Detection of a heat- and acid-stable 'progesterone'-binding protein in the rat lung

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Using a modified charcoal method, we could detect a steroid-binding component in rat lung cytosol which specifically binds R5020, progesterone, and some of its natural derivatives. The concentration of binding sites is high (30–40 pmol/mg protein), the affinity is moderate, the K_d of the R5020 complex being $\sim 10^{-7}$ M. Proteolytic enzymes and sulfhydryl reagents destroyed the binding sites indicating the protein nature and the requirement for disulfide bonds. The protein sedimented in the 2 S range thus had an M_r of 10 000–15 000. Further characteristics are the extreme heat (30 min at 100°C) and acid (pH 1) stability. These properties and the fact that it was not detected in serum, distinguish this binding protein from receptors and specific serum steroid binders.

Rat *Lung* *Cytosol* *Progesterone* *Binding protein*

1. INTRODUCTION

The mechanism of steroid hormone action is dependent on a complex interaction of steroids with proteins such as enzymes, special receptor proteins [1], serum-binding proteins [2], and tissue specific-binding proteins, like the sterol carrier proteins in the liver [3]. The latter seem to be important for a regular metabolic conversion of squalene to cholesterol. In addition to these steroid-binding proteins whose functions are relatively well understood, steroid-binding entities of which the physiological functions are not yet understood have been described [4–6]. Here, we describe the first experiments, to characterise an unusual, soluble, mainly progesterone-binding protein in the adult rat lung.

2. MATERIALS AND METHODS

2.1. *Materials*

The radioactive ligand [^{3}H]R5020 (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione), spec. act. 87 Ci/mmol, unlabelled R5020, and R1881 (17-β-hydroxy-17α-methyl-4,9,11-estratriene-3-one) were purchased from NEN; ORG 2058 (16α-ethyl-21-hydroxy-9-norpreg-4-ene-3,20-dione) was obtained from Amersham; diethylstilbestrol (DES) and cholesterol from Serva; all other steroids were purchased from Makor Chemicals. The enzymes papain (21 units/mg), and non-specific protease type 6 from *Streptomyces griseus* (5 units/mg) were purchased from Sigma; α-chymotrypsin (45 units/mg) from Serva; and protease V8 from *Staphylococcus aureus* (500 units/mg) was obtained from Miles Lab.

2.2. *Preparation of rat lung cytosols*

Lungs from male and female adult Sprague–Dawley rats were homogenized in 10 mM Tris–HCl, 1.5 mM EDTA buffer (pH 7.4) (TE-buffer) at 0°C using an Ultra-Turrax. After centrifugation at 105 000 × g the supernatant was treated with dextran-coated charcoal (DCC) for 30 min at 4°C. The final concentration of charcoal was 0.5% and dextran 0.05%. DCC was removed from the cytosol by centrifugation at 2000 × g for 15 min. With this procedure most of the unbound

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and low affinity-bound steroids were removed. Cytosol which has been treated in this maner will be termed '0°-cytosol' to distinguish it from '65°-cytosol', which was prepared by heating the '0°-cytosol' for 2 h at 65°C and subsequent centrifugation for 30 min at 105 000 × *g*.

2.3. *Binding studies*

[^{3}H]R5020, a synthetic ligand for the determination of progesterone (prog.) receptors, was used because it has the highest affinity of all steroids tested for this binding component. The spec. act. of commercially available [^{3}H]R5020 was decreased to 4.35 Ci/mmol with unlabelled R5020. The incubation conditions for all subsequent binding measurements were, unless otherwise noted, as follows: 0.2 ml aliquots of cytosol were incubated with defined concentrations of steroids (see below) for 1 h at 4°C. Removal of the unbound steroid was achieved by adding 0.5 ml of a DCC suspension (0.5% Norit A; 0.05% Dextran) and shaking for 2.5 min at 4°C followed by centrifugation at 1500 × *g* for 5 min. Under these conditions about 99% of the free steroid was removed with only minimal dissociation of the steroid–protein complex (fig. 1).

2.3.1. Saturation analysis

Cytosol was incubated with increasing amounts of [^{3}H]R5020 (40–320 nM) alone, or in the presence of 32 μM unlabelled R5020. The upper limit of solubility for R5020 in cytosol was 4×10^{-5} M. The values of specific binding were plotted as in [7], providing the equilibrium dissociation constant (K_d), and concentration of binding sites/mg protein. Protein was determined as in [8].

2.3.2. Time course of association

To determine the minimum incubation time required to reach equilibrium, cytosol was incubated with 160 nM [^{3}H]R5020 ± 16 μM R5020, and after the appropriate time-intervals DCC was added.

2.3.3. Time course of dissociation

After incubation of 8 ml of cytosol with 160 nM [^{3}H]R5020 for 1 h at 4°C, unlabelled R5020 or prog. was added to reach a final concentration of 40 μM and incubated. Aliquots (0.2 ml) were removed at different time-intervals and treated with DCC as above.

2.3.4. Specificity studies

Cytosol was incubated for 1 h with 320 nM [^{3}H]R5020 alone, or together with unlabelled steroids in ratio of 1:1, 1:10 and 1:100. In some cases only a 100-fold excess of unlabelled steroids was used as competitor.

2.4. *Stability of the binding component towards heating, acid–alkali treatment, disulfide reducing reagents and proteolytic enzymes*

The measurement of binding activity after the following treatments was performed with 160 nM [^{3}H]R5020 ± 16 μM unlabelled R5020.

2.4.1. Temperature stability

'65°-Cytosol' was heated for different periods of time at 100°C and recentrifuged at 105 000 × *g* for 20 min.

2.4.2. Exposure to extreme acidic or basic milieu

'0°-Cytosol' and '65°-cytosol' were adjusted to pH 1 and pH 12 with HCl and NaOH, respect., incubated for 1 h at 25°C, and re-titrated to pH 7.

2.4.3. Influence of S–S reducing reagents

'65°-Cytosol' was treated with either 10 mM α-monothioglycerol (α-MTG), β-mercaptoethanol (β-merc.) or dithiothreitol (DTT) for 10 min either at 100°C, and then cooled on ice, or just kept on ice.

2.4.4. Enzyme digestion

'65°-Cytosol' was incubated with either papain (1 mg/ml) together with 10 mM EDTA and 10 mM cysteine [9] for 2 h at 37°C, or with α-chymotrypsin (1 mg/ml), non-specific protease (1 mg/ml) or protease V8 [10] (0.5 mg/ml), both at 37°C for 24 h. The cytosols were made in 50 mM with respect to Tris; the enzymes were dissolved in 50 mM Tris–HCl (pH 7.4).

2.5. *Sucrose density gradient centrifugation*

Concentrated '65°-cytosol' (2 ml) or '0°-cytosol' (2 ml) were layered on top of a linear gradient of 2.5–17.5% sucrose and centrifuged for 20 h at 300 000 × *g* at 4°C. Myoglobin was used as a marker protein. Two drop fractions from the gradient were diluted with 150 μl of TE buffer and incubated with 160 nM [^{3}H]R5020 alone. In a parallel gradient, fractions were incubated with

160 nM [^{3}H]R5020 + 16 μM unlabelled R5020. This post-labelling method was necessary because of the relatively rapid dissociation of the protein–steroid complex.

3. RESULTS

3.1. *Establishing conditions for the binding assay*

A standardisation of the binding assay procedure has been important for all subsequent experiments. Fig. 1 shows that specific binding did not decrease by more than ~5% by DCC treatment for 2.5 min at 4°C.

After 1 h the association of [^{3}H]R5020 to the binding component reaches equilibrium (fig. 2). The dissociation of the steroid–protein complex in the presence of a 250-fold excess of R5020 or prog. occurred within 1 h.

3.2. *Determination of* K_d *and no. of binding sites*

In cytosol with conc. ~2 mg protein/ml, saturation was achieved at ~320 nM [^{3}H]R5020 (fig. 3). Scatchard plot-analysis of the specific binding provided a K_d of 1.33×10^{-7} M (6×10^{-8}–2×10^{-7}) with no apparent differences between '0°-cytosol' and '65°-cytosol'. The no. of binding sites was ~30–40 pmol/mg protein in '0°-cytosol', and 80–100 pmol/mg protein in '65°-cytosol', the latter value being due to loss of non-specific protein during heating procedure.

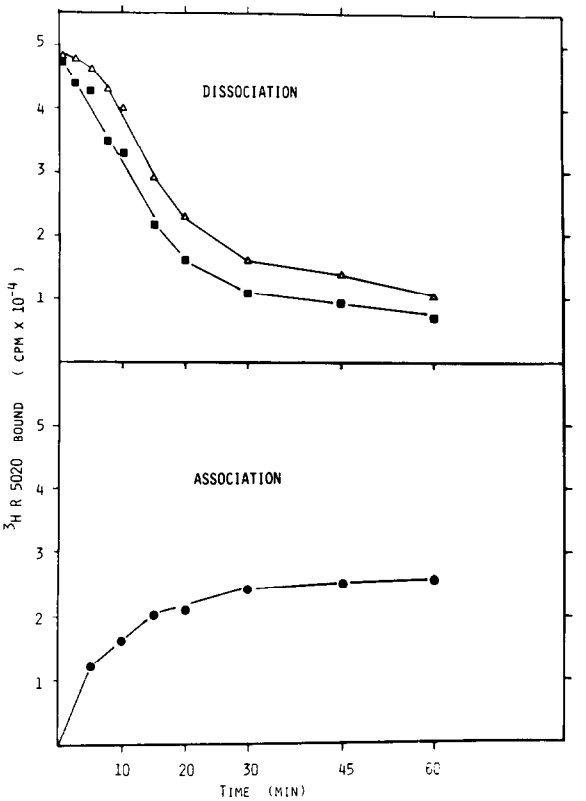

Fig. 2. Dissociation: Preincubated '65°-cytosol' (160 nM [^{3}H]R5020 at 4°C for 1 h) was further incubated with 40 μM (■) R5020 or (△) prog. Association: Specific binding of [^{3}H]R5020 in '65°-cytosol' as a function of time.

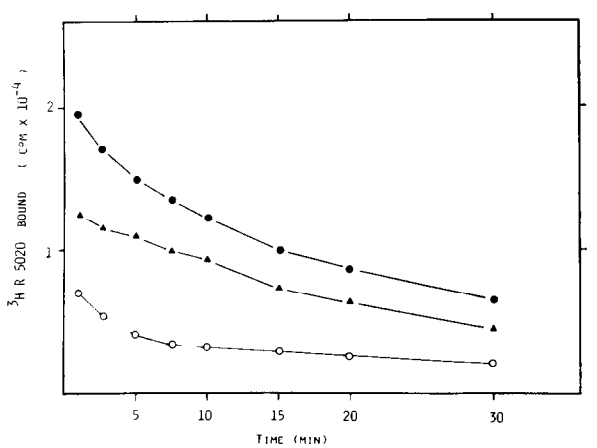

Fig. 1. Dissociation of [^{3}H]R5020 from its binding site in '0°-cytosol' in the presence of DCC: (●) total; (○) non-specific; (▲) specific binding.

3.3 *Specificity*

At least 3 determinations have been performed with each of the following cytosols: '0°-cytosol' and '65°-cytosol' from male and from female rat lung. No significant difference in the affinity of the different steroids for the [^{3}H]R5020 binding entity was observed. Fig. 4 shows an example of the specificity studies with female '65°-cytosol'. The competition experiments revealed two surprising facts: Concerning the natural steroids, prog. and its immediate derivatives (5β-pregnane-3,20-dione, 11-desoxycorticosterone-21-hydroxyprogesterone, 17α-hydroxyprogesterone and to an equal extent 4-androstene-3,17-dione) are the most potent competitors. Neither the synthetic gestagen, ORG 2058 (another ligand with high affinity to the prog. receptor) nor estrogens, androgens or glucocorti-

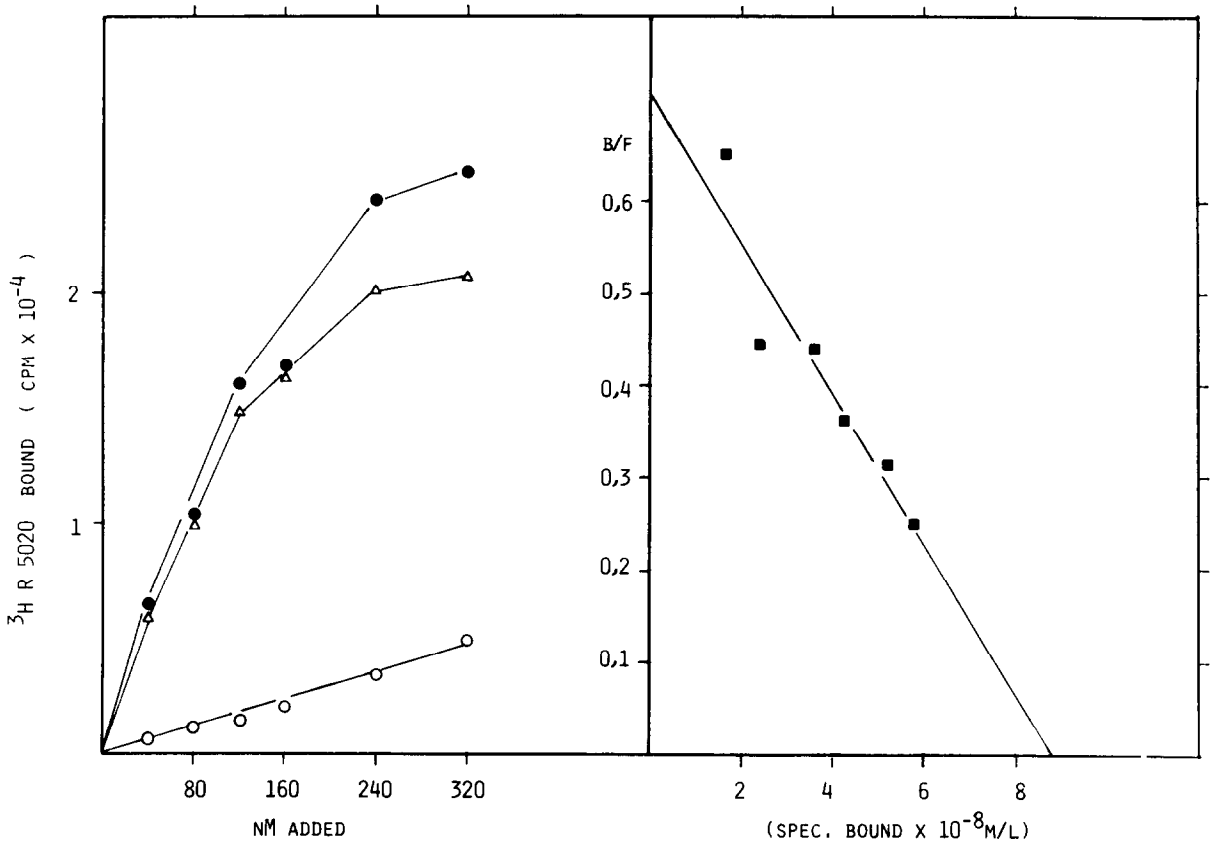

Fig. 3. Saturation analysis of the [^{3}H]R5020 binding in '65°-cytosol' and Scatchard plot-analysis: (●) total; (○) non-specific and (△) specific binding.

coids displayed significant affinity to the binding component. However, synthetic estrogens such as DES and 17α-ethinylestradiol showed competitive properties similar to prog. or 5β-pregnane-3,20-dione.

3.4. *Stability of the rat lung-binding component against heat, acid and alkali, S–S reducing reagents, and proteolytic enzymes*

Fig. 5 shows the time course of inactivation of binding at 100°C. After 4 h a complete loss of binding activity could be observed. When '0°-cytosol' and '65°-cytosol' were exposed to extreme acidic or alkaline milieus, followed by re-titration to neutral pH, we recovered > 95% of binding activity in the acid-treated cytosol, but none after exposure to alkali (not shown). The disulfide reducing reagents α-MTG, β-merc. and DTT were very effective in reducing the binding activity when the cytosol was incubated at 100°C for 10 min (table 1), but only DTT decreased the specific binding at 0°C. The fact that neither α-MTG nor β-merc. inactivated the binding activity at 0°C indicates that the loss of binding at 100°C was not a non-specific effect due to the alteration of the incubating milieu by the sulfhydryl reagents. We therefore conclude that disulfide bonds are essential for steroid binding. Reduction of binding activity as a result of the use of different proteolytic enzymes to prove its protein nature is demonstrated in table 2. It has not yet been determined if the binding protein contains carbohydrates.

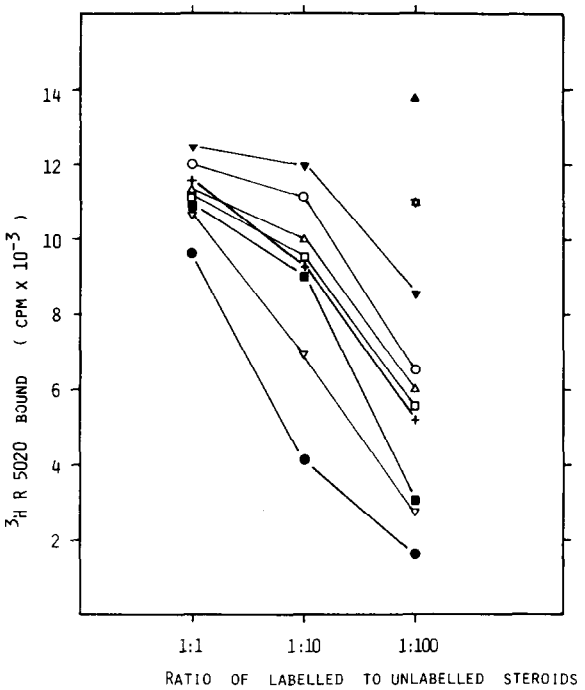

Fig. 4. Specificity of [^{3}H]R5020 binding measured in '65°-cytosol': 0.2 ml of cytosol was incubated with 320 nM [^{3}H]R5020 alone, or diluted with following unlabelled steroids in ratios of 1:1, 1:10 and 1:100: (●) R5020; (▽) prog.; (■) DES; (□) 5β-pregnane-3,20-dione; (△) R1881; (○) DOC; (▼) 17α-hydroxyprogesterone; (▼) 4-androstene-3,17-dione and (+) 17α-ethinylestradiol. Only a 100-fold excess was applied for (✡) estrone, estradiol, estriol, testosterone, 5α-dihydrotestosterone, cortisol, corticosterone, dexamethasone, triamcinolone and pregnenolone, and (▲) cholesterol.

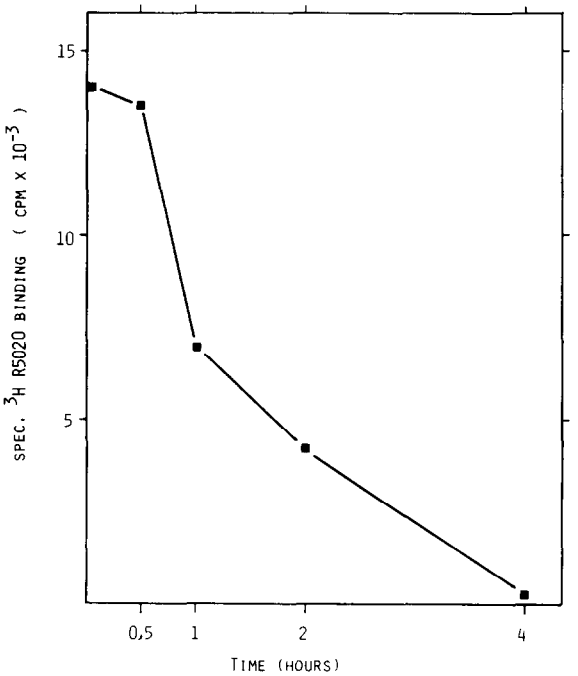

Fig. 5. Specific binding of [^{3}H]R5020 after heating '65°-cytosol' for different periods of time at 100°C.

3.5. *Molecular property on sucrose density gradient (fig. 6)*

A first estimate of the M_r-value of the binding protein was obtained from sucrose density gradient analysis (M_r 10 000–15 000). The binding entity displayed a sedimentation coefficient somewhat below myoglobin (2 S). Analysis of '0°-cytosol' and '65°-cytosol' showed identical results.

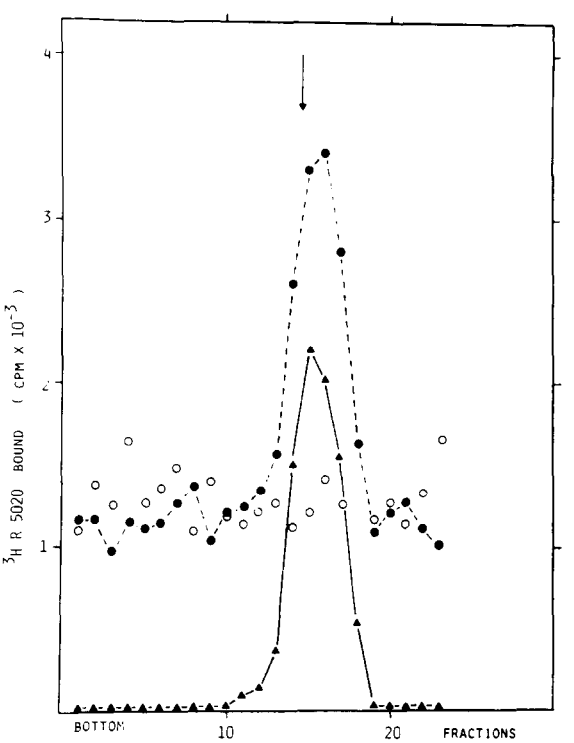

Fig. 6. Post-labelling sucrose density gradient analysis of [^{3}H]R5020 binding entity: (●) total; (○) non-specific and (▲) specific [^{3}H]R5020 binding; the arrow indicates the sedimentation property of myoglobin (2 S).

Table 1

Influence of 3 different S–S reducing reagents on the binding activity for [^{3}H]R5020 in '65°-cytosol'

Reagent	Control 1 (0°C)	Control 2 (100°C)	Reagent (0°C)	Reagent (100°C)
	(nM [^{3}H]R5020 specifically bound)			
β-Merc.	21.5	24.5	20.6	7.5
DTT	22.6	23.7	8.2	8.2
α-MTG	23.8	24.2	23.1	8.2

Before measuring the binding activity, cytosols were treated as follows: control 1, kept on ice; control 2, heated for 10 min and cooled on ice; reagent (0°C), kept on ice with reagent for 10 min; reagent (100°C), heated with reagent for 10 min and cooled on ice. Results represent the mean of 2 determinations

Table 2

Influence of 4 different proteolytic enzymes on the binding activity for [^{3}H]R5020 in '65°-cytosol'

Enzyme	Control incub.	Enzyme incub.	Inactivation (%)
	(nM [^{3}H]R5020 specifically bound)		
Papain (1 mg/ml)	53.7[a]	2.8	95
α-Chymotrypsin (1 mg/ml)	77.1	5.8	93
Protease non-spec. (1 mg/ml)	76.5	0.0	100
Protease V8 (0.5 mg/ml)	75.8	26.4	65

[a]Results represent the mean of 2 determinations

4. DISCUSSION

The steroid binding protein described is characterized by extreme heat and acid stability, lability against mercaptanes, and by its relatively low M_r. We could detect this type of [^{3}H]R5020-binding in the rat lung only, but not in organs of this animal such as thymus, kidney, liver, heart or brain nor in serum. All these findings clearly distinguish this binding entity from any classical steroid receptors which are characterized by high affinity (K_d 10^{-10}–10^{-9} M) and specificity towards its correspondent steroid as well as by low capacity (< 2 pmol/mg protein).

Its characteristics and the fact that we could not detect it in serum distinguish our binding protein from serum steroid binders. Also, its properties are different from the sterol-carrier proteins [11,12] which have a relatively high affinity for cholesterol only, but not for prog. The biological function of this protein is not yet understood. Although its affinity towards prog. is moderate, its binding capacity is about 100-times greater than for steroid receptors. In addition, it could not be excluded that the binding protein is accumulated in specific cell types of the lung or cell compartments such as membranes, thus reaching high local concentrations necessary to exert biological effects despite the low physiological prog. levels and the low affinity of the binding site. Thus it could serve to accumulate prog. in the cell or to prevent its binding to the glucocorticoid receptor in the lung [13]. The latter possibility may be important, because there are reports that prog. interacts with the glucocorticoid–receptor complex either by direct binding or by enhancing the dissociation of glucocorticoids from their receptors [14–16].

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