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THE UPTAKE AND OVERFLOW OF RADIOLABELLED β-ADRENOCEPTOR BLOCKING AGENTS BY THE ISOLATED VAS DEFERENS OF THE RAT

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1 A comparison of uptake into and overflow from the isolated vas deferens of the rat has been made between [^{3}H]-noradrenaline ([^{3}H]-NA), [^{14}C]-D-sorbitol and three radio-labelled β-adrenoceptor blocking agents, [^{14}C]-practolol, [^{14}C]-(±)-propranolol and [^{3}H]-penbutolol.

2 The accumulation of[^{3}H]-NA after 30 min incubation was reduced by desmethylimipramine (DMI) 1×10^{-8}M and was also reduced in vasa from rats pretreated with 6-hydroxydopamine (6-OHDA). This was not so with [^{14}C]-D-sorbitol.

3 6-OHDA pretreatment of the rats reduced the uptake of [^{3}H]-penbutolol after 30 min incubation but not that of [^{14}C]-propranolol or [^{14}C]-practolol. DMI 1×10^{-8}M did not alter the tissue uptake of [^{14}C]-propranolol, [^{14}C]-practolol or [^{3}H]-penbutolol.

4 Electrical stimulation of vasa preloaded with [^{3}H]-NA caused a significantly greater increase in [^{3}H]-NA overflow than during the resting, unstimulated periods. No such increase in overflow was observed with [^{14}C]-sorbitol or any of the three β-adrenoceptor blocking agents used.

5 The β-adrenoceptor blocking agent penbutolol was shown to possess adrenergic neurone blocking activity in the isolated vas deferens of the rat.

6 It is concluded that any effect that practolol or (±)-propranolol have on noradrenergic neurones is brought about without the need for these drugs to gain access to the interior of the neurone.

Introduction

The neurotransmitter noradrenaline (NA) is taken up by noradrenergic nerves (Iversen, 1967). Following electrical stimulation, this stored NA is subsequently released into the surrounding extracellular space (Langer, 1970). These facts are also true for the adrenergic neurone blocking drug guanethidine (Chang, Costa & Brodie, 1964; 1965; Boullin, 1966; Boullin, Costa & Brodie, 1966). Furthermore, the uptake of both NA and guanethidine is reduced by desmethylimipramine (DMI) (Brodie, Chang & Costa, 1965; Iversen, 1967).

The adrenergic neurone blocking action of some β-adrenoceptor blocking agents has been described in some detail by Day, Owen & Warren (1968), Barrett & Nunn (1970), Mylecharane & Raper (1970) and Eliash & Weinstock (1971). Dollery, Lewis, Myers & Reid (1974) have suggested that propranolol is taken up into central noradrenergic neurones via the uptake$_1$ process and that there it causes a release of endogenous NA.

In view of these findings and suggestions, it was decided to investigate the possibility that β-adrenoceptor blocking agents may be taken up into peripheral noradrenergic neurones, as is guanethidine, and that electrical stimulation may cause their release. An attempt has been made to compare the uptake and overflow patterns of some β-adrenoceptor blocking agents with those of the neurotransmitter NA and the extracellularly distributed carbohydrate, D-sorbitol. Studies have also been made on the adrenergic neurone blocking effect of penbutolol.

Some of these results have been presented to the British Pharmacological Society (Lewis, 1974a).

Methods

Uptake studies

Male Wistar rats weighing 100–120 g were killed by cervical dislocation. Both vasa were removed and immersed in cold modified Holman's solution of the following composition (mM): NaCl 120, $NaHCO_3$ 25, NaH_2PO_4 1, KCl 5, $CaCl_2$ 2.5, glucose 11, sucrose 10, ascorbic acid 0.11 and disodium edetate 0.04. Each vas was stripped of mesentery,

blotted on filter paper and weighed. Pairs of vasa were incubated for 5, 10, 20 and 30 min in 2.5 ml of Holman's solution at 37°C gassed with 5% CO_2 in O_2 and containing one of the following: (a) [^{14}C]-sorbitol, 1.4×10^{-5}M in carrier sorbitol to 3.56×10^{-2}M; (b) [^{3}H]-(−)-NA, 1.23×10^{-8}M in carrier NA to 2.96×10^{-6}M; (c) [^{14}C]-practolol, 3.15×10^{-5}M; (d) [^{14}C]-(±)-propranolol, 1.52×10^{-5}M; (e) [^{3}H]-penbutolol (Hoe 893d), 8.2×10^{-7}M.

Each of the above incubations was repeated in the presence of DMI, added to give a final concentration of 1×10^{-8}M in each medium. Finally, the experiment was replicated at the 30 min incubation time for all media using vasa from rats pretreated with 6-hydroxydopamine (6-OHDA) according to the 14-day schedule of Thoenen & Tranzer (1968).

Clearance of [^{14}C]-sorbitol

After incubation of some vasa with [^{14}C]-sorbitol for 30 min the tissues were washed for 5–30 min in 50 ml gassed, modified Holman's solution at 37°C which was changed every 5 minutes. Eighty-two per cent of [^{14}C]-sorbitol taken up by the tissue was cleared by the first two 5 min washes. It was assumed that this represented clearance of the extracellular space; consequently in the uptake studies where [^{14}C]-sorbitol was not used, all tissues were given two 5 min washes in non-radioactive Holman's solution to clear the extracellular space. All results are based on the assumption that 18% of the drug remained in the extracellular space.

Sampling and counting

Following incubation, a 100 µl aliquot of the incubation medium was taken and added to 10 ml of scintillation fluid containing Triton X-100. Vasa were blotted individually, dissolved in 1 ml of Protosol solution at 54°C for 18 h and dispersed in 10 ml of scintillation fluid (without Triton X-100). All samples were counted by standard technique in a SL30 Liquid Scintillation Counter. The accumulation of each drug in the tissue was expressed as counts per minute (ct/min) per mg tissue/ct/min per µl medium, and was termed the 'drug space'. Quenching of samples and efficiency of counting did not vary significantly between samples, therefore ct/min were not corrected to disintegrations per minute.

Overflow studies

Fifteen male Wistar rats weighing 250–500 g were killed by cervical dislocation, the vasa deferatia removed, the mesentery stripped and the organs immersed in cold, modified Holman's solution. After 15 min the tissues were blotted, weighed and incubated for 30 min in modified Holman's solution containing one of the radiolabelled drugs as listed under uptake studies, except for [^{3}H](−)-NA where a concentration of 6×10^{-6}M was used.

Six vasa were used for each incubation. Each vas, after incubation was mounted between two longitudinal platinum wire electrodes of 2 cm length in a 5 ml organ bath and allowed to equilibrate at a tension of 0.3 g for 30 min after [^{3}H]NA and for 10 min after the other drugs. After equilibration, the bath fluid was changed and 5 min later 0.5 ml transferred to a scintillation vial, the bath fluid changed again and field stimulation applied 10 s later, for 10 s, using pulses of 1 ms duration, supramaximal voltage, 25 Hz, delivered from an SRI-6053 square wave electronic stimulator. Contractions were recorded with a force displacement gauge coupled to a Devices pen recorder. After 5 min a 0.5 ml sample of the bathing fluid was collected as before. The 5 min periods containing 10 s stimulation were repeated four times.

To each 0.5 ml sample were added 10 ml of Triton scintillant and the sample counted using standard technique. The results were expressed as counts per minute/mg tissue (ct min^{-1} mg^{-1} tissue).

Electron microscopy and fluorescence histochemistry

Morphological evidence for the effectiveness of 6-OHDA-induced sympathectomy in rat vasa has been evaluated by use of electron microscopic and fluorescence histochemical techniques.

Eight male Wistar rats weighing 100–120 g were used for this part of the investigation. Four were treated with 6-OHDA using the 14 day schedule of Thoenen & Tranzer (1968) and four were injected with distilled water to act as controls. At the end of 14 days all animals were killed by cervical dislocation and exsanguination. Both vasa from each rat were prepared for electron microscopic and fluorescence histochemical examination. Standard procedure was used for the preparation of tissues for electron microscopic studies. Glutaraldehyde (5%) and osmium tetroxide (1%) were used as fixatives and after dehydration in ethanol, tissues were impregnated and finally embedded in araldite before sectioning on an LKB ultramicrotome. Sections were contrasted with lead citrate and viewed using a Phillips 300 electron microscope.

Tissue for histofluorescence examination were subjected to the formaldehyde condensation procedure for demonstrating adrenergic nerves by the method of Spriggs, Lever, Rees & Graham (1966). The mounted specimens were viewed under a Zeiss Universal fluorescence microscope.

The adrenergic neurone blocking effect of penbutolol

Vasa from male Wistar rats weighing 250–300 g were set up for transmural stimulation in the manner described for the overflow studies but in a 50 ml bath.

Isotonic contractions were recorded with a lever system coupled to a 'Follograph' (EOS Industrial Electronics Ltd., see Tonks & Williams, 1972). Electrical stimulation of the intramural sympathetic nerve endings was with pulses of 1 ms duration, supramaximal voltage and at frequencies varying from 5 to 20 Hz for periods of 10 s and delivered from an SRI-6053 square wave electronic stimulator.

Responses to NA (2.96×10^{-5}M) and to transmural stimulation of various frequencies were recorded in untreated vasa and also 30 min after the addition of several concentrations of penbutolol to the bath.

Chemicals and drugs

Scintillation fluid was made up of scintillation grade 2,5-diphenyloxazole 5 g, 4-di(2-(5-phenyloxazolyl) benzene 0.5 g and toluene 1 litre; Triton X-100, 500 ml was also added for aqueous samples. Protosol was obtained from the New England Nuclear Chemical Co. The following drugs were used: desmethylimipramine (Geigy Pharmaceuticals); [^{3}H]-(−)-NA (Radiochemicals, Amersham) specific activity 4.8 mCi/mg for uptake studies and 16.5 mCi/mg for overflow studies; [^{3}H]-penbutolol (Hoechst Pharmaceuticals) specific activity 295.5 μCi/mg; [^{14}C]-practolol (ICI Ltd.) specific activity 12.2 μCi/mg; [^{14}C]-(±)-propranolol (ICI Ltd.) specific activity 14.66 μCi/mg; [^{14}C]-D-sorbitol (Radio chemicals, Amersham) specific activity 9.3 mCi/mg.

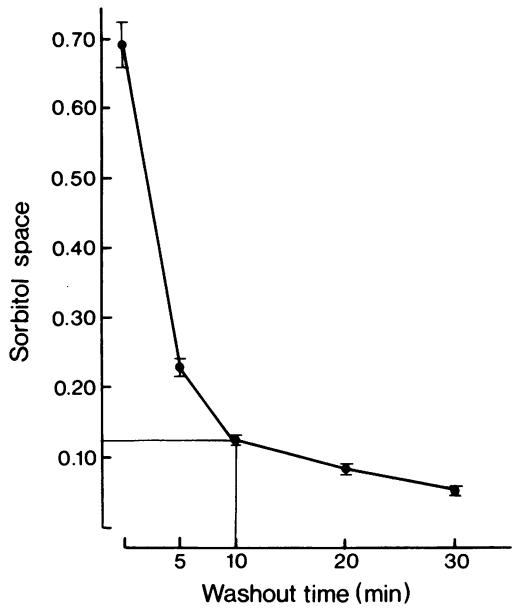

Figure 1 [^{14}C]-sorbitol clearance time from isolated vas deferens of rat. Horizontal axis: Washout times (min); vertical axis: Sorbital space (μl/mg tissue). The graph shows clearance of [^{14}C]-sorbital from the isolated rat vas deferens following an initial incubation of 30 min with]^{14}C]-sorbitol; 82% clearance had occurred after two 5 min washout periods.

Results

Counting efficiencies

The efficiency of counting of ^{14}C in Triton scintillant was $92.97 \pm 0.61\%$ ($n = 18$) and in Protosol scintillant $91.05 \pm 0.46\%$ ($n = 18$). The efficiencies for tritium were $39.33 \pm 0.5\%$ ($n = 18$) in Protosol (means ± s.e. means).

Uptake studies

Uptake of [^{14}C]-sorbitol. Uptake of [^{14}C]sorbitol increased with time, saturation occurring between 20 and 30 min (Table 1). Neither the presence of DMI, 1×10^{-8}M, nor pretreatment of rats with 6-OHDA affected the uptake pattern. Two 5 min wash periods cleared 82% of the sorbitol from the tissue. (Figure 1).

Uptake of [^{3}H]-noradrenaline. The uptake curve for [^{3}H]-NA by the isolated rat vas is shown in Figure 2. There was a rapid accumulation in the initial 5 min followed by a slower phase and saturation had not occurred at 30 minutes. DMI, 1×10^{-8}M significantly reduced the uptake of [^{3}H]-NA at 30 min (Student's t test, $P < 0.02$) but was not effective at 5, 10 or 20 minutes. Pretreatment of rats with 6-OHDA also reduced the uptake of [^{3}H]-NA at 30 min ($P < 0.005$) the only time of incubation tested.

Uptake of [^{14}C]-practolol. The accumulation of [^{14}C]-practolol by the isolated vas deferens is shown in Table 1. There was a continual increase in the accumulation of [^{14}C]-practolol by the rat vasa at successive incubation times. The amount of [^{14}C]-practolol bound by the tissue was less than that of NA at each incubation. The presence of DMI in a concentration of 1×10^{-8}M did not alter significantly the accumulation curve. Pretreatment of rats with 6-OHDA did not alter significantly the accumulation of [^{14}C]-practolol at 30 minutes.

Uptake of [^{14}C]-(±)-propranolol. The accumulation pattern of [^{14}C]-(±)-propranolol is shown in Table 1. There was no evidence of saturation even at the 30 min incubation time. The amount bound by the tissue was greater than that of NA at each incubation time. DMI, 1×10^{-8}M did not alter the accumulation curve. 6-OHDA pretreatment of the rats did not alter significantly the uptake of [^{14}C]-(±)-propranolol at 30 minutes.

Uptake of [^{3}H]-penbutolol. Table 1 shows the accumulation of [^{3}H]-penbutolol by the rat isolated vas

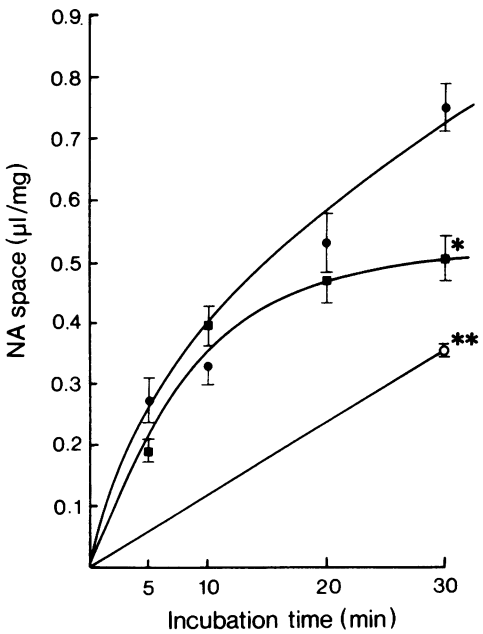

Figure 2 Uptake of [^{3}H]-(−)-noradrenaline by isolated vas deferens of the rat. Horizontal axis: Incubation time (min); vertical axis: noradrenaline (NA) space (µl/mg tissue). (●) Control uptake in untreated rat vasa; (○) uptake in vasa of rats pretreated with 6-hydroxydopamine; (■) uptake of NA in presence of desmethylimipramine 1×10^{-8} M. Each point is mean of results from a minimum of 4 vasa vertical lines show s.e. mean. $^*P < 0.02$; $^{**}P < 0.005$ (Student's t test).

deferens. [^{3}H]-penbutolol accumulation by the tissue was greater than that of NA at each interval. The presence of DMI in a concentration of 1×10^{-8}M did not alter the accumulation curve but pretreatment of rats with 6-OHDA significantly reduced accumulation at 30 min ($P < 0.01$).

Overflow studies

Tritium overflow following transmural stimulation of the isolated rat vas deferens was significantly greater than the non-stimulated overflow after incubation with [^{3}H]-NA (sign test, $P < 0.001$), see Figure 3. This was not so for [^{14}C]-sorbitol (Figure 3), [^{14}C]-practolol, [^{14}C]-(±)-propranolol or [^{3}H]-penbutolol.

Electron microscopy and fluorescence histochemistry

6-OHDA-induced morphological alterations in the adrenergic nerve endings in the vas deferens of the rat were similar to those previously described by Thoenen & Tranzer (1968) and Malmfors (1971). Electron microscopic studies showed the presence of degenerative changes in the majority of adrenergic nerve endings whereas the surrounding Schwann cell and smooth muscle cells remained intact.

The histofluorescence technique showed a marked decrease in the number and intensity of adrenergic nerve varicosities, findings which were consistent with the electron microscopic studies.

The adrenergic neurone blocking effect of penbutolol

In six experiments penbutolol (1.46×10^{-6} to 8.76×10^{-6}M) caused a progressive impairment of the

Table 1 'Drug space' (µl/mg tissue) of rat vasa deferentia after incubation with [^{14}C]-sorbitol, [^{14}C]-practolol, [^{14}C]-propranolol and [^{3}H]-penbutolol in control vasa, vasa incubated in presence of desmethylimipramine (DMI, 1×10^{-8} M) and in vasa of rats pretreated with 6-hydroxydopamine (6-OHDA)

		Incubation time (minutes)			
Drug	*Condition*	*5*	*10*	*20*	*30*
[^{14}C]-sorbitol	Control	0.347 ± 0.02	0.497 ± 0.02	0.848 ± 0.08	0.690 ± 0.03
	DMI	0.403 ± 0.02	0.604 ± 0.05	0.709 ± 0.07	0.579 ± 0.12
	6-OHDA				0.794 ± 0.09
[^{14}C]-practolol	Control	0.070 ± 0.004	0.153 ± 0.010	0.281 ± 0.009	0.379 ± 0.014
	DMI	0.059 ± 0.001	0.134 ± 0.010	0.240 ± 0.022	0.329 ± 0.017
	6-OHDA				0.357 ± 0.008
[^{14}C]-propranolol	Control	1.79 ± 0.07	2.92 ± 0.39	4.49 ± 0.51	6.31 ± 0.12
	DMI	2.12 ± 0.14	3.51 ± 0.08	5.30 ± 0.32	5.94 ± 0.60
	6-OHDA				5.55 ± 0.54
[^{3}H]-penbutolol	Control	2.30 ± 0.14	5.11 ± 0.17	7.05 ± 0.55	10.45 ± 0.52
	DMI	2.57 ± 0.07	6.13 ± 0.79	7.33 ± 0.35	10.47 ± 0.37
	6-OHDA				8.06 ± 0.81

Figures are means ± s.e. mean of at least four observations.

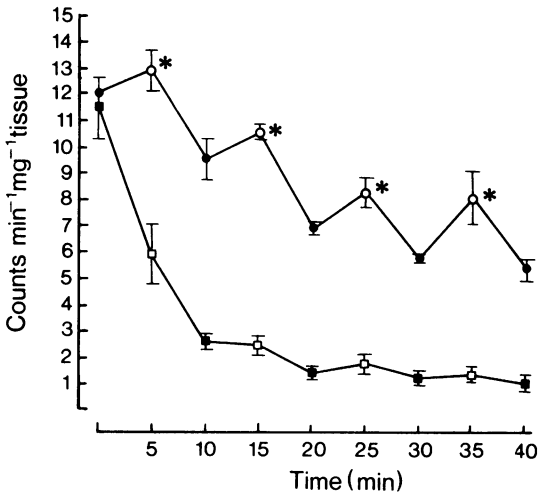

Figure 3 Overflow pattern of [^{14}C]-D-sorbitol (■-□), and [^{3}H]-(−)-noradrenaline (●-○) from the isolated vas deferens of the rat at rest (closed symbols) and following transmural stimulation (open symbols). Each point is the mean of results from 6 vasa. Vertical lines show s.e. mean. Vertical axis: counts min^{-1} mg^{-1} tissue; horizontal axis: Sampling times (min). * Significant increase in overflow.

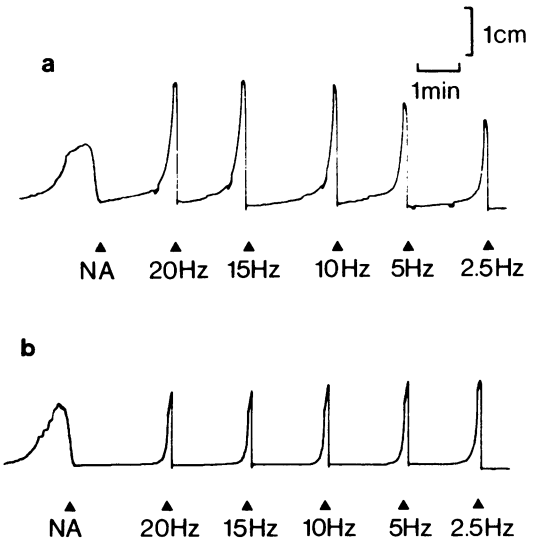

Figure 4 The effect of noradrenaline (NA, 2.96×10^{-5}M) and transmural stimulation (2.5 to 20 Hz supramaximal voltage) on the contractions of the isolated vas deferens of the rat. (a) Shows control responses and (b) responses in the presence of penbutolol 8.76×10^{-6}M.

responses to sympathetic nerve stimulation whilst the responses to added NA were either unaffected or occasionally increased. The result of a typical experiment using penbutolol in a concentration of 8.76×10^{-6}M is shown in Figure 4.

Discussion

The carbohydrate, D-sorbitol, is known to be distributed exclusively in the extracellular space (Morgan, Henderson, Regan & Park, 1961) and the present results show that this distribution is not altered significantly by the presence of DMI or pretreatment with 6-OHDA. In contrast, NA is actively accumulated by adrenergic neurones by the uptake$_1$ process (Iversen, 1967) and as can be seen from the results, this accumulation is significantly reduced at 30 min by the uptake$_1$-blocking agent, DMI and by pretreatment with 6-OHDA, a drug known to destroy adrenergic neurones (Thoenen & Tranzer, 1968). The accumulation of practolol or propranolol was not affected significantly by DMI or pretreatment of the animals with 6-OHDA. One may conclude, therefore, that neither of these drugs is being taken up into noradrenergic neurones.

The retention of [^{3}H]-penbutolol by the rat isolated vas deferens was greater at each incubation than that of any of the other drugs studied. The accumulation of this β-adrenoceptor blocking agent at the 30 min incubation time (the only time tested) was significantly less after pretreatment of the rats with 6-OHDA. However, its accumulation was not affected by DMI, 1×10^{-8}M. It would appear, therefore, that penbutolol is partly accumulated by the rat vas deferens by uptake into noradrenergic neurones like NA but its route of entry into neurones may not be via the uptake$_1$ process. The markedly greater affinity of penbutolol for the tissue relative to NA implies either other sites of binding for this drug than those that are available for NA or a greater affinity of penbutolol for the same binding sites.

Stimulation of the sympathetic nerves in the vas did not enhance the overflow of the β-adrenoceptor blocking agents as it did that of NA, and in this respect their overflow patterns resembled that of sorbitol.

Propranolol has previously been shown to possess adrenergic neurone blocking activity (Barrett & Nunn, 1970) and from the present investigation penbutolol has also been shown to exert this activity in the isolated vas deferens of the rat. Practolol, however, has no adrenergic neurone blocking activity in acute studies (Barrett & Nunn, 1970) but in chronic dosage does reduce the blood pressure response of the pithed rat to spinal cord stimulation (Lewis, 1974b). Penbutolol behaves like guanethidine in being taken up by adrenergic neurones but differs from guanethidine

in that its uptake into neurones is not blocked by DMI nor is it released from sympathetic neurones following electrical stimulation. However, practolol and propranolol appear not to be taken up into noradrenergic neurones and one must conclude that any adrenergic neurone blocking effects which they possess are exerted from outside the neurone. This effect would seem to be produced by a mechanism which does not involve either their uptake or subsequent release from adrenergic neurones.

In conclusion, it appears that any effect on the adrenergic neurone brought about by practolol and (±)-propranolol occurs without these drugs gaining access to the interior of the neurone. It may be that the mechanism of the adrenergic neurone blocking action of penbutolol is different, as it is accumulated in the neurone, whereas the other β-adrenoceptor blocking agents are not.

I should like to thank Dr S.M. Egan and Dr T.L.B. Spriggs for their assistance during the preparation of this paper. I should also like to thank ICI Pharmaceuticals for their gifts of [^{14}C]-practolol and [^{14}C]-(±)-propranolol, and Hoechst Pharmaceuticals for their gift of [^{3}H]-penbutolol.

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