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Double stimulation modulates afterhyperpolarization phase following action potentials evoked in rat motoneurones

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Abstract. The influence of a pair of stimuli running in time sequence between 5–10 ms (a doublet) on the basic parameters of antidromic action potentials was studied in rat motoneurones. Electrophysiological experiments were based on stimulation of axons in the sciatic nerve and intracellular recording of antidromic action potentials from individual motoneurones located in L4–L5 segments of the spinal cord. The following parameters were analyzed after application of a single stimulus and a doublet: amplitude and duration of the antidromic spike, amplitude, total duration, time to minimum, half-decay time of the afterhyperpolarization (AHP). It was demonstrated that application of a pair of stimuli resulted in: (1) a prolongation of action potentials, (2) a prolongation of the total duration and half-decay time of the AHP, (3) a decline of the time to minimum of the AHP, (4) an increase of the AHP amplitude of the spike evoked by the second stimulus. Significant differences in AHP parameters were found either in fast or slow motoneurones. We suppose that doublet-evoked changes in the AHP amplitude and duration are linked to intrinsic properties of individual motoneurones and may lead to the prolongation of the time interval to subsequent motoneuronal discharges during voluntary activity.

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INTRODUCTION

The doublet is usually described as a pair of impulses, running at an interval shorter than 10 ms. This has been most commonly observed at the beginning of a firing pattern in spinal motoneurones, being the simplest form of natural repetitive activation of motor cells in mammals (Bawa and Calancie 1983, Kirkwood and Munson 1996). Burke and coauthors (1970, 1976) have demonstrated in feline motor units that one additional pulse at a short interval leads to force enhancement ("the catch-like property") in both fast and slow motor units, although the more pronounced effect has been observed in the first part of a tetanus in slow units. Investigations of Stein and Parmiggiani (1979) and Zajac and Young (1980) have revealed in the cat that the initial interpulse interval in a range between 5 and 10 ms is optimal for reaching the maximum tetanic tension during stimulation of motor units by two pulses. Studies on effects of an initial doublet in the rat extensor digitorum longus (EDL) and soleus (Sol) muscles (Hennig and Lömo 1987) have confirmed previous observations in cats. These authors have shown that forces produced by motor units reach higher levels when the stimulation train starts with a doublet. In freely moving rats doublets have been observed in the fast EDL muscle; therefore, it has been suggested that generation of doublets is limited to fast fatigable units, which are recruited during strong contractions in order to produce the extra tetanic forces. On the other hand, experiments with intracellularly applied stimulating currents have shown that motoneurones innervating slow motor units are able to fire with initial doublets, as was shown by Spielmann and colleagues (1993). Moreover, Celichowski and Grottel (1998), investigating the influence of a doublet in the rat medial gastrocnemius (MG) motor units, have reported that after stimulation with a pair of pulses, the force and area under the force record significantly increase in all types of units. Slow units appear to be more sensitive to this kind of stimulation than fast ones.

It has been suggested in several studies that the firing rate of motoneurones depends to a certain degree on the afterhyperpolarization period (AHP), which follows the action potential, when recorded from neuronal cell bodies or dendrites. The AHP develops as a result of a significant increase of potassium conductance, based on activity of calcium dependent types of potassium channels (Barrett et al. 1980, Delgado-Lezama and Hounsgaard 1999). As a consequence of this process, the excitability of motoneurones becomes very low after the spike generation and gradually increases during the AHP period (Kernell 1984). The AHP duration amounts to 30–116 ms in the rat (Bakels and Kernell 1993a,b) or 50–200 ms in the cat motoneurones (Eccles et al. 1958, Cope et al. 1986). It has also been demonstrated in studies with a technique of steady current injection that the interval between subsequent motoneuronal spikes is almost equal to the AHP duration (Kernell 1965, 1984). Therefore, the AHP period has been acknowledged as the main factor responsible for setting the maximal frequency of motoneuronal discharges.

Until now, the influence of an initial doublet with respect to the AHP parameters of single motoneurones has not been investigated *in vivo*. Therefore, the purpose of this electrophysiological study performed on rat motoneurones was to compare the AHP duration and amplitude after the doublet and after a single stimulus, either in fast or slow types of motoneurones. We applied the electrophysiological method of intracellular recording of action potentials from motoneurones, as evoked by antidromic activation by single and double stimuli.

METHODS

Preparation

Experimental procedures were accepted by the Local Ethics Committee and followed the Polish Law on the Protection of Animals, and EU guidelines. Eleven male Wistar rats weighing between 470 and 520 g were used in this study. Animals were anaesthetized with pentobarbital sodium (Morbital, Biowet Puławy; initial dose 60 mg/kg i.p., additional doses of 10 mg/kg, i.p., supplemented after the first three hours). The depth of anesthesia was controlled by monitoring withdrawal reflexes during preparation and the heart rate (300–360 bpm) by ECG recording.

Each experimental session started with preparation of the right femoral vein (to insert a cannula for drug administration) and intubation of the trachea for artificial ventilation. The left sciatic nerve was dissected free and cut distally for electrical stimulation, and laminectomy was performed over lumbar segments of the spinal cord. At the level of L4–L5 spinal segments,

the dura was removed and several small holes in the pia were made to allow introduction of recording glass micropipettes. In two additional experiments, the dorsal roots of L4–L5 spinal nerves were cut in order to provide control results confirming lack of influence of afferent input on AHP parameters measured in motoneurones. All exposed areas of the hind limb and the lumbar spinal cord were covered by warm paraffin oil (37° ± 1°C).

Rats were immobilized in a stereotaxic frame. The muscles were paralyzed in order to minimize respiratory movements during recordings, through intravenous administration of Pancuronium bromide (Pancuronium, Jelfa; first dose of 0.4 mg/kg, supplementary doses of 0.2 mg/kg, applied every 30 minutes). The animals were sacrificed by an overdose of pentobarbital sodium (180 mg/kg) at the end of the experiment.

Stimulation and recording

A single stimulus and a doublet were applied on the sciatic nerve with 0.1 ms duration at a frequency of 3–5 Hz, using a bipolar silver wire electrode connected to a square pulse stimulator (GRASS Company Instruments, model S88). The strength of stimulation pulses amounted up to 0.5 V (in experiments with cut dorsal roots of spinal nerves) or set around two-times threshold for the most excitable fibers in the sciatic nerve, as assessed from the spinal cord surface recordings in the dorsal root entry zone (in experiments with intact dorsal roots).

In order to record intracellular antidromic action potentials (APs) from motoneurones, glass micropipettes with tips broken to 1.5–2.0 μm in diameter (resistance 5–10 MΩ) were filled with 2 M potassium citrate solution and inserted into the grey matter of the L4–L5 segments of the spinal cord. The antidromic character of each AP was recognized on the basis of the constant latency of the spike, and its "all-or-none" appearance. Additionally, in experiments with intact dorsal roots of spinal nerves, a silver ball-tipped monopolar electrode was placed near the dorsal root entry zone in order to record incoming afferent volleys. The central latency of each motoneuronal spike was then assessed on the basis of the first positive peak of the afferent volley to differentiate from synaptically evoked spikes (with latencies usually exceeding 1 ms).

Recordings were amplified (Axon Instruments, Axoclamp model 2B), monitored and displayed on an oscilloscope screen during the experimental session, and stored on a computer disc by an analogue-to-digital 12-bit converter (model RTI-800, sampling rate of 20 kHz).

Experimental protocol and data analysis

The following pattern of stimuli was applied to the sciatic nerve after intracellular penetration of a motoneurone: (1) a single pulse, (2) a 5 s interval, (3) two pulses in the shortest possible time sequence necessary to evoke the second action potential (this time was manually adjusted and varied between 5 and 10 ms).

Five superimposed single recordings were recorded and averaged in a custom-designed laboratory computer program (Analog). Only motoneurones with resting membrane potentials of at least –40 mV and the amplitudes of antidromic responses over 45 mV were considered for further measurement (Gardiner and Kernell 1990).

The following parameters of antidromic action potentials were measured automatically (and manually verified), and compared between motoneurones and between single and double stimuli recordings: (1) the amplitude of the antidromic spike (AP amplitude), (2) its duration (AP duration), (3) the amplitude of the afterhyperpolarization (AHP amplitude), (4) the total AHP duration, (5) the AHP time to minimum, (6) the AHP half-decay time (HDT).

The AP amplitude was measured in millivolts from the baseline to the maximum positive peak; the AP duration was the time (in milliseconds) between the start and the end of the action potential peak at the level of the baseline; the AHP amplitude was the voltage from the baseline to the negative peak; the AHP duration was the time from the spike end to post-hiperpolarization return to the baseline potential; the AHP time to minimum was the time from the spike end to the AHP negative peak; the HDT was the time from the AHP negative peak to the half of this value. Figure 1 presents methods of measurements of the above parameters on records of a single spike (A) and a doublet (B).

Motoneurones collected during the study were divided according to the half-decay times, which were shorter or equal to 20 ms for fast motoneurones and

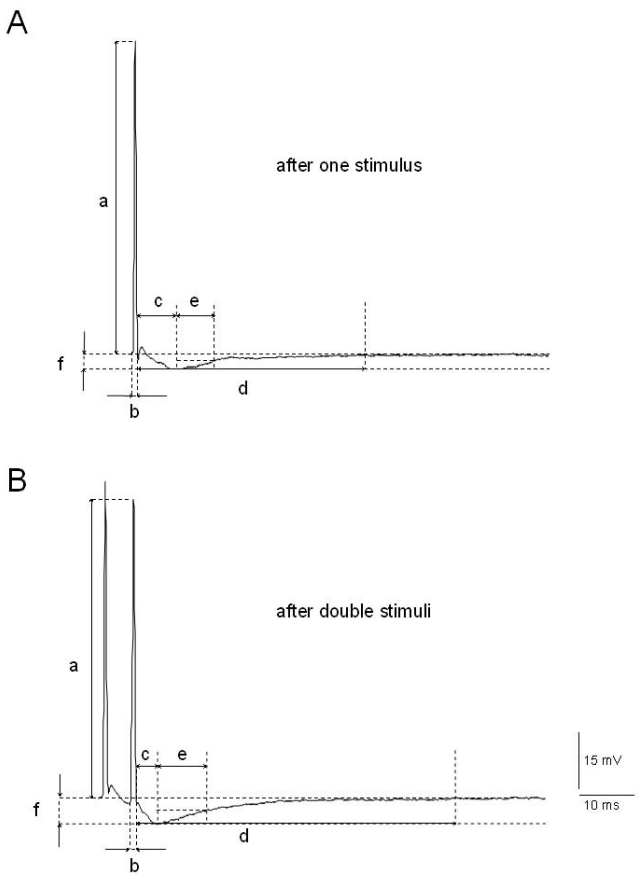

Fig. 1. Basic electrophysiological parameters of action potentials (AP) recorded from a single motoneurone following application of a single stimulus (A) and double stimuli (B). (a) the AP amplitude; (b) the AP duration; (c) the AHP time to minimum; (d) the AHP total duration; (e) the AHP half decay time; (f) the AHP amplitude.

longer than 20 ms for slow motoneurones. This classification was based on results of Gardiner (1993), who described the half decay-time of the AHP as the precise index to distinguish these two groups of rat motoneurones. The HDT were also used for motoneuronal classification in several further studies concerning changes of rat motoneuronal properties after physical training (Beaumont and Gardiner 2002, 2003) and tetrodotoxin-induced paralysis (Cormery et al. 2000). Moreover, the same criterion was useful for division of the cat medial gastrocnemius motoneurones, though due to the longer AHP course, the border value of the HDT amounted to 30 ms (Cope et al. 1986, Eccles et al. 1958, Zengel et al. 1985).

Statistical comparisons of the measured parameters between fast and slow types as well as between the effects of single and double stimuli were made with unpaired and paired Student's *t*-tests, respectively.

RESULTS

Intracellular antidromic action potentials were recorded from 90 motoneurones. Fifty-nine motor cells were determined as fast, while the remaining thirty-one motoneurones were considered as slow. Table I presents mean values and ranges of the measured parameters for both groups of neurones.

No significant differences were found between the basic AP properties (amplitude and duration) of fast and slow motoneurones, but AHP parameters (amplitude, total duration and half-decay time) were significantly different between the two groups. In slow motoneurones the mean AHP amplitude was larger by 24.4% ($P<0.05$), the mean AHP total duration longer by 18.1% ($P<0.001$), and the mean HDT longer by 34.9% ($P<0.001$) with respect to values in fast motoneurones.

The application of two stimuli did not change significantly the AP amplitude, but led to a significant prolongation of the AP duration in both groups of motoneurones (a mean increase of 16.6% and 23.3%, for fast and slow motoneurones, respectively). In all cases ($n=90$) application of the doublet evoked the following changes: an increase of the AHP amplitude (a mean increase of 34.6% and 22.4%, for fast and slow motoneurones, respectively), a prolongation of the AHP total duration (a mean increase of 15.2% and 14.2%, for fast and slow motoneurones, respectively), a decrease of the AHP time to minimum (a mean decrease of 34.4% and 37.3%, for fast and slow motoneurones, respectively), a prolongation of the HDT (a mean increase of 18.8% and 10.4%, for fast and slow motoneurones, respectively). Comparison of statistical significance of differences of the above parameters between fast and slow motoneurones is presented in Table I.

Figure 2 presents two examples of antidromic action potentials evoked by a single and by double stimuli in one fast (A–C) and one slow (D–F) motoneurone. Superpositions of AHP traces reflect the typical influence of the doublet that concern a shortening of the first phase of the AHP (AHP – time to minimum) with a parallel increase of the AHP amplitude, as well as a prolongation of the second phase (AHP–HDT), resulting in an increase of the total AHP duration.

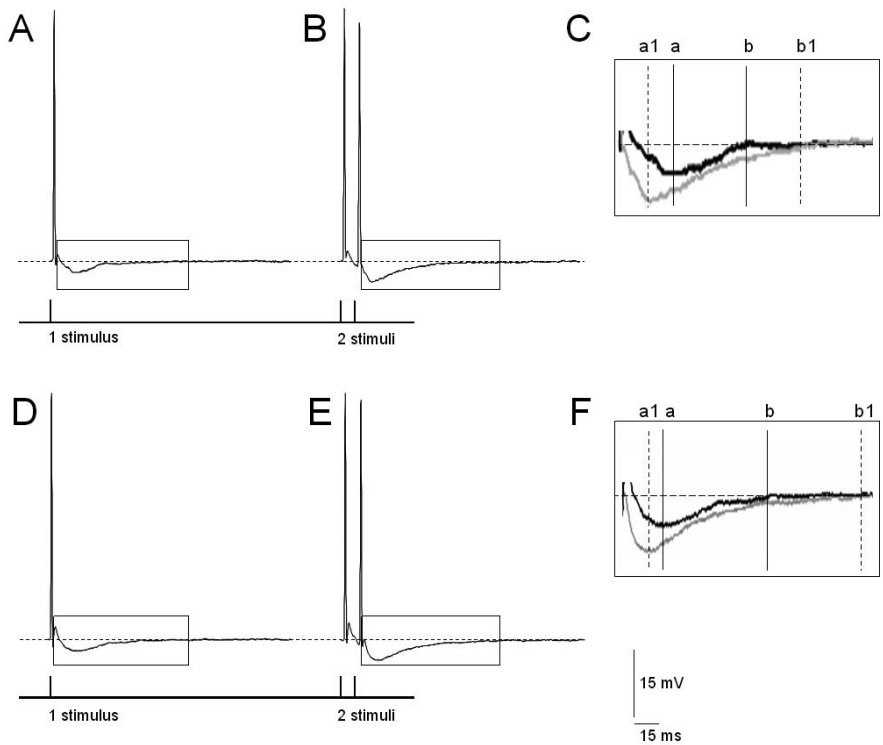

Fig. 2. Comparison of effects of a single stimulus and a doublet in fast (A–C) and slow (D–F) motoneurones. Right-panel (C, F) shows enlarged and superimposed afterhyperpolarization periods [boxes in (A), (B), (D), (E)] recorded after stimulation by single pulse (black lines) and the doublet (grey lines) in fast (C) and slow (F) motoneurones. Vertical lines indicate the AHP times to minimum (a, a1) and the total duration (b, b1) after one stimulus (solid lines) and after the doublet (dashed lines). Calibration bars concern (A), (B), (D) and (E).

DISCUSSION

AHP parameters

Data presented in this study have been collected from motoneurones without identification of the muscle innervated by them because we have stimulated the whole sciatic nerve. In the rat, motoneurones of the tibialis anterior, medial gastrocnemius, lateral gastrocnemius, soleus and plantaris are all located in L4–L5 spinal segments (Nicolopoulos-Stournaras and Iles 1984). The basic parameters of the AHP measured in this study are comprised in the ranges of values that have been previously reported for rat motoneurones of the tibialis anterior and medial gastrocnemius muscles. For the tibialis anterior, Bakels and Kernell (1993b) have obtained the total duration and amplitude of the AHP in the ranges of 32–74 ms and 0.39–4.96 mV, respectively, while Cormery and others (2000) have reported the AHP half-decay time amounting to 19.02 ± 0.8 ms and the AHP amplitude to 3.21 ± 0.19 mV. For the medial gastrocnemius motoneurones, the total duration of the AHP and HDT as well as the AHP amplitude have ranged between 30–116 ms, 6.4–25.8 ms and 0.9–8.0 mV, respectively (Bakels and Kernell 1993a). Gardiner and Kernell (1990) have reported mean values of the total duration of AHP and HDT in these motoneurones amounting to 46 ± 10.1 ms and 11.6 ± 2.8 ms, respectively.

Evident differences in time courses of the AHP between fast and slow motoneurones have been revealed in this paper. The significantly longer AHP total duration has been observed for slow motoneurones (mean 52.1 ms) in comparison to fast motoneurones (mean 42.7 ms). Moreover, a significant difference between the fast and slow motoneurones studied has been found with respect to the AHP amplitude (mean 3.4 mV and 4.5 mV, for fast and slow motoneurons, respectively). These observations are also in agreement with previous reports

Table I

Basic electrophysiological properties (mean values ± SD, and ranges) of the sample of motoneurones studied and statistical significance of differences

	Fast motoneurons (n=59)		Slow motoneurons (n=31)		Statistical significance of differences		
	1 stimulus	2 stimuli	1 stimulus	2 stimuli	fast vs. slow motoneurones (unpaired Student's t-test)	one stimulus vs. a doublet in fast motoneurones (paired Student's t-test)	one stimulus vs. a doublet in slow motoneurones (paired Student's t-test)
AP amplitude (mV)	54.3 ± 9.7 (45.579.3)	53.6 ± 8.1 (45.7–73.9)	55.3 ± 6.5 (45.8–71)	57.2 ± 7.4 (44.8–74.8)	ns	ns	ns
AP duration (ms)	2.5 ± 0.7 (1.4–3.7)	3.0 ± 0.6 (1.7–4.3)	2.3 ± 0.6 (1.4–3.6)	3.0 ± 0.6 (2.0–4.3)	ns	***	***
AHP amplitude (mV)	3.4 ± 1.0 (1.3–5.6)	5.2 ± 2.0 (2.5–14)	4.5 ± 1.6 (2.0–8.3)	5.8 ± 2.5 (2.5–11.2)	*	***	*
AHP duration (ms)	42.7± 9.0 (26.9–61.9)	50.4 ± 9.6 (34.2–77.6)	52.1 ±13.6 (35.4–85.4)	60.7 ± 16.3 (41.8–106)	***	***	*
AHP time to minimum (ms)	9.9 ± 3.3 (4.1–17.2)	6.5 ± 2.2 (3.5–12.3)	9.1 ± 3.5 (4.1–15.4)	5.7 ± 1.4 (3.8–10.1)	ns	***	***
AHP half-decay time (ms)	14.7 ± 2.5 (8.6–18.7)	18.1 ± 3.7 (9.9–26.3)	22.6 ± 1.7 (20.2–6.8)	25.2 ± 3.2 (20.6–36.1)	***	***	***

Data presented separately for fast (the half-decay time <20 ms) and slow (the half-decay time >20 ms) motoneurones. (AP) the action potential (spike); (AHP) the afterhyperpolarization period. ***P<0.001, *P<0.05, ns – not significant.

of Bakels and Kernell (1993a) and Cormery and colleagues (2000). We assume that basic AHP parameters of the sample of motoneurones studied are a reliable basis for analysis of their changes after double stimuli.

Effects of the doublet

We have demonstrated in this study that application of two stimuli in a short interval significantly changes parameters of the AHP in all the motoneurones investigated (Table I). After the doublet, statistically significant increase of the AHP amplitude, half-decay and total duration times, corresponding to the decrease of the AHP time to minimum, seem to be linked to intrinsic properties of motoneurones.

Results obtained in the part of experiments with cut dorsal roots of spinal nerves have enabled us to eliminate an influence of afferent input on AHP parameters measured in motoneurones. No differences between electrophysiological parameters measured in both types of experiments have been found. Therefore, we assume that modulation of the studied AHP parameters by postsynaptic afferent influences may be minimized.

A likely explanation of the shortened time to minimum, larger amplitude of the AHP and its longer duration, observed in motoneurones after the doublet might be higher outflow of potassium ions. This might result

either from the persistent activity of the calcium dependent potassium channels or summation of the effects of the K^+ outflow after the first and the second stimulus, since action potentials in the doublet follow each other at an interval that is considerably shorter than duration of the potassium conductance increase.

It is widely accepted that the AHP duration determines rate of firing of mammalian motoneurones (Kernell 1984). It has been reported by Kernell (1965) that the subsequent action potential evoked in a motoneurone can be generated after return of the AHP evoked by the previous stimulus to the level of a resting potential. Our results have shown that following the doublet, the time course of the AHP in a motoneurone becomes longer, so time to the next potential should be also prolonged. We suppose that this increase of the time interval between spikes may act as a protective mechanism for muscle fibers of motor units from generating too high frequency of motoneuronal discharges. It has been shown in numerous papers (Burke et al. 1970, 1976, Stein and Parmiggiani 1979, Zajac and Young 1980) that the initial doublet creates the optimum pattern for the most effective generation of force in motor units. Therefore, the prolonged AHP might temporarily restrict the rate of motoneuronal firing after application of two stimuli.

The mechanism of AHP prolongation after the doublet stimuli should be considered as an intrinsic property of a single motoneurone, which reduces its ability to generate a firing rate independent of external influences. However, it should be stressed that the prolongation of the AHP described here is not the only phenomenon that restricts the motoneuronal firing rate. The motoneuronal activity (output) could also be regulated by recurrent inhibitory actions from Renshaw cells activated by axon collaterals of motoneurones located in the same motor nucleus or from synergic motoneurones (Hultborn et al. 1988). Moreover, motoneurones receive strong synaptic input from Ia interneuron's mediating reciprocal inhibition (Hultborn et al. 1979, Jankowska and Roberts 1972) and are activated by Ib afferents from Golgi tendon organs during muscle fiber contraction (Jami 1992). The Ib afferents form di-synaptic connections to motoneurones and evoke inhibition of alpha-motoneurones by Ib inhibitory interneurones (Jankowska 1992). Thus, all three mechanisms mentioned above: the activity of Renshaw cells, the contraction-evoked inhibitory effects of Ib afferents, and the presented modulation of the AHP course take part in adjusting motoneuronal firing rate to the level that is optimal for muscle fibers.

Our investigations have been performed under pentobarbital anesthesia that can interfere with rhythmic properties of neurones through direct effects as well as through suppression of various kinds of synaptic activity and actions (Kernell et al. 1999). However, it seems that such effects are more significant and noticeable during constant-intensity sustained or intermittent stimulations of motoneurones leading to the adaptation process (Kernell and Monster 1982, Spielmann et al. 1993). In our experimental procedures, one or two stimuli have been applied in a short 5–10 ms period, so we can assume that anesthesia barely influences the results. However, electrophysiological parameters recorded from anesthetized animals should be regarded only as representing "basic discharge properties" of motoneurones (Kernell et al. 1999). One cannot exclude that during natural physiological activity of motor units the AHP parameters might be additionally modified by postsynaptic influences from afferent and descending pathways.

CONCLUSION

The basic electrophysiological parameters of action potentials measured in fast and slow motoneurones are significantly changed after the doublet. The most significant change appears to be the prolongation of the AHP, and this seems to be independent from postsynaptic influences from the spinal neuronal network. We suppose that doublet-evoked modulations of the AHP parameters are linked to intrinsic properties of individual motoneurones and may lead to the prolongation of the time interval to subsequent motoneuronal discharges during voluntary activity.

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