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Atypical characteristics of the β-adrenoceptor mediating cyclic AMP generation and lipolysis in the rat adipocyte

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1 The characteristics of the rat epididymal adipocyte β-adrenoceptor have been examined using lipolysis and cyclic AMP accumulation in adipocytes as well as adenylate cyclase activity in fat cell membranes.

2 The pA_2 values corrected for binding to bovine serum albumin of the selective antagonists betaxolol (β_1-selective) and ICI 118.551 (β_2-selective) against noradrenaline or fenoterol-stimulated lipolysis were indicative of an atypical β-adrenoceptor associated with the lipolytic response.

3 Antagonism of isoprenaline-stimulated cyclic AMP accumulation in whole cells and adenylate cyclase activity in membranes yielded pA_2 values to betaxolol, ICI 118.551 and (−)-propranolol, which suggested that the atypical β-adrenoceptor was coupled to adenylate cyclase.

4 Comparisons of the K_i values obtained in binding studies using [^{125}I]-cyanopindolol with pA_2 values obtained in adenylate cyclase experiments suggest that the typical β_1-receptor identified with radioligand binding studies is not the only receptor site mediating stimulation of adenylate cyclase activity and lipolysis.

Introduction

The rat adipocyte β-adrenoceptor was initially classified as β_1 in the subdivision proposed by Lands *et al.* (1967). Thus, it was shown that the rank order of potency of a series of agonists to stimulate lipolysis, demonstrated a high correlation with cardiac but not bronchial and vascular responses which were classified as β_2 in nature. More recently, however, doubts have been cast on the precise nature of the lipolytic β-adrenoceptor since it has often been shown that there are marked differences between cardiac and adipose tissue, based upon the antagonism of functional responses to β-agonists by several β-adrenoceptor blocking antagonists (Stanton, 1972; Harms *et al.*, 1974; 1977; Harms & Van der Meer, 1975; De Vente *et al.*, 1980). Furthermore, certain novel β-adrenoceptor agonists have been found to be more potent at stimulating lipolysis than conventionally used agonists and to have a rank order of potency that does not correlate with either a β_1 or β_2 classification in white or brown adipocytes (Arch *et al.*, 1984; Wilson *et al.*, 1984). Collectively, these results suggest that a hybrid or atypical β-adrenoceptor present on fat cells may mediate lipolysis.

β-Adrenoceptors with somewhat atypical characteristics have been found in certain non-mammalian tissues such as avian and amphibian erythrocytes using direct radioligand binding techniques (Dickinson & Nahorski, 1981) and in amphibian heart using functional responses (O'Donnell & Wanstall, 1982). In contrast, the atypical rat adipocyte β-adrenoceptor has been shown only by analysis of the lipolytic response since recent analysis of binding data in isolated adipocyte membranes revealed the presence of a binding site possessing the properties of a typical β_1-adrenoceptor (Bojanic & Nahorski, 1983). This poses the question of whether the β_1-binding site is the one responsible for the functional lipolytic response or if, in addition, there exists an atypical

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β-receptor recognition site which is the main lipolytic mediator and which has not been identified using radioligand binding studies. Alternatively, it should be considered that results obtained from functional analysis could include tissue artifacts or that other mediators were involved in the lipolytic response. We have therefore attempted to examine the putative atypical functionally-linked β-adrenoceptor by assessing lipolysis and cyclic AMP accumulation in adipocytes as well as adenylate cyclase activity in washed membranes.

Methods

Animals

Male Wistar rats weighing 150–200 g for lipolysis and 300–350 g for cyclic AMP estimations were killed and isolated adipocytes prepared as described previously (Bojanic & Nahorski, 1983), except that 1% bovine serum albumin was used for lipolysis and cyclic AMP accumulation experiments.

Lipolysis

Adipocytes were suspended in Krebs-bicarbonate buffer ($CaCl_2$ 1.25 mM, $Na_2S_2O_5$ 50 μg ml^{-1}), pH 7.4, containing 1% demineralized bovine serum albumin with appropriate drugs in Teflon vessels using a shaking water bath at 37°C and gassed with 5% CO_2 in O_2. After 90 min, the incubations were terminated by solvent extraction and the amount of free fatty acids released determined according to Ko & Royer (1967). All incubations were performed in duplicate.

Protein binding

Binding of betaxolol and ICI 118.551 to 1% bovine serum albumin was studied by equilibrium dialysis for 16–18 h at 20°C using Teflon compartments separated by cellulose acetate membranes (Technicon nr. 105–110). Samples were taken from the albumin-free compartment and analysed fluorimetrically using a Perkin-Elmer 300 spectrofluorimeter. Excitation wavelengths were 271 and 272 nm, with the emission peaks appearing at 299 and 301 nm with betaxolol and ICI 118.551 respectively.

Cyclic AMP accumulation in adipocytes

Adipocytes were incubated as described above except that 1% of Fraction V bovine serum albumin was used and the incubations terminated after 8 min by heating the vessels in a boiling water bath for 5 min, followed by centrifugation. Cyclic AMP in the supernatant was assayed by slight modifications of the protein kinase binding method of Brown *et al.* (1971) or radioimmunoassay of acetylated cyclic AMP (Skomedal *et al.*, 1980).

Adenylate cyclase in adipocyte membranes

Fat cell ghosts were prepared by lysing the isolated adipocytes in 20 volumes of ice-cold 2 mM Tris-maleate, pH 7.4, 2 mM EGTA, followed by centrifugation at 50,000 *g* for 15 min at 4°C. The fat 'cake' and supernatant were discarded and the pellet resuspended using a glass-Teflon homogenizer in 80 mM Tris-maleate, pH 7.4, 4 mM $MgSO_4$, 0.2 mM EDTA, 1 mM IBMX (assay buffer), followed by centrifugation. The final pellet was then resuspended in assay buffer at a protein concentration of 1–2 mg ml^{-1} and stored at −50°C. Adenylate cyclase activity was determined by incubating membranes (10–20 μg protein) in assay buffer containing 1 mM ATP, 100 μM GTP, 1 mM ascorbic acid, and an ATP regenerating system consisting of 20 mM phosphocreatine and 100 u ml^{-1} creatine phosphokinase in a total volume of 250 μl for 10 min at 37°C. The reaction was stopped by placing the tubes in a boiling water bath for 5 min, followed by centrifugation, and the amount of cyclic AMP produced determined by the protein binding assay (Brown *et al.*, 1971).

Dose-response curves and analysis of data

In all assays antagonists were preincubated with tissues for 10 min before the addition of agonists in whole cell experiments, or ATP in membrane adenylate cyclase experiments. The pA_2 values of the antagonists were determined according to Arunlakshana & Schild (1959) using four different concentrations of antagonist. All responses were expressed as a percentage of the control maximum response to isoprenaline. Schild slopes were analysed using the *t* test and were considered significantly different from unity at confidence intervals of 95% or greater.

Drugs and chemicals

[^{3}H]-cyclic AMP (58 Ci $mmol^{-1}$) was obtained from Amersham International. (−)-Isoprenaline bitartrate, (−)-noradrenaline bitartrate, isobutyl methyl xanthine (IBMX), collagenase type II, GTP, cyclic AMP, creatine phosphokinase, phosphocreatine and bovine serum albumin fraction V were purchased from Sigma, collagenase was also obtained from Worthington. ATP was purchased from Boehringer Mannheim. Demineralized bovine serum albumin was obtained from Organon Teknika (Oss, The Netherlands). The following compounds were kindly donated by the following companies: (±)-betaxolol (Synthelabo), (±)-ICI 118.551 (erythro-1-(7-

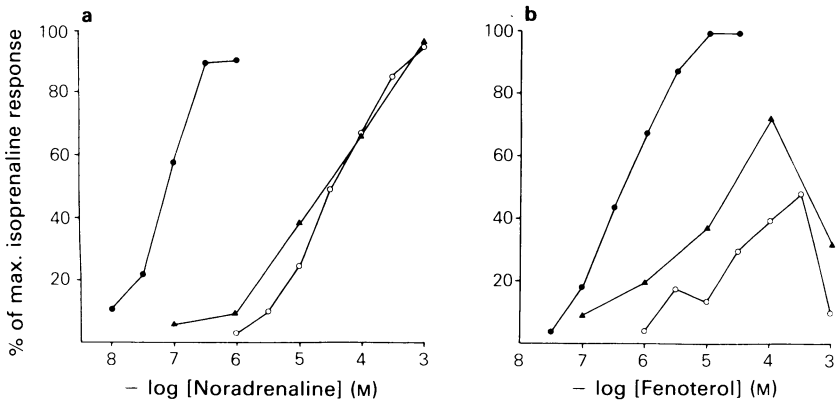

Figure 1 Dose-response curves of (a) noradrenaline- and (b) fenoterol-stimulated (●) lipolysis, (▲) cyclic AMP accumulation and (○) cyclic AMP production in membranes. The results are the means of three to eight experiments performed in duplicate and standard errors are excluded for clarity.

methylindan-4-yloxy)-3 isopropylaminobutan-2-ol), (+)- and (−)-propranolol (ICI, Macclesfield), fenoterol (Boehringer Ingelheim). All other chemicals were of analytical reagent grade.

Results

Dose-response curves to noradrenaline and fenoterol

Figure 1 shows the effect of the agonists noradrenaline (β_1-selective) and fenoterol (β_2-selective) on lipolysis, cyclic AMP accumulation and adenylate cyclase activity in membranes. Both drugs were potent stimulators of lipolysis, producing similar maximal responses to isoprenaline. Noradrenaline was also a full agonist stimulating cyclic AMP accumulation and adenylate cyclase activity in membranes maximally with respect to isoprenaline, although the concentration needed for half maximal stimulation was higher. Fenoterol on the other hand, maximally stimulated cyclic AMP production to a lesser extent than isoprenaline.

Lipolysis

Betaxolol (β_1-selective antagonist) and ICI 118.551

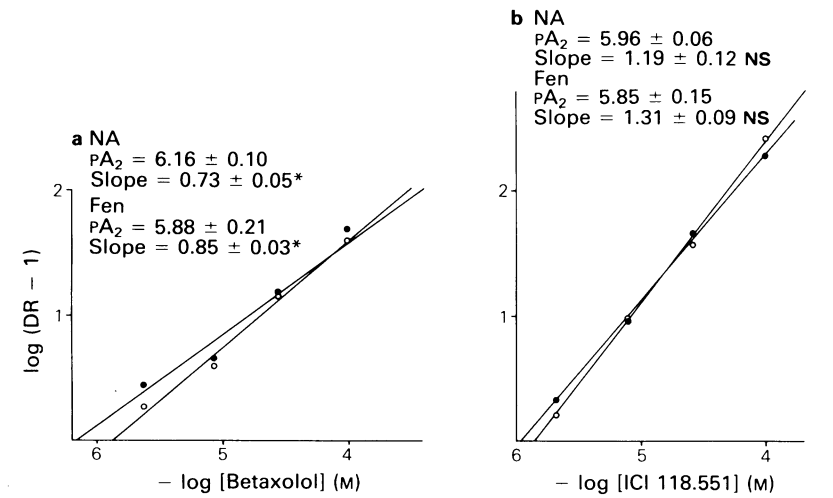

Figure 2 Schild plots of (a) betaxolol and (b) ICI 118.551 antagonism of (●) noradrenaline (NA) or (○) fenoterol (Fen) stimulated lipolysis. The pA_2 values and slopes were calculated by linear regression and represent the mean ± s.e. of four separate experiments performed in duplicate. * Slopes significantly different from unity, $P < 0.05$.

(β_2-selective antagonist) induced dose-dependent shifts to the right of either noradrenaline or fenoterol lipolytic dose-response curves. Before constructing the Schild plots, total antagonist concentrations applied were transformed into free drug concentrations by correction for protein binding which, particularly for the more lipophilic antagonists, may decrease the affinities considerably (Zaagsma *et al.*, 1977). With both antagonists, binding to 1% albumin was found to be linearly related to the concentration; from 1 μM to 0.1 mM, it steadily decreased from 27.7 to 2.8% in the case of betaxolol, and from 39.2 to 5.4% with ICI 118.551. Analysis of Schild plots (Figure 2) revealed that the slopes and pA_2 values obtained were similar irrespective of the agonist used. The pA_2 values obtained are neither indicative of a β_1- or β_2-receptor as both antagonists inhibited lipolysis with low affinity, betaxolol being approximately two orders of magnitude weaker than its affinity for typical β_1-receptors (Boudot *et al.*, 1979; Bojanic & Nahorski, 1983) and ICI 118.551 more than one hundred fold weaker than at β_2 sites (O'Donnell & Wanstall, 1980; Dickinson & Nahorski, 1981; Carswell & Nahorski, 1983; Bojanic & Nahorski, 1983).

The slopes of the Schild plots obtained with betaxolol against both agonists were significantly below unity and do not suggest simple competitive interactions. Since betaxolol concentrations were corrected for protein binding, other factors which could influence this analysis (see Discussion) must be taken into account.

Cyclic AMP accumulation in adipocytes and adenylate cyclase in membranes

All the antagonists used produced dose-dependent shifts to the right of the isoprenaline dose-response curve with both whole cell and membrane models. Figure 3 shows antagonism by betaxolol of isoprenaline-stimulated adenylate cyclase in adipocyte membranes. Although the maximal stimulation for cyclic AMP generation appeared to decrease somewhat with higher antagonist concentrations, the dose-response curves remained parallel to one another at all concentrations examined, and the EC_{50} of isoprenaline calculated was the concentration required to reach 50% of the reference isoprenaline dose-response curve. The apparent decrease in maximal stimulation at high agonist/antagonist concentrations may be due to interference either in the enzyme system or protein binding assay rather than to non-competitive interactions which would not render the curves parallel to each other.

The corresponding Schild plot yields a pA_2 value for betaxolol that is atypical for a β_1-receptor. Results in Table 1 show the pA_2 values and Schild slopes for the other antagonists used. In all cases, the values from whole cells and membranes agree closely and again do not correspond to either a classical β_1- or β_2-receptor, indicating that the atypical β-receptor is linked to adenylate cyclase and associates cyclic AMP in the atypical lipolytic response to adrenoceptor agonists.

Relationship between affinities of antagonists at [^{125}I]-cyanopindolol binding sites and pA_2 values against isoprenaline-stimulated adenylate cyclase in adipocyte membranes

The affinities of the four drugs studied using receptor binding techniques with [^{125}I]-cyanopindolol (Bojanic & Nahorski, 1983) have been compared to the

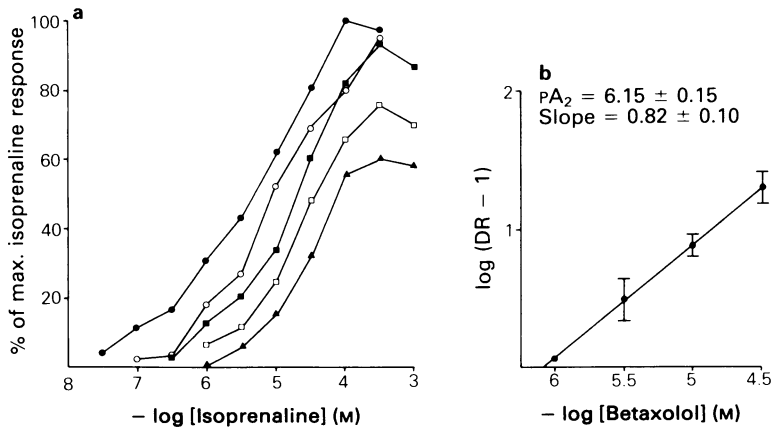

Figure 3 (a) Dose-response curves of isoprenaline-stimulated adenylate cyclase activity in adipocyte membranes in the absence (●) or presence of betaxolol, 10^{-6} M (○), 3×10^{-6} M (■), 10^{-5} M (□) and 3×10^{-5} M (▲). (b) The corresponding Schild plot shows the mean values and the vertical lines s.e.mean of three separate experiments.

Table 1 pA_2 values and Schild slopes (in parentheses) of β-adrenoceptor antagonists against isoprenaline-stimulated cyclic AMP accumulation in whole cells and adenylate cyclase in membranes

Effector response	*Betaxolol (β_1-selective)*	(n)	*ICI 118.551 (β_2-selective)*	(n)	*(−)-Propranolol*	(n)	*(+)-Propranolol*	(n)
Cyclic AMP accumulation	6.15 ± 0.20 (0.84 ± 0.12)	(4) NS	6.35 ± 0.24 (1.11 ± 0.08)	(5) NS	7.35 ± 0.23 (1.22 ± 0.18)	(5) NS	6.39 ± 0.27 (0.98 ± 0.11)	(6) NS
Cyclic AMP accumulation (corrected for protein binding)	6.30 ± 0.21 (0.79 ± 0.11)	NS	6.59 ± 0.26 (1.01 ± 0.07)	NS				
Adenylate cyclase	6.15 ± 0.15 (0.82 ± 0.10)	(3) NS	6.43 ± 0.16 (0.91 ± 0.15)	(4) NS	7.81 ± 0.27 (0.68 ± 0.11)	(5) *	6.78 ± 0.17 (0.50 ± 0.04)	(3) **

NS = slops not significantly different from unity; $*P < 0.05$, $**P < 0.01$.

apparent pA_2 values against isoprenaline-stimulated cyclase (Figure 4). Clearly, there is a poor correlation between the two approaches. Betaxolol displayed the greatest difference in affinity followed by (−)-propranolol and ICI 118.551. (+)-Propranolol on the other hand, had a very similar affinity for both systems.

Discussion

Catecholamine-stimulated lipolysis in the rat has been classified as a β_1-receptor-mediated response (Lands *et al.*, 1967). In agreement with this classification, we have recently identified high affinity specific binding sites on rat adipocyte membranes that possess all the properties of a classical β_1-adrenoceptor (Bojanic & Nahorski, 1983). There is, however, considerable alternative evidence to suggest that the rat adipocyte β-adrenoceptor is atypical (Stanton, 1972; Harms *et al.*, 1974; 1977; Harms & Van der Meer, 1975; De Vente *et al.*, 1980; Wilson *et al.*, 1984). This evidence has been based entirely on analysis of lipolytic responses to agonists and antagonism by selective and non-selective β-adrenoceptor blockers. In the present study, we have confirmed and extended these observations and, in view of the close relationship between cyclic AMP production and lipolysis (Butcher *et al.*, 1965) we have also demonstrated that this atypical receptor appears to mediate cyclic AMP generation in whole cells and membranes. To our knowledge this is the first example of a β-adrenoceptor not conforming to β_1 or β_2 classification linked to adenylate cyclase.

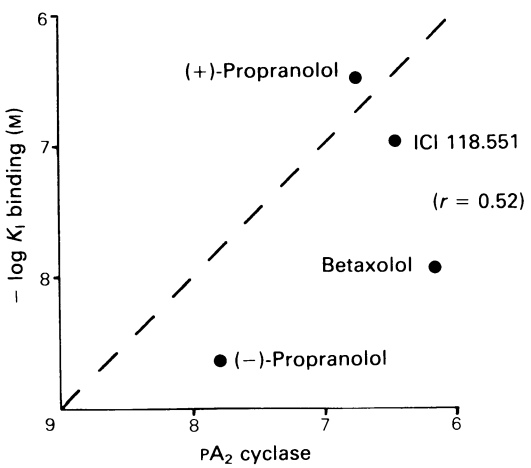

Figure 4 Comparison of pA_2 values obtained from inhibition of isoprenaline-stimulated adenylate cyclase activity versus K_i values obtained from ligand binding studies (Bojanic & Nahorski, 1983). The correlation coefficient (r) was calculated by linear regression of the four points.

The availability of the highly selective β_1- and β_2-receptor antagonists, betaxolol and ICI 118.551, has allowed us to strengthen further the proposal made by one of us (De Vente *et al.*, 1980) that the rat adipocyte possesses a hybrid or dualistic β-adrenoceptor rather than a typical β_1 or β_2 site or, indeed, a mixture of both. Noradrenaline (β_1-selective) and fenoterol (β_2-selective) are full lipolytic agonists as indeed are other β_2-agonists such as salbutamol (Frisk-Holmberg & Östman, 1977) and hexoprenaline (Lipshitz & Vinik, 1978). Therefore, the co-existence of β_1- and β_2-subtypes in adipocytes would be revealed when analysing the effect of selective β_1- and β_2-receptor antagonists on the lipolytic responses to these agonists (Zaagsma *et al.*, 1979; 1983; O'Donnell & Wanstall, 1980; Carswell & Nahorski, 1983). As no difference in antagonist pA_2 values was found against different agonists, it can be argued that the receptor response on adipocytes cannot be mediated by both β_1- and β_2-adrenoceptors. Furthermore, this receptor (or receptors) cannot be

classified as either a β_1- or β_2-adrenoceptor since the pA_2 values with betaxolol or ICI 118.551 are much weaker than those selectively expressed at each subtype (see O'Donnell & Wanstall, 1980; Dickinson *et al.*, 1981; Zaagsma *et al.*, 1983; Bojanic & Nahorski, 1983). Although the previous conclusions are justifiable, analysis of this receptor(s) is complex. The Schild slopes with betaxolol are significantly below unity and are not indicative of a simple competitive interaction between agonist and antagonist. This complication is not due to binding of the antagonist to bovine serum albumin present with the fat cell suspension, since each antagonist concentration was corrected for protein binding.

The results from the present study further demonstrate that with cyclic AMP accumulation in whole cells and, indeed, adenylate cyclase in membranes, the pA_2 values of betaxolol and ICI 118.551 are atypically low and similar to those obtained with lipolysis. Furthermore, the non-selective antagonists (+)- and (−)-propranolol, in agreement with previous studies (Harms *et al.*, 1977), display a degree of stereoselectivity that is smaller than that seen at typical β-adrenoceptors. Schild slopes for both isomers were significantly different from 1 in adenylate cyclase assays, not suggesting simple competitive interactions. However, although the pA_2 values found should not be extrapolated to K_B values, the value for (−)-propranolol is still far too low for classical β-receptors which are apparently identified in binding studies within the same tissue (Bojanic & Nahorski, 1983). This suggests that the reason for the atypical pA_2 values is not one of simple distribution and accessibility of the drug for the receptor, since one would anticipate that both isomers of propranolol (with identical physico-chemical properties) should gain equal access to the receptor.

In contrast to the present results, other well characterized systems (Kaumann & Birnbaumer, 1974; Minneman *et al.*, 1979; Carswell & Nahorski, 1983; Robberecht *et al.*, 1983; Waelbroeck *et al.*, 1983) show a good correlation between drug affinities identified in binding studies and those found using adenylate cyclase or classical functional studies. However, in the rat white fat cell, the receptor subtype labelled directly is β_1 (Bojanic & Nahorski, 1983) but the effector response is atypical.

It is possible that the rat adipocyte contains a heterogeneous population of β_1- and atypical β-receptors with the β_1-subtype not normally being well coupled to adenylate cyclase and, therefore, not involved directly in functional responses. An example of this phenomenon is to be found in human atria which possess both β_1- and β_2-receptors as identified with radioligand binding studies while only the β_2-receptor is directly coupled to the adenylate cyclase (see Robberecht *et al.*, 1983; Waelbroeck *et al.*, 1983). An alternative possibility is that the atypical β-receptor is quantitatively more abundant than the β_1-receptors and therefore in dominant control of lipolysis. It is also possible that endogenous mediators i.e. adenosine (Schwabe *et al.*, 1973) and prostaglandins (Bergstrom, 1967; Fredholm, 1978) could interact with effector responses such as lipolysis and cyclic AMP accumulation, and may exert an effect on the efficiency of coupling of the β_1-receptors. Indeed, these mediated effects would be only observed in whole cell systems where the receptor reserve is large, and it could be of interest to examine responses in whole cells under conditions in which the production and action of these mediators is suppressed. However, the atypical β-adrenoceptor is observed in washed membrane preparations where adenosine and prostaglandin production is minimal. Therefore, the atypical β-adrenoceptor is clearly involved in mediating all the effector responses studied.

The ratio of typical β_1- to atypical β-receptors may be important in determining the overall 'β-adrenoceptor mediated response'. Preliminary evidence from hamster white adipocytes (that appear to lack high affinity β-adrenoceptor binding sites identified with [^{125}I]-cyanopindolol and have a very atypical cyclase response) and human adipocytes, that have a high density of β-adrenoceptor binding sites and a lipolytic response more characteristic of a β_1-receptor (Harms, 1976), support this suggestion. Present studies using selective irreversible antagonists for typical β_1- and β_2-receptors may provide better evidence for or against this concept.

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