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Reproducible Withdrawal Contractions of Isolated Guinea-pig Ileum after Brief Morphine Exposure: Effects of Clonidine and Nifedipine

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Abstract—Guinea-pig ileum stored for 30 min in Krebs solution and then mounted in Tyrode solution gave reproducible contracture responses to naloxone after brief exposure to morphine. The preparation lasted for several hours and a variety of pharmacological tests could be made. Clonidine, an α_2-adrenoceptor agonist, and nifedipine, a calcium channel antagonist, both known to interfere with tolerance and physical dependence, inhibited naloxone withdrawal contractures in a dose related way. Their action seemed to be receptor-mediated since yohimbine and Bay k 8644, respectively, reversed their inhibitory effect.

The guinea-pig ileum has been extensively used as a model to study opiates and opioid peptides, the effects of which on the enteric nervous system are believed to mimic those on the central nervous system (Paton 1957; Kosterlitz & Waterfield 1975; North et al 1980; Szerb 1982; Garzòn et al 1987).

Attempts have also been made to induce in the preparation changes having the characteristics of the acute and chronic effects of opiates on the whole animal. Several studies have shown that the isolated ileum or the longitudinal muscle-myenteric plexus preparations from guinea-pigs dependent on morphine respond to naloxone with a powerful contraction (Ehrenpreis et al 1972; Schulz & Herz 1976; Gintzler 1980; Chahl & Thornton 1987).

Since the ileum can be made dependent in-vitro by incubation with opiates, this indicates that the enteric nervous system alone is responsible for the development of dependence (Lujan & Rodriguez 1981; Collier et al 1981a). It has been reported that the development of ileum dependence occurs after just 0·5–2 min exposure to opioids, when naloxone or washout induces withdrawal contractures (Chahl 1983, 1986).

That observation offered a simple model for the study of acute dependence, facilitating investigations on incipient molecular mechanisms involved in dependence development and on the expression of the abstinence syndrome. However, only responses with enkephalin were reproducible after washout and tissue preparations could no longer be used after morphine and naloxone challenge (Chahl 1986). The present work was undertaken to set up a simple and rapid preparation of ileum with reproducible responses to a challenge with naloxone after brief exposures to morphine. We also tried to establish whether this model possessed some of the characteristics of intact animals. To do this we studied the effects of clonidine, an α_2-adrenoceptor agonist which is known to antagonize some withdrawal symptoms in animals and man, and nifedipine, a calcium channel antagonist. There is evidence that calcium plays a role in the antinociceptive action of opiates as well as in the development of tolerance and physical dependence (Harris et al 1976; Bongianni et al 1986; Shah et al 1987a) and binding sites for calcium blocking drugs have been found in intestinal smooth muscles (Bolger et al 1983).

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Materials and Methods

Methods

The experimental procedure was that of Chahl (1986) with modifications. Male guinea-pigs (300–350 g) purchased from Morini (Italy) were killed by a blow on the head. The terminal portion of the ileum, discarding the 10 cm nearest the caecum, was kept in a Petri dish with Krebs solution (mM: NaCl 118, KCl 4·7, $CaCl_2$ 2·5, KH_2PO_4 1·18, $MgSO_4$ 7, H_2O 1·2, $NaHCO_3$ 25, glucose 11·1) for 30 min and then washed free of faecal matter. Two to four segments, 2–3 cm long, from the same animal were set up under 1 g tension in 5 mL organ baths containing Tyrode solution (mM: NaCl 137, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·05, NaH_2PO_4 0·4, $NaHCO_3$ 11·9, glucose 5). The baths were maintained at 37°C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The segments were allowed to equilibrate for 40–60 min without washing and the response to acetylcholine (ACh) was determined two or three times (10^{-6} M) so that responses could be expressed as percentages of the ACh maximum. After a further 20–30 min, morphine was added to the baths and 5 min later the ileum was challenged with naloxone. Tension changes were recorded by an isotonic force transducer (Ugo Basile, 7006 Italy).

Atropine (2×10^{-6}M), clonidine ($3{\cdot}5\text{–}3 \times 10^{-9}$M), nifedipine ($1\text{–}5 \times 10^{-8}$M) were added to the bath 1 min before naloxone, while yohimbine (2×10^{-8}M) and Bay k 8644 (methyl 1,4-dihydro-2,6-diemthyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (10^{-7}M) were added 30 s before clonidine and nifedipine, respectively. After the response to naloxone, the preparations were washed, so the opioid antagonist was in contact with tissues for not more than 1–2 min.

Drugs

Drugs used were: morphine hydrochloride and naloxone hydrochloride, SIFAC (Milan, Italy), clonidine hydrochlor-

ide and yohimbine hydrochloride, Sigma Chemical Company (St. Louis, USA), nifedipine, Pfizer (New York, USA). All drugs were dissolved in deionized water and ethanol (1:1) to make up 1 mg mL^{-1} solution from which further dilutions were prepared before use.

Results

Time course of naloxone-precipitated contractures of morphine-dependent ileum

The addition of morphine (10^{-6} M) to the bath produced relaxation and inhibition of movements of the preparation (Fig. 1). Following a 5 min exposure to the opiate, naloxone (10^{-7} M) induced a strong contracture (about 60% of the ACh maximum). After washout and resting periods of about 10 min, a further addition of naloxone (10^{-6} M) elicited contractions decreasing with time up to 30 min by when contracture to naloxone was not easily distinguished from spontaneous tissue activity. Naloxone did not produce effects on "naive" preparations or those washed after morphine contact.

Reproducible contractures to naloxone after morphine contact

A typical tracing of contracture responses of the ileum to repeated challenges with morphine and naloxone is shown in Fig. 2. Under our conditions the first contact with morphine followed by naloxone induced a response which was not the maximum obtainable (as in the tracing) for the doses used. However, after washout and a 30 min interval between tests to allow the dependence state to fade out (Fig. 1), a further 5 min exposure to morphine 10^{-6} M followed by naloxone 10^{-6} M elicited reproducible responses.

Contractures were blocked by atropine injected into the bath 1 min before naloxone. This also occurred after many challenges with morphine and naloxone, indicating no tachyphyllaxis (ACh mediated contractions). Following washing and the 30 min resting period, the ileum responded again to morphine and naloxone with the same intensity. The

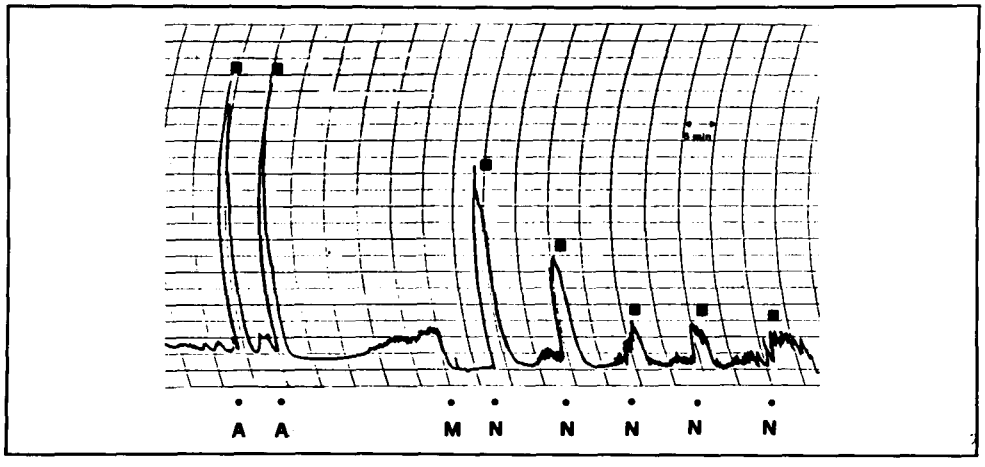

FIG. 1. Time course of guinea-pig ileum contracture to naloxone 10^{-7} M (N) after 5 min exposure to morphine 10^{-6} M (M). A = acetylcholine 2×10^{-6} M, ■ washout.

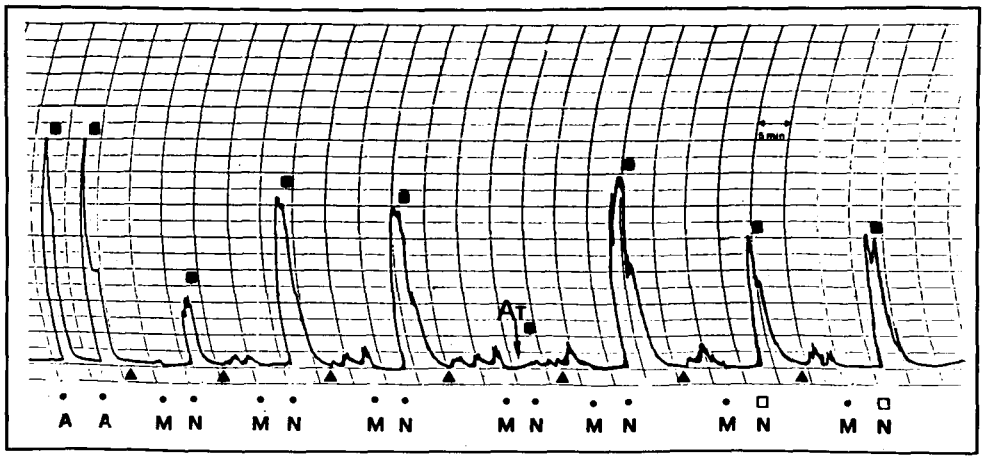

FIG. 2. Reproducible contractions of guinea-pig ileum to naloxone (N) ● 10^{-6} M □ 10^{-7} M after 5 min exposure to morphine 10^{-6} M (M) and effect of atropine 2×10^{-6} M (AT). A = acetylcholine 2×10^{-6} M, ■ washout, ▲ 25–30 min intervals between two tests.

highest contractures for both morphine and naloxone (10^{-6} M) was 68·3 ± 3·18 of the maximum contracture to ACh (36 preparations from 15 animals); no further increase was obtained by raising concentrations of agonist and antagonist. By the use of a lower concentration of naloxone, the contracture intensity in the same tissue preparation was smaller but reproducible.

A more detailed analysis of the responsiveness of the ileum preparation to different doses of naloxone following a constant concentration of morphine 10^{-6} M is shown in Table 1; contractures are expressed as the percentage of that produced by naloxone 10^{-6} M. Naloxone-induced contractures were dose-dependent but the intensity of the response, while generally constant in segments from the same animal, could differ between animals. Ilea from most animals (Table 1, A) responded to naloxone 3×10^{-7} M with almost maximal contractures and a concentration of 5×10^{-8} M gave 12·4 ± 9·84% of the maximum. However, even at the latter concentration some ilea gave an almost maximal contraction (Table 1, B).

Table 1. Responsiveness of ilea from different animals (A, B) to various concentrations of naloxone following 5 min exposure to 10^{-6} M morphine. In brackets number of preparations.

Naloxone concn (M)	% of response to naloxone 10^{-6} M	
	A (10)	B (4)
3×10^{-7}	96·8 ± 3·20	100 ± 00
1×10^{-7}	53·8 ± 10·4	100 ± 00
5×10^{-8}	12·4 ± 9·48	95·7 ± 3·61
$2{\cdot}5 \times 10^{-9}$	—	8·7 ± 5·15

A variation in sensitivity of ilea from different animals was also observed with morphine. The response elicited by naloxone (10^{-6} M), in most tissues, was dose-related to the

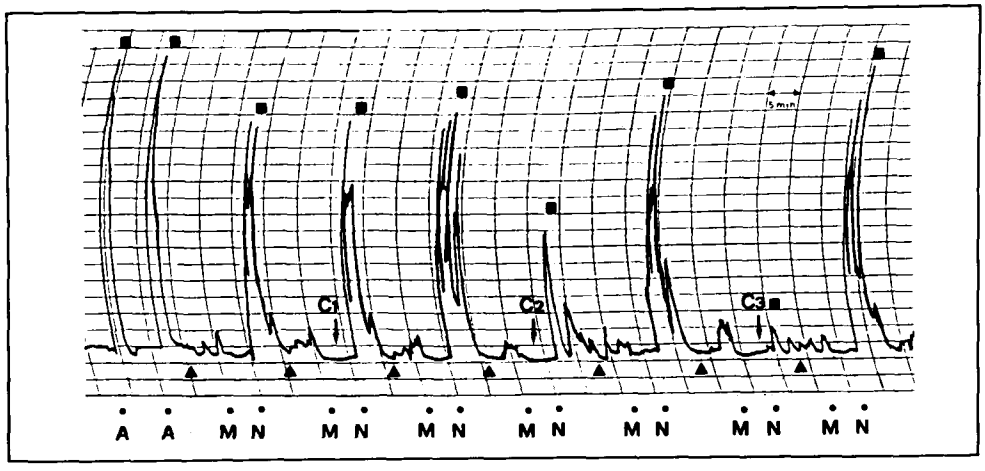

FIG. 3. Dose-dependent inhibition by clonidine $3{\cdot}5 \times 10^{-9}$ M (C_1), 15×10^{-9} M (C_2), 35×10^{-9} M (C_3) of guinea-pig ileum contractions to naloxone 10^{-7} M (N) after 5 min exposure to morphine 10^{-6} M (M). A = acetylcholine 2×10^{-6} M, ■ washout, ▲ 25–30 min intervals between two tests.

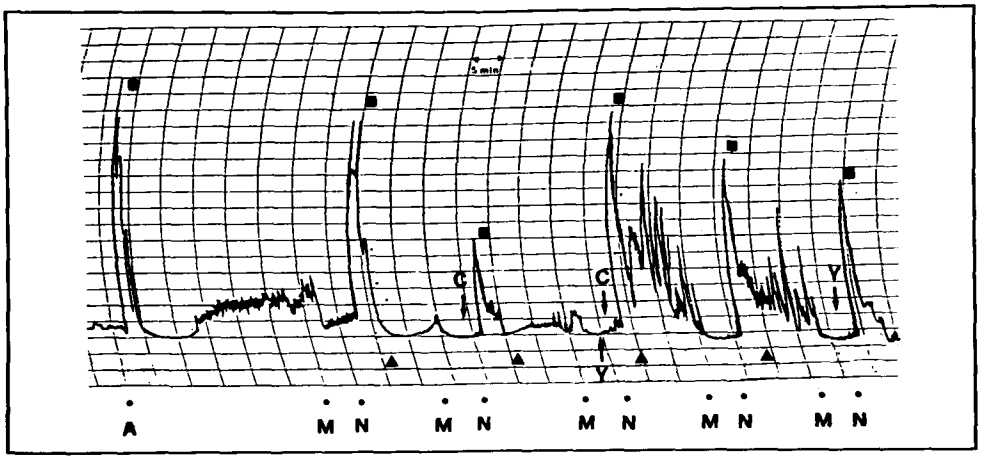

FIG. 4. Clonidine 2×10^{-8} M (C) inhibition antagonism by yohimbine 2×10^{-8} M (Y) of naloxone 10^{-7} M (N) contracture of ileum exposed for 5 min to morphine 10^{-6} M (M). A = acetylcholine 2×10^{-6} M, ■ washout, ▲ 25–30 min intervals between two tests.

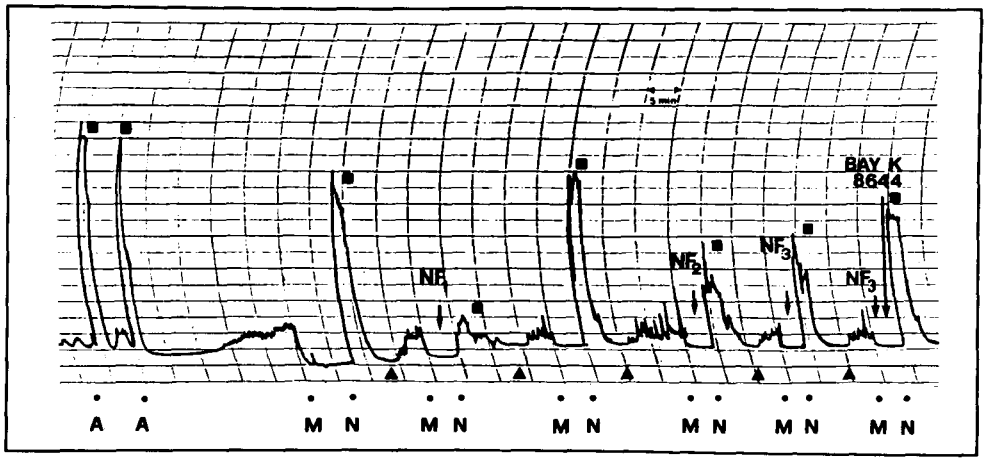

FIG. 5. Dose-dependent inhibition by nifedipine 5×10^{-8} M (NF_1), $2{\cdot}5 \times 10^{-8}$ M (NF_2), 1×10^{-8} M (NF_3) of guinea-pig ileum contracture to naloxone 10^{-6} M (N) after 5 min exposure to morphine 10^{-6} M (M) and nifedipine 1×10^{-8} M (NF_3) antagonism by Bay k 8644 1×10^{-7} M. A = acetylcholine 2×10^{-6} M, ■ washout, ▲ 25–30 min intervals between two tests.

amount of morphine in the range of 5×10^{-8} to 10^{-6} M. However, at the lowest concentration some ilea gave contractions that were about maximal.

Effects of clonidine and yohimbine

The dose-dependent inhibition by clonidine of the guinea-pig ileum contraction to naloxone 10^{-6} M is shown in Fig. 3. Some ilea responded to naloxone with phasic and tonic contractions. Low concentrations of clonidine ($3{\cdot}5 \times 10^{-9}$ M), injected 1 min before naloxone had little effect on the phasic contractions and none on the tonic effects elicited by opiate antagonists. Higher concentrations inhibited both the phasic and tonic contractions. Nevertheless, ileum preparations responded similarly and with the same intensity to morphine and naloxone after repeated tests.

The effect of clonidine seemed to be specific and receptor mediated, since yohimbine, an α_2-adrenoceptor antagonist, injected into the bath 1 min before clonidine blocked its inhibiting effect (Fig. 4). Yohimbine alone had no effect on the intensity of naloxone withdrawal contractures.

Experiments repeated with naloxone concentrations lower than those used in Fig. 4, and giving smaller submaximum contractures, confirmed that yohimbine counteracted the effects of clonidine but did not affect the naloxone response of dependent ilea when administered alone.

Effects of nifedipine and Bay k 8644

Fig. 5 shows a dose-dependent inhibition by nifedipine of the ileum withdrawal contraction. A concentration of 10^{-8} M of the drug inhibited the response by about 50%. Bay k 8644 reversed the effect of nifedipine, 10^{-8} M, but not completely.

Discussion

The results clearly demonstrate that brief contacts with morphine induce a high degree of dependence in the guinea-pig isolated ileum which can be observed only by naloxone precipitated withdrawal, thus confirming a previous study (Chahl 1986).

Under our conditions, the first challenge with morphine followed by naloxone normally elicited a sustained withdrawal contracture which was reproducible for up to 6 h. The effects of opiate agonists and antagonists are reversible and even after 30 min intervals the ileum preparations showed the same sensitivity in further tests, this also occurred after administration to the bath of drugs such as atropine, clonidine, yohimbine and nifedipine.

In pilot experiments on withdrawal responses of guinea-pig ileum to morphine, we noted that Krebs solution (Lujan & Rodriguez 1981) was useful in short experiments as motor activity was increased with time; on the other hand the responses in Tyrode solution alone were not reproducible (Chahl 1986).

The preincubation of ilea in Krebs solution for 30 min or longer, and the subsequent perfusion in Tyrode solution, alters organ behaviour so that the response to the first or second contact to morphine-naloxone is the highest possible and best reproducible for the doses used, and is unchanged by excessive spontaneous activity. Although it is not clear how brief exposure to Krebs solution changes the tissue responsiveness, it seems that some constituents, probably the K^+ ions which are present at much higher concentrations in Krebs than in Tyrode solution, are able to alter the ileum response permanently. It is well established (McFadzean 1988) that ionic mechanisms play an essential role in opioid actions.

Acute physical dependence, that is the precipitated opioid withdrawal symptoms produced by the administration of an opioid antagonist after brief exposure to an opioid agonist, is a relatively unexplored means of investigating the complex and as yet incompletely known processes leading to the development of opiate dependence and to manifestations of the abstinence syndrome (Valeri et al 1989). Studies in animals and man have shown that this phenomenon is characterized by as many signs and symptoms as those observed in long term treatment, thereby confirming its similarity to chronic physical dependence (Martin & Eades 1961; Smits 1975; Eisenberg & Sparber 1979; Eisenberg 1983;

Bickel et al 1988). This approach is a useful means of examining the incipient stages involved in the response to opioid administration and of avoiding more complex adaptive changes resulting from chronic exposure. However, in the whole animal, many variables may confuse the analysis of the biochemical mechanisms underlying dependence even after a single dose of opioids. The study of acute dependence in a simpler system than the isolated organ would be a more suitable tool to illustrate the first molecular processes leading to this condition.

In this context it is particularly attractive to be able to obtain contractions which are completely atropine sensitive without the involvement of non-cholinergic ones, as reported in other experimental conditions where more complex adaptive changes could have occurred (Tsou et al 1982; Collier et al 1981a; Chahl 1983, 1985a; Chahl & Thornton 1987). We therefore focused on those pharmacological characterizations of this cholinergic withdrawal response which seem the simplest obtainable from an isolated preparation in a dependent state.

Clonidine is an α_2-adrenoceptor agonist which shares with opiates an antinociceptive activity (Fielding et al 1978; Browning et al 1982), the ability to reduce the release of acetylcholine from the final motor neurons of the guinea-pig ileum (Drew 1978; Browning et al 1982) and to counteract the effects of opiate withdrawal (Gold et al 1978, 1982). This last property has been widely studied in animals (Tseng et al 1975; Meyer & Sparber 1976) but little attention has been given to the in-vitro preparation (Collier 1981b). Clonidine at low concentrations and in a dose-related manner reduces naloxone-induced ileum contractions as in the whole animal. A similar result has been obtained by Chahl (1985b) with Met-enkephalin. The clonidine effect is receptor mediated since yohimbine is able to counteract its inhibitory effect. The present observation that yohimbine does not increase withdrawal contractures, even to doses of opiate agonists and antagonists which give submaximal responses, is in accordance with some in-vivo studies (Nakaki et al 1980; Van der Laan 1985), but in contrast with other data (Dwoskin et al 1983). Besides the obvious differences between in-vitro and in-vivo studies, it must be observed that in the guinea-pig ileum the ultimate response to α_2-adrenergic and opiate agonists is via the cholinergic neuron, but a comprehensive understanding of the events occurring when these receptors are activated or inhibited is still lacking (Del Tacca et al 1988) as also is knowledge of what takes place at different points between receptor interactions and acetylcholine release.

Among the many factors that could modulate acetylcholine release, the effect of calcium is an example because of its fundamental role in many physiological and pharmacological events. Evidence has accumulated that calcium antagonistic activity could mediate some actions of morphine (Ramaswamy et al 1986; Del Pozo et al 1987; Shah et al 1987b; Ramkumar & El-Fakahany 1988) as well as some events determined by α_2-adrenoceptor activation (Bartfai & Vizi 1986). Calcium channel blockers have been shown to inhibit the expression of the naloxone-precipitated abstinence syndrome in-vivo, suggesting a role for calcium channels in morphine abstinence (Harris et al 1975, 1976; Bongianni et al 1986; Baeyens et al 1987).

Our results showing the inhibition by low doses of nifedipine of naloxone-precipitated withdrawal further stress the similarity to abstinence in-vivo and in-vitro, after brief exposure to opiate. Similar results have been published during the preparation of this paper with verapamil and cis-diltiazem (Barrios & Baeyens 1988). The mechanism underlying these effects is not clear.

As the guinea-pig ileum cholinergic response may be modulated by various neuronal activities (Drew 1978; Garzon et al 1987), a more detailed study is needed to ascertain at what level the effects of calcium channel agonists and antagonists are exerted in morphine withdrawal contractures. For this purpose the present working procedure could be a useful tool since this isolated ileum preparation is simple and versatile. Comparable responses, in the same preparation, can be obtained with different substances in a relatively short time. Working at submaximal response it is possible to ascertain factors and drugs capable of inhibiting or exacerbating the withdrawal contraction in-vitro.

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