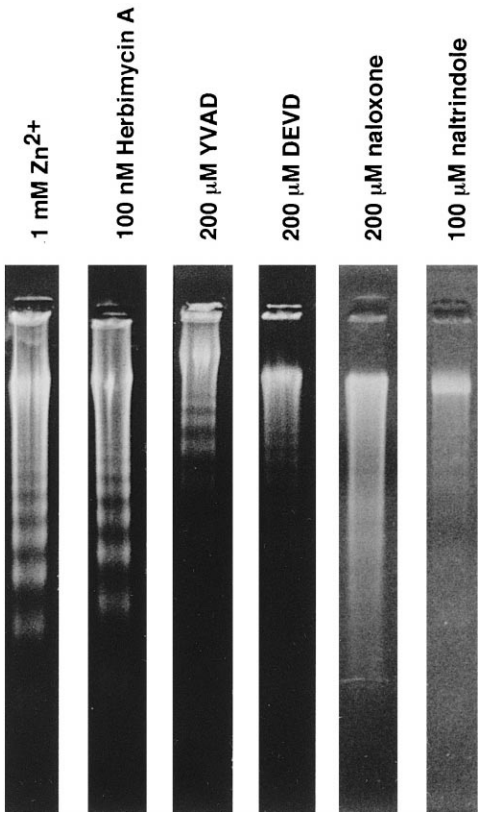

Fig. 4. The effect of apoptosis-signal inhibitors and opioid receptor antagonists. $ZnCl_2$ was used as a DNA endonuclease inhibitor. Herbimycin A was used a tyrosine-kinase inhibitor. YVAD and DEVD were used as caspase 1 and caspase 3 inhibitors, respectively. Naloxone and naltrindole were used as opioid receptor antagonist and δ-opioid receptor specific antagonist, respectively. None of these inhibitors and antagonists induced morphological or cell viability changes by themselves nor did their vehicles.

concentration of Zn^{2+} inhibited the DNA fragmentation caused by 100 μM buprenorphine hydrochloride in NG108-15 cells. Herbimycin A is a potent cell-permeable tyrosine-kinase inhibitor and is reported to inhibit anti-CD3 monoclonal antibody-induced apoptosis of thymocytes at 200 nM (Migita et al., 1994). We used five concentrations of herbimycin A, the lowest being 1 nM and the highest being 100 nM, to examine its effect on buprenorphine hydrochloride-induced apoptosis and found it failed to inhibit DNA fragmentation. Inhibitors of caspase 1 (ICE/CED-3) and caspase 3 (CPP32/Yama/apopain), YVAD and DEVD respectively, had almost no effect on buprenorphine hydrochloride-induced DNA fragmentation even at concentrations as high as 200 μM.

Inhibition by the opioid receptor antagonists naloxone and naltrindole was also examined. Concentrations of naloxone as high as 200 μM had no effect on DNA fragmentation due to buprenorphine hydrochloride-induced apoptosis. Naltrindole is an δ-opioid receptor-specific antagonist. The specific binding of naltrindole to the δ-opioid receptor is reported to be saturated at a concentration of 2

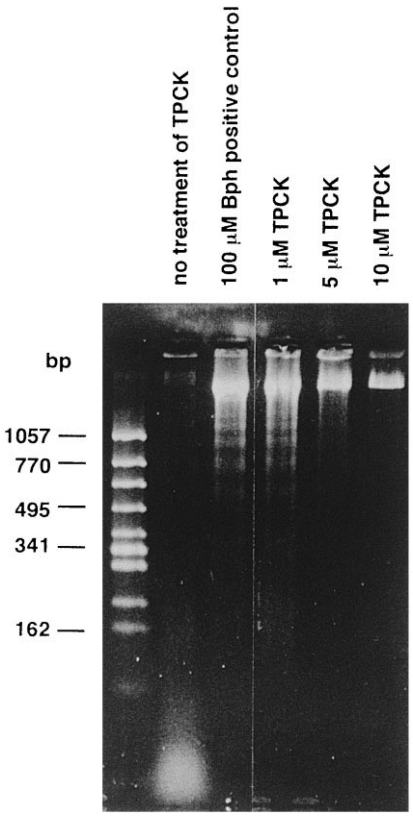

Fig. 5. Inhibition by TPCK of buprenorphine hydrochloride-induced apoptosis in NG108-15 cells. Various concentrations of TPCK (0, 1, 5 and 10 μM) were added to the culture 30 min before addition of 100 μM buprenorphine hydrochloride. The NG108-15 cells were subsequently incubated for 6 h. The DNA fragmentation assay was conducted as described in the text. The leftmost lane is a molecular weight marker. Isopropanol was used as the vehicle.

nM in NG108-15 cells (Ho et al., 1997). The effect of four different concentrations of naltrindole, ranging from 1 nM to 100 μM, on buprenorphine hydrochloride-induced apoptosis was examined, but no effect on DNA fragmentation was observed. We also confirmed that 1 mM $ZnCl_2$, 100 nM herbimycin A, 200 μM YVAD and DEVD, 200 μM naloxone and 100 μM naltrindole do not induce apoptosis in NG108-15 cells by themselves nor do their vehicles.

Fig. 5 shows the inhibition of apoptosis by TPCK in NG108-15 cells treated with 100 μM buprenorphine hydrochloride. TPCK in various concentrations was added to the cultures 30 min before administration of buprenorphine hydrochloride and cultures were incubated for an additional 6 h. The effect of TPCK on apoptosis was evaluated by DNA fragmentation, microscopic observation and Trypan blue dye exclusion. Isopropanol, the solvent of TPCK, did not induce any apoptotic morphological changes or DNA fragmentation (data not shown) in NG108-15 cells. One micromolar TPCK did not inhibit apoptosis, and hence, the DNA ladder was observed. At a concentration of 5 μM TPCK, relatively small amounts of DNA fragmentation were observed, and the DNA ladder completely disappeared at a concentration of 10 μM TPCK. Microscopic observation of the 10 μM TPCK pretreated samples showed that almost all of the NG108-15 cells were detached from the culture dish and floating in the media. We also checked the viability of a 10 μM TPCK pretreated sample by trypan blue dye exclusion. The number of viable cells in the 100 μM buprenorphine hydrochloride positive control was also counted: 98.5% cells were dead in both the 100 μM buprenorphine hydrochloride positive control and the 10 μM TPCK pretreated samples. From the above two observations, the disappearance of the DNA ladder and the very low viability of the cells, we presumed that 10 μM TPCK pretreatment caused necrosis, resulting in the death of NG108-15 cells, instead of causing apoptosis. We also confirmed that 10 μM TPCK by itself has no effect on the viability or cell morphology of NG108-15 cells (data not shown).

Figs. 6 and 7 indicate detailed results of inhibition experiments using YVAD and DEVD against buprenorphine hydrochloride-induced apoptosis. YVAD and DEVD

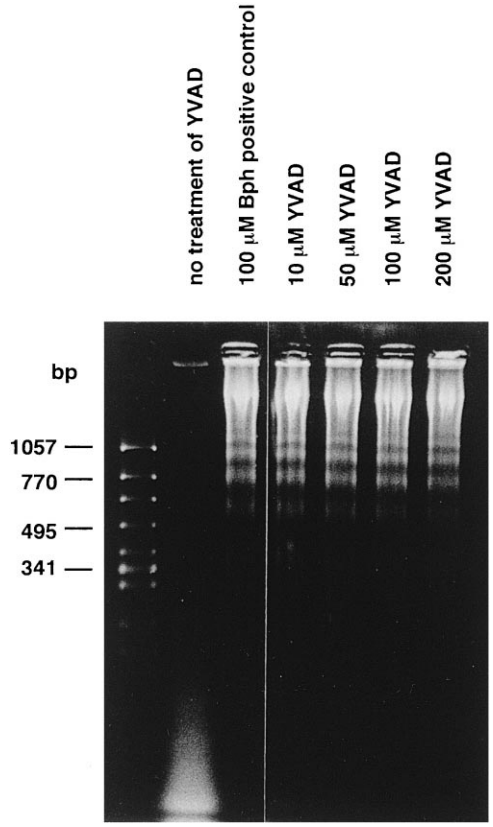

Fig. 6. Inhibition by Ac-YVAD-CHO (YVAD) of buprenorphine hydrochloride-induced DNA fragmentation in NG108-15 cells. Various concentrations of YVAD (10, 50, 100 and 200 μM) were added to the culture 1 h before addition of 100 μM buprenorphine hydrochloride. The culture was then incubated for an additional 6 h. The DNA fragmentation assay was conducted as previously described. The leftmost lane is the molecular weight marker.

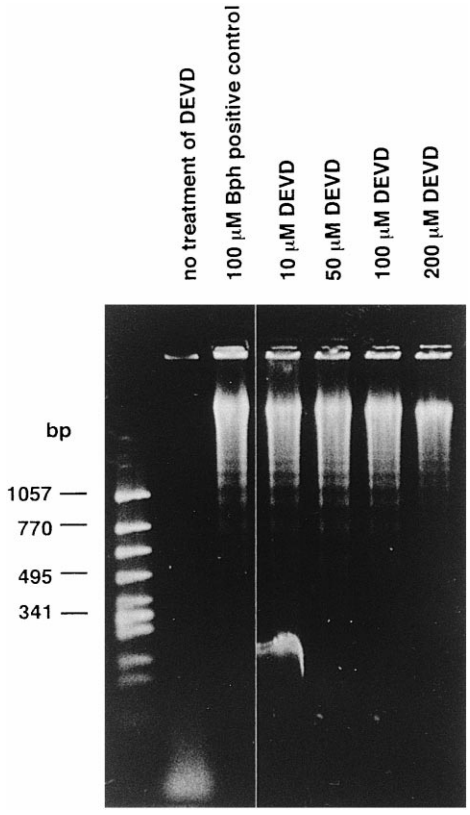

Fig. 7. Inhibition by Ac-DEVD-CHO (DEVD) of buprenorphine hydrochloride-induced DNA fragmentation in NG108-15 cells. Various concentrations of DEVD (10, 50, 100 and 200 μM) were added to the culture 1 h before addition of 100 μM buprenorphine hydrochloride. The culture was then incubated for an additional 6 h. The DNA fragmentation assay was conducted as previously described. The leftmost lane is the molecular weight marker.

are both synthetic tetrapeptides and are widely believed to be specific inhibitors of caspase 1 and caspase 3 (Patel et al., 1996). Although the cell types and apoptosis inducers used differ from those in our study, micromolar order concentrations of YVAD and DEVD were used as apoptosis inhibitors (Martin et al., 1996; Liu et al., 1997). We tested four different concentrations of YVAD and DEVD, ranging from 10 through 200 μM. Fig. 6 shows that at concentrations as high as 200 μM the caspase 1 inhibitor YVAD had no effect on the DNA fragmentation resulting from buprenorphine hydrochloride-induced apoptosis. Also there was little morphological difference between the buprenorphine hydrochloride-administrated positive control and YVAD pretreated cells. We did observe a slight decrease in DNA-ladder intensity in the lane containing the 200 μM DEVD pretreated sample (Fig. 7). Therefore, we next used a 300 μM concentration of DEVD. We observed an even greater inhibition of DNA fragmentation. In addition, we also observed a cytotoxic effect of DEVD on NG108-15 cells, probably as a result of the high concentration of DEVD itself (data not shown). It is not clear at present whether the effect of DEVD on buprenorphine hydrochloride-induced DNA fragmentation was due to specific inhibition of caspase 3 or nonspecific cytotoxity.

4. Discussion

Buprenorphine hydrochloride is a derivative of a morphine alkaloid and is a widely used analgesic with narcotic antagonist activity (Martin, 1983). From the physicochemical point of view, buprenorphine hydrochloride is a hydrophobic compound and has a relatively high octanol/water partition coefficient (Ohtani et al., 1995). Therefore it is thought to easily pass through the blood–brain barrier, allowing incorporation into nerve cells. It is commonly known that buprenorphine hydrochloride binds to μ or κ receptors, acting as either an agonist or sometimes an antagonist (Martin, 1979; Tyers, 1980). However, δ-receptors are known to be expressed exclusively on the surface of NG108-15 cells (Roerig et al., 1992; Baumhaker et al., 1993; Ho et al., 1997). These facts suggest that the buprenorphine hydrochloride-induced apoptosis of NG108-15 cells most likely does not occur through signal transduction pathways resulting from physiological binding of buprenorphine hydrochloride to the δ-opioid receptors expressed on NG108-15 cells. The experiments with naloxone and naltrindole (δ-receptor specific antagonist) as inhibitors of this buprenorphine hydrochloride-induced apoptosis (Fig. 4) also support the idea that buprenorphine hydrochloride binding to the opioid receptor do not contribute to buprenorphine hydrochloride-induced apoptosis. Instead, this particular apoptotic signal probably originates from an as yet unknown direct interaction between buprenorphine hydrochloride and certain molecules on the cell membrane, in the cytosol or nuclei of NG108-15 cells.

An interesting feature of the buprenorphine hydrochloride-induced apoptosis of NG108-15 cells is the short time lag between administration of buprenorphine hydrochloride and the appearance of the DNA ladder. As shown in Fig. 2, as early as 4 h after addition of buprenorphine hydrochloride to the culture, a clear and intense DNA ladder was observed. Induction of apoptosis caused by anti-tumor reagents in U937 cells, a human leukemia cell line, took much longer. It is reported that 3 μM adriamycin took 18 h and 10 μg/ml of mitomycin C took 12 h before a DNA ladder was observed (Mashima et al., 1995). Even apoptosis induced by serum starvation in NG108-15 cells, requires almost 24 h before the DNA ladder is observed (Kugawa, unpublished data). However, other 'fast' apoptotic signal transduction pathways, like the Fas pathway, exist. Usually the Fas pathway takes less than an hour to induce apoptosis (Chow et al., 1995). Another example of fast apoptosis is the p53-bax pathway (Miyashita and Reed, 1995).

Whether the apoptosis caused by buprenorphine hydrochloride in NG108-15 cells is transcription- and/or translation-dependent was also investigated. We treated NG108-15 cells with actinomycin D or cycloheximide and found that these inhibitors did not influence apoptosis-specific DNA ladder formation (Kugawa, unpublished data). While a detailed investigation may still yield further results, the apoptosis caused by buprenorphine hydrochloride in NG108-15 cells is more likely to be due to signals, such as phosphorylation, modification and proteolysis of certain protease(s), or a Fas-like fast signal transduction system (Enari et al., 1995), which are generated by the buprenorphine hydrochloride-cell interaction.

Low-molecular-weight DNA fragmentation, changes in cell morphology and chromatin condensation observed by electron or fluorescence microscopy are all accepted indicators of apoptosis (Wyllie, 1980; Cohen and Duke, 1984; Tepper and Studzinski, 1992). With regard to apoptosis-specific signal transduction, DNA endonucleases which are responsible for DNA fragmentation could be located downstream of caspase 1-like proteases as DNA fragmentation is thought to be one of the final steps of the death signal cascade. Among identified DNA endonucleases that function in apoptosis in mammalian cells, some are known to be inhibited by Zn^{2+} (Gaido and Cidlowski, 1991; Ribeiro and Carson, 1993; Shiokawa et al., 1994; Tanuma and Shiokawa, 1994). Interestingly, concentrations of Zn^{2+} as high as 1 mM did not inhibit apoptosis caused by 100 μM buprenorphine hydrochloride (Fig. 4). It is important to emphasize that the DNA endonuclease responsible for apoptosis induced by buprenorphine hydrochloride in NG108-15 cells could be classified as another type of DNA endonuclease whose activity is not inhibited by Zn^{2+}.

The caspase 1 family proteases have recently been implicated as important players in the cell death cascade of apoptosis (Wang et al., 1994; Enari et al., 1996; Chinnaiyan et al., 1997). It is also well known that the cysteine protease activity of caspase 1 is inhibited by the synthetic peptide YVAD (Thornberry et al., 1992). Similarly, the activity of caspase 3, which is located just below caspase 1 in the apoptosis signal cascade, is inhibited specifically by the synthetic peptide DEVD (Nicholson et al., 1995). Figs. 6 and 7 show the results of treatment with the synthetic peptides YVAD (Fig. 6) and DEVD (Fig. 7) on buprenorphine hydrochloride-induced apoptosis in NG108-15 cells. The maximum concentration of YVAD and DEVD used as inhibitors of apoptosis was 200 μM, which is consistent with other apoptosis studies. As shown in both Figs. 6 and 7, DNA fragmentation was not inhibited by YVAD at 200 μM while DEVD showed only a slight inhibition at 200 μM, as described in Results. These results indicate that caspase 1 and caspase 3 are most likely not responsible for the buprenorphine hydrochloride-induced apoptosis in NG108-15 cells. These data and the results shown in Fig. 4 strongly suggest that the apoptosis observed in this study is caused by unidentified proteases which are not classified in the caspase 1 or 3 families because DNA fragmentation was inhibited by TPCK. The results shown in Fig. 5 also support this possibility. After 6 h of treatment with 100 μM buprenorphine hydrochloride, 1.5% of NG108-15 cells were found to be viable. However, additional pretreatment with 10 μM TPCK did not change the viability of NG108-15 cells. Interestingly, after 6 h incubation, 100 μM buprenorphine hydrochloride caused apoptosis whereas 10 μM TPCK pretreatment caused necrosis-like cell death in NG108-15 cells. As TPCK is known to be a serine protease inhibitor, these results suggest that an unidentified serine protease plays an important role in the buprenorphine hydrochloride-induced apoptosis signal cascade in NG108-15 cells.

Though technically difficult, identification of this serine protease is critical for understanding the signal cascade of buprenorphine hydrochloride-induced apoptosis in NG108-15 cells. A molecular-biology-based strategy such as differential subtraction cloning could be used in future studies to help elucidate this signal cascade.

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