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Halothane Effects on ATP Content and Uridine Uptake and Phosphorylation in Tetrahymena pyriformis

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The effects of halothane on uptake and phosphorylation of uridine, and on cellular ATP content were studied in *Tetrahymena pyriformis*, a ciliate protozoan. Exposure to halothane inhibited the accumulation of ^{14}C-uridine into the following acid-soluble intracellular pools: UTP, UDP, UMP, and an unidentified compound. Halothane did not alter ATP content of intact cells. It is concluded that inhibition by halothane of uridine incorporation into RNA of *T. pyriformis* is due to effects on uridine uptake and/or phosphorylation and not due to inhibition of the RNA polymerase reaction or reduction of ATP content. (Key words: Anesthetics, volatile: halothane. Cells: replication; uridine. Metabolism: RNA).

HALOTHANE INHIBITS cell division and the incorporation of precursors into nucleic acids and protein of *Tetrahymena pyriformis* as demonstrated by our earlier investigations.[1,2] Although thymidine and uridine incorporation was inhibited by halothane, the anesthetic did not inhibit DNA or RNA synthesis when these processes were assayed in isolated nuclei using the nucleoside triphosphates as precursors.[1] It was concluded that halothane does not directly inhibit nucleic acid synthesis (*i.e.*, the nucleic acid polymerase reactions) and that the inhibition of precursor incorporation observed in intact cells was due to a different mechanism.

Incorporation of nucleosides into DNA and RNA by intact cells requires several processes, including membrane transport of the nucleosides into the cell, phosphorylation of the nucleosides to the nucleoside triphosphates, intracellular and intranuclear transport of the precursors to their sites of incorporation, as well as the DNA and RNA polymerase reactions. Inhibition of any of these sites could block thymidine or uridine incorporation into nucleic acids. Additionally, inhibition of cellular energetics may decrease ATP content, which also could reduce precursor incorporation by blocking energy-dependent membrane transport and/or phosphorylation of the nucleosides. The objectives of the present study were twofold. First, in order to determine if halothane inhibits uridine transport and/or phosphorylation, the effect of the anesthetic on formation of uridine nucleotides (from exogenous uridine) was investigated using intact *T. pyriformis*. Secondly, the effect of halothane on ATP content of *T. pyriformis* was determined.

Materials and Methods

Cultures of *T. pyriformis*, strain GL, were grown to logarithmic growth phase,[3] and exposed to air (control) or halothane (in air) in plexiglass exposure chambers as described previously.[1]

Uridine uptake and phosphorylation were determined by measurement of acid-soluble nucleotide pools. Nine milliliters of a *T. pyriformis* culture ($7–9 \times 10^4$ cells/ml) were placed in a sterilized petri dish (65 mm diameter) within each exposure chamber. The chambers were closed and the gas flows started. After a 15-min equilibration period, 9 μCi (180 μl) of (2-^{14}C)-uridine (65 mCi/mmol)‡ was added to each petri dish. At time zero (immediately after uridine addition) and at 30-min intervals for 3 hr, 1-ml samples were withdrawn from each petri dish, the cells separated from the medium, and the nucleotide pools extracted as described by Freeman and Moner.[4] Twenty-five microliters of the resulting supernatant, containing the soluble nucleotides, then were chromatographed on plastic-backed PEI cellulose sheets. Co-chromatography with nonradioactive uridine, uridine 5′-monophosphate (UMP), uridine 5′-diphosphate (UDP), uridine 5′-triphosphate (UTP), and UDP-glucose (UDPG) was utilized for identification of the labeled nucleotides. One-dimensional chromatography was carried out with a step-wise elution using distilled water for 5 min, followed by 0.5 M LiCl-2 N acetic acid, run to a distance of 14.5 cm. The position of the compounds after chromatography was determined with a UV lamp. The chromatogram then was cut into 0.5-cm segments, each segment placed in a scintillation vial, and the radioactivity determined as described previously.[1] In each experiment where nucleotide pools were determined, the cell population (from a culture in a duplicate petri dish without radioactive uridine) and uridine incorporation into RNA were determined as described previously.[1]

For determination of cellular adenosine 5′-triphosphate (ATP) content, 12 ml of a *T. pyriformis* culture ($5–6 \times 10^4$ cells/ml) were placed in a sterilized petri dish (65 mm diameter) within each exposure chamber. The chambers were closed, the gas flows started, and after a 15-min

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equilibration period (time zero) 1.0-ml samples removed in duplicate at 0, 60, 120, and 180 min for determination of ATP content. The cell sample immediately was mixed with 10 ml of cold (0° C) Ringer's phosphate buffer,[5] centrifuged at 600 *g* for 2 min, the supernatant aspirated, and the cell pellet mixed with 1.0 ml of 0.5 M perchloric acid. The mixture was placed on ice for 30 min, centrifuged at 10,000 *g* for 4 min at 4° C, the supernatant removed and mixed with 0.075 ml of 5 N KOH, and this mixture placed on ice for an additional 30 min. Following centrifugation at 10,000 *g* for 4 min, the supernatant was used for determination of ATP content. Assay of ATP was performed using the luciferase enzyme system as described by Kimmich *et al.*[6] In each experiment, samples also were removed from each petri dish for determination of the cell population by the method described previously.[1]

Each experiment was performed four times. Where duplicate samples were assayed (ATP determinations), the average of the two values was used for statistical analysis. Statistical analysis of the data from ATP determinations was done using Student's *t* test for unpaired data, accepting $P < 0.05$ as significant. For statistical analysis of nucleotide pool data, pairwise comparisons were done using the Bonferroni method of multiple comparisons at each time period and controlling the overall alpha level at 0.05. Figures were drawn by eye, attempting to minimize the distances of the means from the fitted curve.

Results

Chromatography of the acid-soluble extract from cells incubated with (2-^{14}C)-uridine resulted in separation of four different compounds, including UTP (R_f 0.11), UDP (R_f 0.31), UMP (R_f 0.50), and a fourth unidentified compound with an R_f value of 0.73. The unidentified compound was not UDPG (R_f 0.43) as determined by cochromatography, but further attempts to identify it were not made. Uridine, which has an R_f value of 0.92 with our solvent system, was not found in the acid-soluble fraction.

Halothane, 1.2 and 2.4%, significantly inhibited formation of the unidentified uridine compound (URIDINE-X) throughout the 3-h exposure period, except for the 30-min sample exposed to 1.2% halothane (fig. 1). At 3 h, the degree of inhibition by halothane, 1.2 and 2.4%, was 38 and 74%, respectively.

Exposure to halothane, 1.2%, significantly inhibited UMP (fig. 2) formation only at 30 min and had no significant effect on UDP (fig. 3) formation throughout the 3-h exposure period. Exposure to halothane, 2.4%, resulted in significant inhibition of UMP (fig. 2) and UDP fig. 3) formation (except UDP at 90 min) throughout the 3 h of exposure. At 3 h, the degree of inhibition of UMP and UDP formation by halothane (2.4%) was 69% and 53%, respectively.

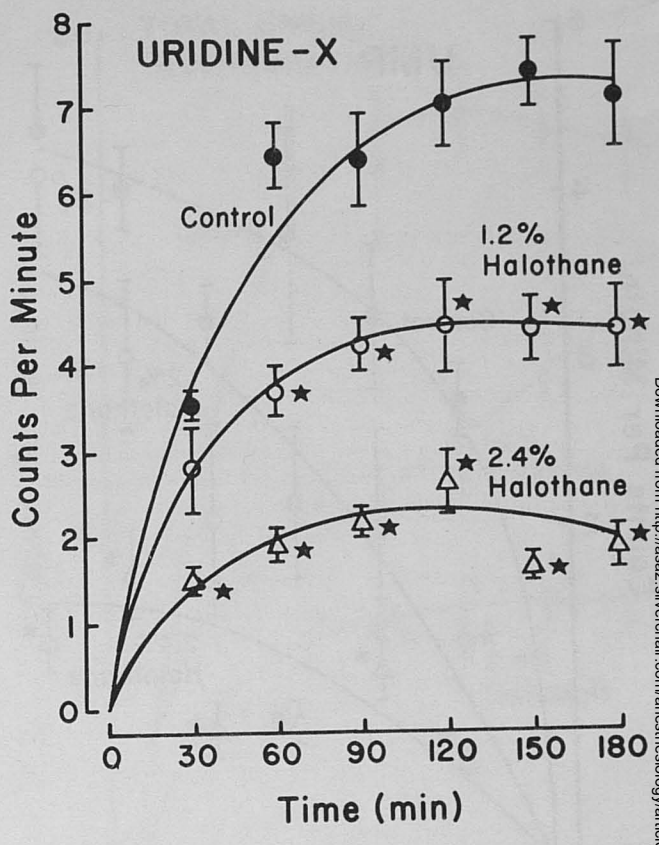

FIG. 1. Effects of halothane on the cellular content of the unidentified uridine compound (URIDINE-X). Cell cultures were exposed to air (control) or halothane at 1.2 or 2.4% in air, and the amount of the unidentified uridine compound formed from (2-^{14}C)-uridine was determined. Vertical bars indicate ±SEM. Stars indicate significant difference from control ($P < 0.05$). Counts per minute are in thousands per 10^5 cells.

Exposure to halothane, 1.2 and 2.4%, resulted in significant inhibition of UTP formation (fig. 4) during the first 2 h. At 2.5 h, significant inhibition was observed only with the higher halothane concentration, and at 3 h neither concentration significantly inhibited UTP formation.

Fig. 5 illustrates the effects of halothane on total uridine incorporation. Significant inhibition was observed at each time interval for both anesthetic concentrations, except for the 3-h sample exposed to 1.2% halothane.

Halothane, 2.4%, had no effect on cellular ATP content during the 3-h exposure period (table 1).

Discussion

Two mechanisms exist for uptake of uridine by *T. pyriformis*. The first is a carrier-mediated transport mechanism of the cell surface. When uptake occurs by this

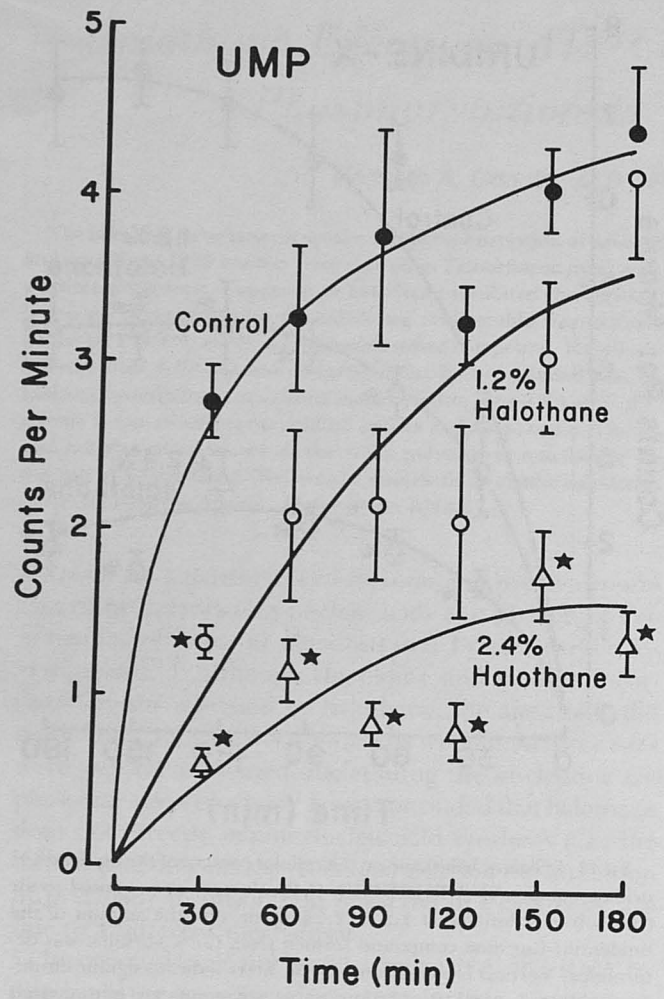

FIG. 2. Effects of halothane on the cellular content of UMP. Cell cultures were exposed to air (control) or halothane at 1.2 or 2.4% in air, and the amount of UMP formed from (2-^{14}C)-uridine was determined. Vertical bars indicate ±SEM. Stars indicate significant difference from control ($P < 0.05$). Counts per minute are in thousands per 10^5 cells.

process, free uridine is not found intracellularly.[4] Thus, the nucleoside apparently is phosphorylated immediately by pyrimidine nucleoside kinase as it enters the cell. As in mammalian cells, uptake by this process most likely occurs by facilitated diffusion[7] and alone does not support rapid growth of *T. pyriformis* (*i.e.*, generation time of 40 h[8]) unless the nucleoside concentration of the growth medium is very high.[9] The second mechanism for uridine uptake is via food vacuole formation, which appears to involve a very efficient transport system[10] (possibly active transport by the vacuole membrane) and supports rapid growth of this organism. Although we did not determine the individual contribution of the two uptake mechanisms, food vacuoles most likely account for the majority of uridine uptake in our test system because of the short generation time (3 h) of our culture.[10] Uridine uptake in our studies, however, is in no way affected by *de novo* synthesis of uridine, since *T. pyriformis* cannot synthesize nucleosides.[11]

Nucleotide pool analysis in our studies revealed that uridine appeared intracellularly as one of four compounds: UMP, UDP, UTP, and an unidentified compound (which was not UDPG as is found in mammalian cells incubated with uridine[7]). This is in contrast to the results of Freeman and Moner,[4] who found that uridine appeared only as UMP and UTP when uptake is by the carrier-mediated transport system. These differences may be accounted for by any of several factors, including the longer incubation period used by us (3 h vs. 2 min), the fact that our experiments were carried out in a nutrient medium instead of an inorganic salt solution, the fact that our cells were not pyrimidine starved (as were theirs),

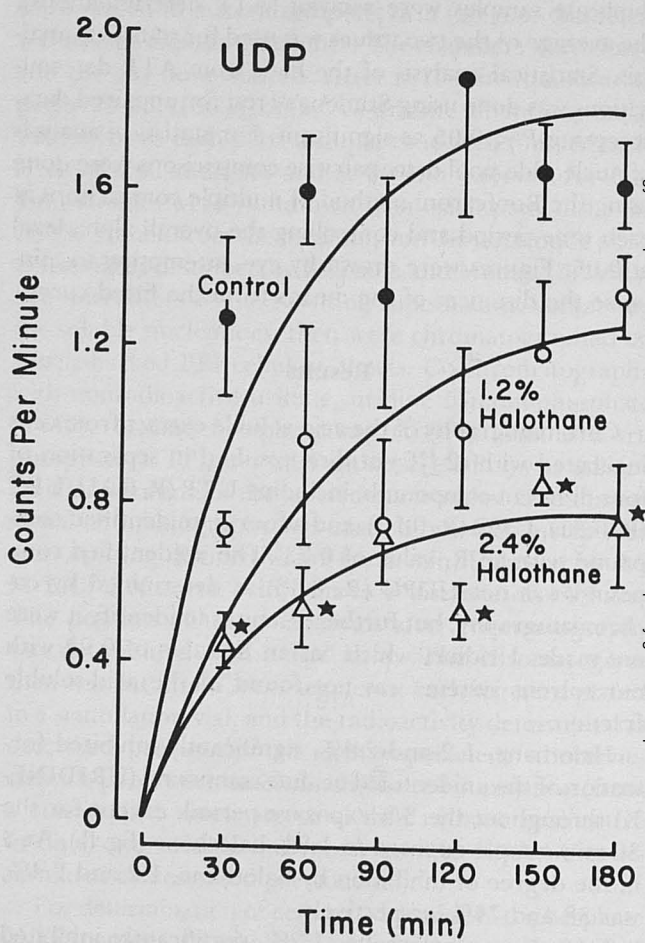

FIG. 3. Effects of halothane on the cellular content of UDP. Cell cultures were exposed to air (control) or halothane at 1.2 or 2.4% in air, and the amount of UDP formed from (2-^{14}C)-uridine was determined. Vertical bars indicate ±SEM. Stars indicate significant difference from control ($P < 0.05$). Counts per minute are in thousands per 10^5 cells.

and uptake in our system most likely occurs primarily via food vacuole formation.

The primary objective of the present study was to further evaluate the mechanism whereby halothane inhibits incorporation of nucleoside precursors into nucleic acids of *T. pyriformis*. Halothane did not reduce the ATP content of *T. pyriformis*. Therefore, inhibition of uridine incorporation into RNA does not appear to be due to reduced ATP availability, which otherwise could result in reduced uptake and/or phosphorylation of uridine. Others, however, have found that halothane (2.0%) inhibits electron transport at the site of NADH dehydrogenase in rat liver mitochondria,[12] an effect that could reduce cellular ATP content if ATP utilization were unchanged. Although it is possible that mitochondria of *T. pyriformis* differ from those of mammalian cells and are not affected by halothane, there is another possible interpretation of our results. Since halothane exposure inhibits cellular processes that require energy (*e.g.*, swimming[13]; growth, division, and precursor incorporation into nucleic acids and protein[1,2]) the reduced requirement for ATP may result in normal cellular content of this compound, despite reduced production. Clarification of this matter, however, would be done best by determining the effects of halothane on electron transport and oxidative phosphorylation in mitochondria isolated from *T. pyriformis*.

Exposure to halothane, 2.4%, substantially reduced incorporation of uridine into all acid-soluble pools. Whether the site of action of halothane is on uridine uptake or uridine phosphorylation is difficult to determine, since the latter process takes place immediately upon entry of the nucleoside into the cell. However, hal-

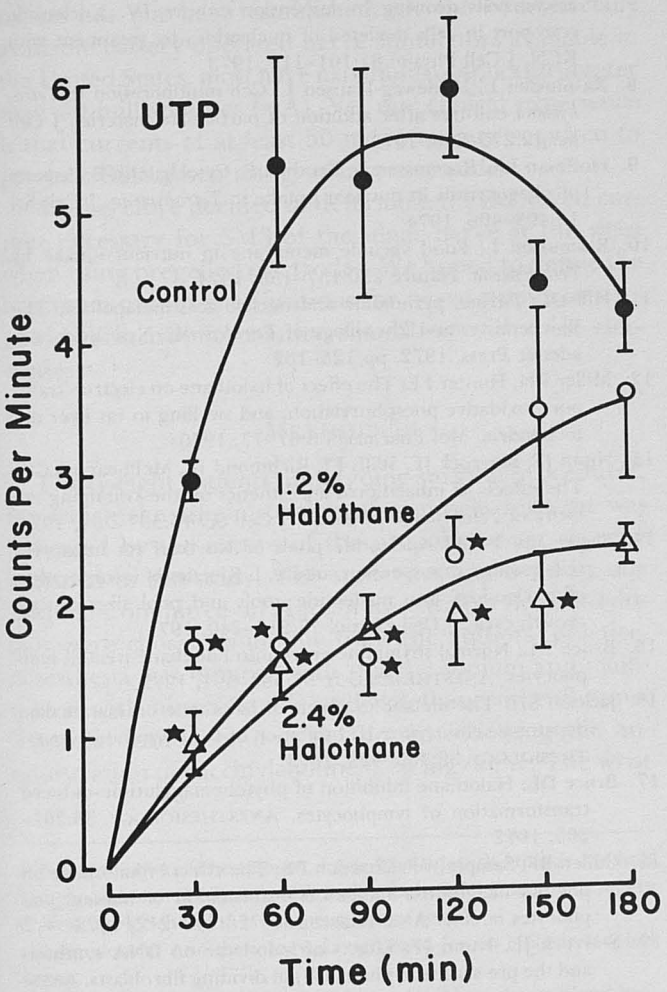

FIG. 4. Effects of halothane on the cellular content of UTP. Cell cultures were exposed to air (control) or halothane at 1.2 or 2.4% in air, and the amount of UTP formed from (2-^{14}C)-uridine was determined. Vertical bars indicate ±SEM. Stars indicate significant difference from control ($P < 0.05$). Counts per minute are in thousands per 10^5 cells.

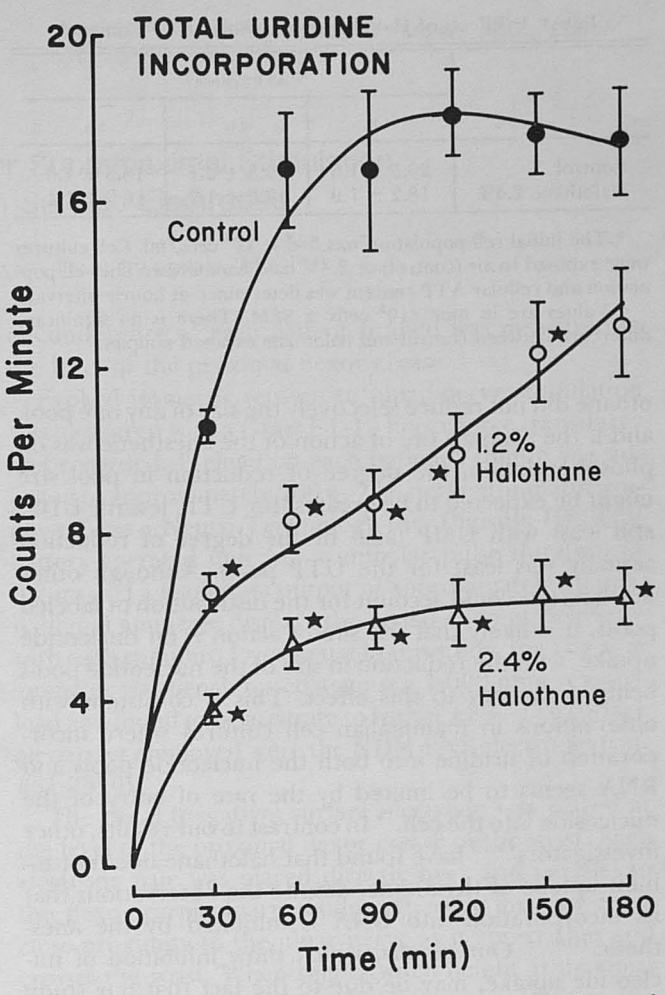

FIG. 5. Effects of halothane on total uridine incorporation. Illustrated is the sum of counts per minute (thousands per 10^5 cells) incorporated into UMP, UDP, UTP, and URIDINE-X. Vertical bars indicate ±SEM. Stars indicate significant difference from control ($P < 0.05$).

TABLE 1. Effects of Halothane on Cellular ATP Content*

	ATP Content†		
	1 h	2 h	3 h
Control	20.5 ± 1.6	19.3 ± 2.1	18.3 ± 1.8
Halothane 2.4%	18.2 ± 1.9	17.0 ± 1.7	18.7 ± 1.4

* The initial cell population was 5–6 × 10^4 cells/ml. Cell cultures were exposed to air (control) or 2.4% halothane in air. The cell population and cellular ATP content was determined at hourly intervals.
† Values are in nmol/10^5 cells ± SEM. There is no significant difference between control and halothane exposed samples.

othane did not reduce selectively the size of any one pool, and if the primary site of action of the anesthetic was on phosphorylation, the degree of reduction in pool size might be expected to be greatest for UTP, less for UDP, and least with UMP (at 3 hr the degree of reduction actually was least for the UTP pool). Although other mechanisms could account for the distribution of labeled pools, it is likely that the site of action is on nucleoside uptake, with the reduction in size of the nucleotide pools being secondary to this effect. This is consistent with observations in mammalian cell cultures where incorporation of uridine into both the nucleotide pools and RNA seems to be limited by the rate of entry of the nucleoside into the cell.[14] In contrast to our results, other investigators[15,16] have found that halothane does not inhibit uptake of thymidine, despite the observations that its incorporation into DNA is inhibited by the anesthetic.[1,16–19] Our results, which show inhibition of nucleoside uptake, may be due to the fact that our study was done with uridine (a precursor for RNA) instead of thymidine; the test system used by us was a protozoan culture instead of a mammalian cell culture; and we evaluated primarily the effects of halothane on uridine uptake by food vacuoles, which is a process that does not occur in mammalian cells.

In conclusion, our studies have shown that halothane does not block uridine incorporation into RNA of *T. pyriformis* by inhibition of the RNA polymerase reaction[1] or reduction of ATP content. The most likely site of action of the anesthetic, based on the present study, is on uridine uptake. One mechanism that could account for this is that halothane inhibits food vacuole formation, which we have yet to investigate. Another possible mechanism of action may be through alteration of membrane structure, which induces conformational changes in membrane transport proteins and modifies their activity.[20] This effect could reduce the efficiency of uridine uptake by membrane carriers.

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