ANIMAL RHODOPSIN AS A PHOTOGENERATOR OF AN ELECTRIC POTENTIAL THAT INCREASES PHOTORECEPTOR MEMBRANE PERMEABILITY

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1. Introduction

In 1971 Stoeckenius, Oesterhelt and Blaurock discovered a new retinal-containing protein called bacteriorhodopsin [1,2]. Three years later its biological function was revealed. It proved to be a photoelectric generator in halobacteria transducing photon energy into a convertible form, i.e., protonic potential [3–5]. Its 7 Å three-dimensional structure and amino acid sequence were described in [6] and [7,8], respectively. Having collated the structural and the sequence data, a model was suggested for the arrangement of the bacteriorhodopsin polypeptide in the membrane of halophilic bacteria [9].

None of the above-mentioned problems has been solved for animal rhodopsin, although this pigment was described almost a century earlier than its bacterial analog. The most dramatic difference in our knowledge about the two rhodopsins is that the function of the animal protein still remains unknown in spite of the success in estimating the reaction sequence of the photocycle and other details of its behaviour in the photoreceptor disc membrane. It is clear that absorption of a photon gives rise to 11-*cis*–all-*trans* isomerization of the retinal in rhodopsin that ultimately results in release of free retinal into solution. However, the biological significance of these photochemical reactions that proceed in the disc membrane is still obscure since per se they cannot cause the next well-characterized event of the process of vision, i.e., the closing of Na^+ channels in another (cell) membrane of the outer segment of rods. One of the popular hypotheses of visual reception [10] postulates that light increases Ca^{2+} permeability of the disc membrane inducing thereby an efflux of Ca^{2+} pre-accumulated inside the discs in the dark. Indeed, there are indications of a dark ATP-dependent Ca^{2+} accumulation in discs [11]. A light-induced efflux of Ca^{2+} from Ca^{2+}-preloaded discs was observed [12,13] (reviewed in [14]). We have found [15,16] that illumination induces a Ca^{2+} release from native frog retina discs without any in vitro Ca^{2+} preloading. It was assumed that these events are a result of the formation of pores through the disc membrane [14]. But why are the pores formed when a photon is absorbed by rhodopsin?

It is well known that in electro-excitable membranes ion channel opening can be a consequence of a membrane potential change. This mechanism might be applied to photoreceptor discs if animal rhodopsin generates, as does bacteriorhodopsin, a photopotential across the disc membrane. We suggested the latter after studying the formation of a photoelectric potential across the photoreceptor disc membrane [17–20].

The first evidence of the electrogenic activity of rhodopsin was obtained when the so-called early receptor potential (ERP) was discovered [21]. This biphasic photoelectric response of the cell membranes of rods and cones was found to appear so rapidly that no other component than rhodopsin can be involved. However, even under strong illumination, the amplitudes of ERP measured with an intracellular electrode were very small, i.e., $\leqslant$3 mV [22]. A small photoeffect (0.5–3 mV) was shown [14,23,24] when a solid Teflon film covered on one side with rhodopsin was illuminated. Takagi and Kishimoto have recorded an ERP-like response in a system consisting of squid outer segment vesicles adsorbed onto a thick monoolein–decane film separating two water solutions (see [14]).

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We have elaborated a method for direct measurement of electrogenic activity of membrane proteins [25–28]. It was applied to the study of bacteriorhodopsin and, later, of animal rhodopsin. Illumination of cattle photoreceptor discs associated with a phospholipid-impregnated millipore filter resulted in the generation of a significant (20–25 mV) electric potential difference [18,19]. The action spectrum of this response coincided with the absorption spectrum of rhodopsin. We had demonstrated a photopotential of the same direction as in the above-mentioned experiments (the disc interior positive) in a suspension of discs, using the method of penetrating ions. The photopotential decay was greatly accelerated by protonophorous uncouplers [20].

Here, we compare the responses of bacterial and animal rhodopsins exposed to: (1) continuous illumination; (2) a very short laser flash inducing a single turnover of the pigment molecules; and (3) an electric field of different directions. Bacteriorhodopsin-containing fragments of *Halobacterium halobium* cytoplasmic membrane or cattle photoreceptor discs were incorporated onto one side of a phospholipid-impregnated collodion (or fluoropore) film. Then electric responses were measured with two electrodes immersed in the bathing solutions on either side of the film.

Striking similarities in many essential features of the flash-induced photoelectric responses of the two rhodopsins were shown. In both cases, electrogenesis was found to consist of 3 main phases, the first phase directed oppositely to the second and the third. The τ value of the fastest phase was <200 ns, whereas the slowest phase was several ms. The amplitudes of the phases were the 1st < 2nd < 3rd. The decay times of the photopotential under a flash of low light intensity were several seconds. The overall amplitudes of the flash-induced photoresponses were usually somewhat lower for the animal rhodopsin (10–25 mV) than for the bacterial one (30–60 mV). With animal rhodopsin, the maximal responses to switching on the continuous illumination were >40 mV.

The main difference in the bacterial and animal systems was found to be in the responses to repeated flashes. The animal rhodopsin generated a maximal photopotential in response to the first flash, each next flash being less effective than the preceding one. Parallel to a progressive decrease in the amplitude, an acceleration of the photopotential decay took place. Addition of 11-*cis* retinal improved the amplitude of the photoresponses but not the decay time. As to the bacteriorhodopsin, it displayed identical amplitudes of the photopotential to the repeated flashes, and the kinetics of the potential decay remained constant. A significant decrease in the decay time of the animal rhodopsin photopotential caused by pre-imposing an external battery-produced electric field across the fluoropore filter if this field was of the same direction as that generated in the light. Pretreatment with an oppositely directed field was without effect. In bacteriorhodopsin, no influence of the electric field pretreatment was demonstrated.

We conclude that both the bacterial and the animal rhodopsins are photoelectric generators. The animal protein is only capable of a single turnover, and a generated photoelectric potential induces a steady increase in the membrane conductance, whereas bacteriorhodopsin operates many times and without any changes in the insulating properties of the membrane in the dark.

2. Materials and methods

Photoreceptor discs from rods of cattle retina and bacteriorhodopsin-containing membrane fragments (sheets) from *Halobacterium halobium* (R_1) were isolated as in [20] and [26], respectively. Discs or sheets were added to one of the two compartments of a Teflon chamber separated by a collodion film or a fluoropore filter impregnated with a decane solution of egg lecithin (70 mg/ml) and octadecylamine (1 mg/ml) or of asolectin (100 mg/ml), respectively [25–27]. Bathing solutions contained 0.1 M NaCl and 5 mM MES–KOH (pH 6.0) and in experiments with fluoropores films 0.025 M $CaCl_2$. After 2–4 h incubation required for incorporating discs or sheets into the film, the system was illuminated with continuous light from a 2.5 kW Xenon lamp, transmitted through an HL Jobin-Yvon monochromator or with a flash generated by an LTIPChl-5 neodimium Q-switched laser of doubled light frequency (λ = 530 nm, $t_{1/2}$ = 15 ns, 10 mJ light impulse energy). Electric potential difference generation was measured with two Ag/AgCl electrodes immersed into solutions on both sides of the film. To prevent light artifacts, the electrodes were covered with black polyethylene. The electrodes were connected with an Analog Devices 48K operational amplifier. From the amplifier, the electric signal was transmitted to a DL 905 transient recorder and further to a Nova 3D computer.

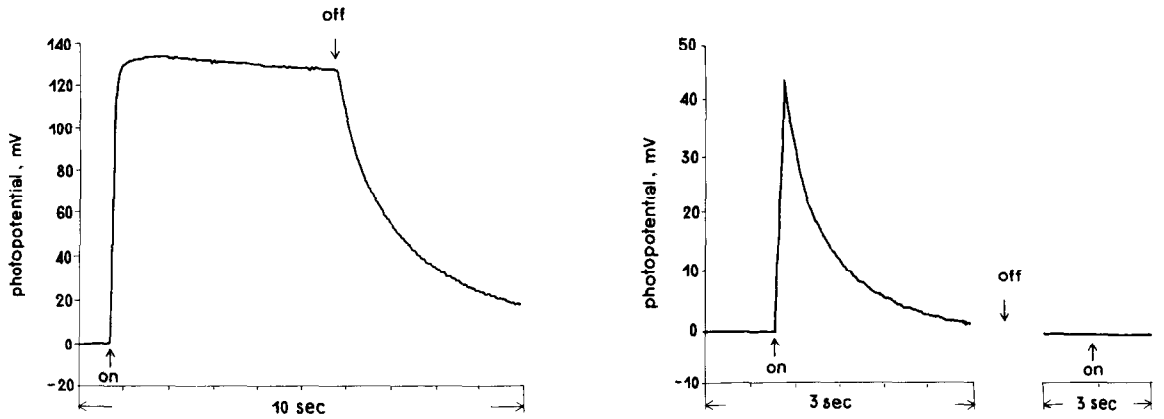


Fig.1. Continuous light-supported generation of electric potential differences in systems 'bacteriorhodopsin sheet–collodion film' (A) and 'photoreceptor disc–collodion film' (B).

3. Results

Fig.1 shows continuous light-induced generation of electric potential differences in the 'rhodopsin disc–collodion film' (B) and 'bacteriorhodopsin sheet–collodion film' (A) systems. Bacteriorhodopsin is shown to generate a steady electric potential as long as illumination continues [27,28]. In agreement with [18,19], animal rhodopsin was also competent in photoelectric potential generation reaching in this experiment 43 mV, however, no steady state level of the response is observed (due to irreversible bleaching of rhodopsin). The potential increase spontaneously changes into a decrease long before the light is switched off. Repeated illumination of the system does not induce measurable photoresponse.

In fig.2, the effects of a laser flash inducing a single turnover of the rhodopsins are given. It is seen that flash-induced responses of the animal and bacterial pigments are much more similar than the responses to continuous light. In both rhodopsins, flash causes very fast ($\tau < 200$ ns) formation of a potential difference whose direction (the rhodopsin-free side of the film negative) is opposite to that observed under continuous illumination. Then a 'positive' photoresponse develops and the membrane potential reaches its maximum in the ms time scale. Both the kinetics and the direction of the electric vector prove similar to the early receptor potential that is also composed of oppositely-directed phases, R1 and R2 [21].

Computer analysis of the above data revealed the existence of at least two exponents in the 'positive' photoresponse, the τ-values of the more rapid electrogenic phase being 50 μs for bacterial and 500 μs for animal rhodopsins. In both cases, the slowest phase took several ms and the amplitude of the first

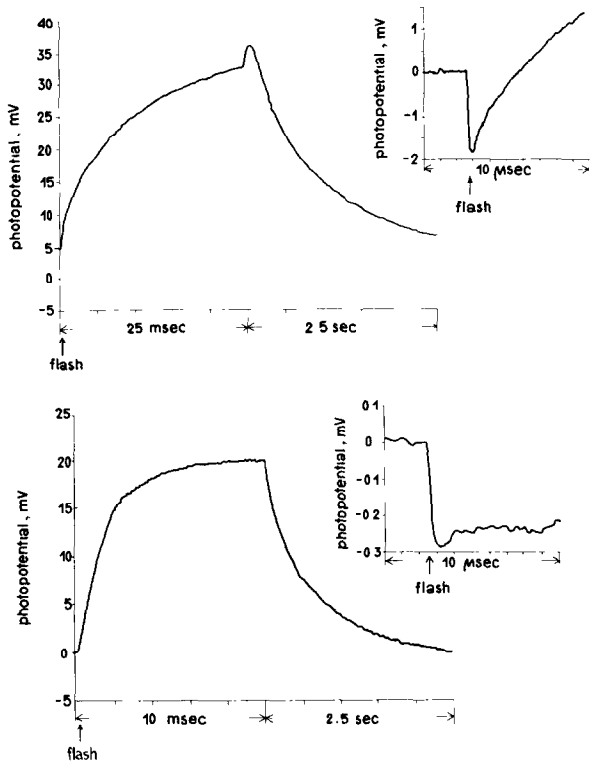


Fig.2. Laser flash-induced electrogenesis mediated by bacterial (A) and animal (B) rhodopsins. Flash energy was 10 mJ. Collodion film.

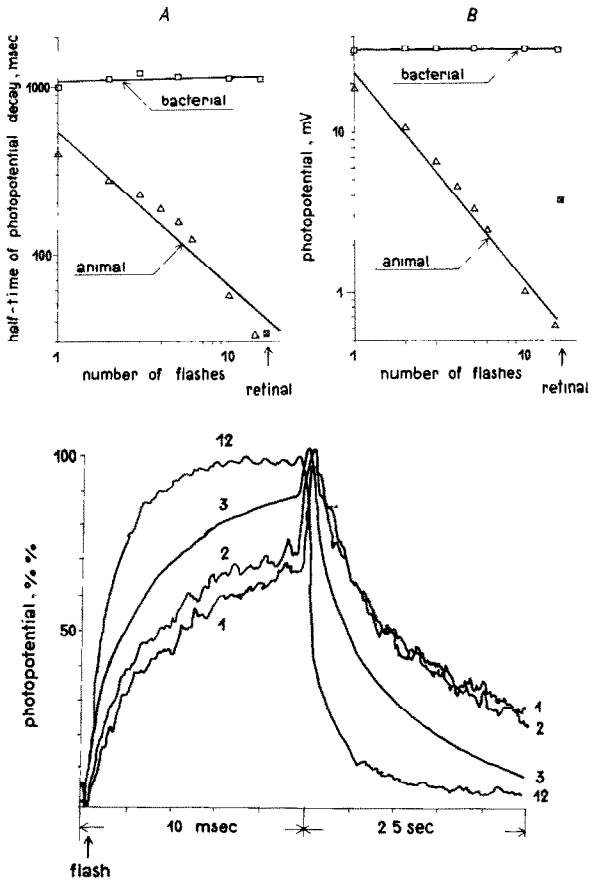


Fig.3. Photoelectric responses of animal and bacterial rhodopsins to repeated laser flashes. Half-times of the photopotential decay (A) and amplitude of photopotential (B) as functions of number of consecutive laser flashes. Symbols (△) and (▣) before and after addition of 2×10^{-5} M 11-*cis* retinal. Flash energy was 10 mJ. (C) Photopotential generations induced in the animal rhodopsin system by weak (1,2) and strong (3,12) laser flashes. Flash energies were 5 μJ (1st and 2nd flashes) and 5 mJ (from 3rd–12th flashes). Collodion film.

phase was the lowest, whereas that of the third phase was the highest.

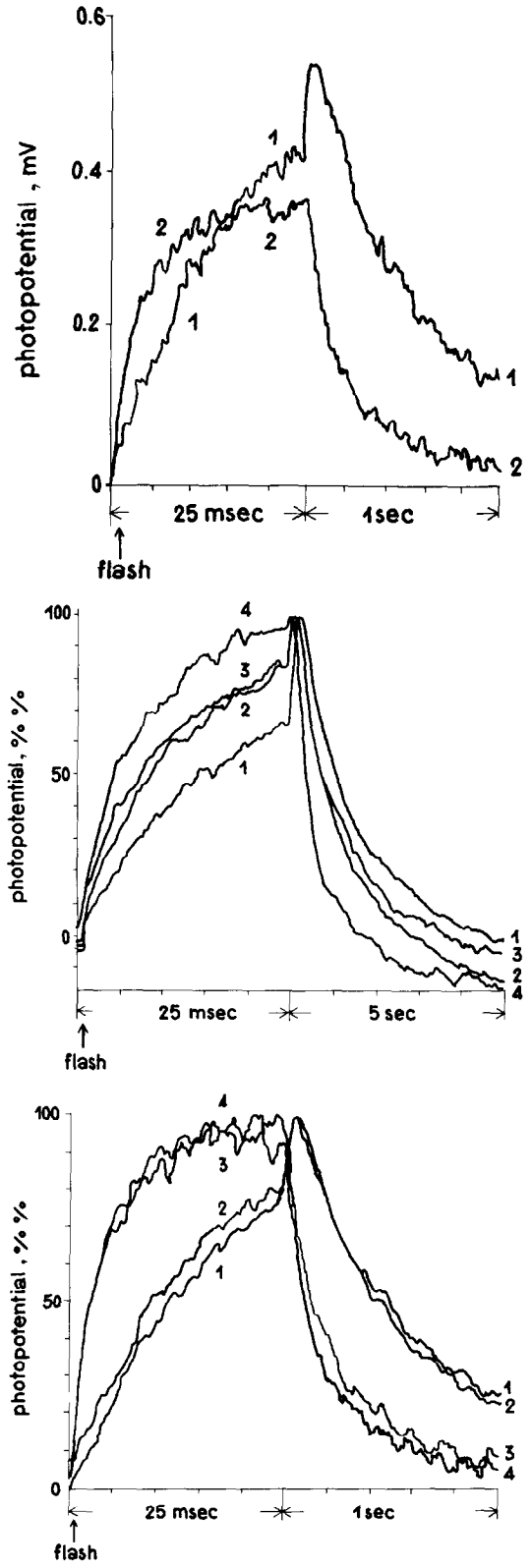
With bacteriorhodopsin, the photoresponses were observed to be independent of the number of flashes. Animal rhodopsin demonstrated an entirely different behaviour. The response to each subsequent flash was of a lower amplitude (a consequence of rhodopsin bleaching). Moreover, the decay times of the photoelectric responses decreased as the number of flashes grew, indicating a rise of the disc membrane conductance (fig.3A,B).

In fig.3C responses of animal rhodopsin to the 1st, 2nd, 3rd and 12th flashes are shown in % of the maximal photopotential value reached after each flash. The energy of the 1st and 2nd flashes was 1000-fold lower than that of the 3rd–12th flashes. It is seen that both the rise and decay times of the photoelectric effect are much shorter in the 3rd (and even more in the 12th) response than in the 1st and 2nd, indicating that the increase in disc membrane conductance develops in the same time scale as the electrogenic response per se.

These data may be compared with the findings of other laboratories about light-induced formation of pores in membranes of discs and rhodopsin proteoliposomes [14,29–31]. Such pores were found to be permeable to Na^+, Cs^+, Ca^{2+}, glycerol and glucose but not to sucrose [29]. (About the light-dependent conductivity increase in rhodopsin-containing planar membranes, see [14,32].) Our observations show that the pores in question open so fast that their formation can, in principle, be involved in the chain of events of photoreception.

The question arises, what is the mechanism of the pore opening mediated by rhodopsin? As shown in fig.3A,B, addition of 11-*cis* retinal to a bleached system results in reconstituting the rhodopsin electrogenic activity but the decay time of photoresponse remains as low as before retinal treatment. Thus, to close a pore, it is not sufficient to reconstitute rhodopsin from opsin and retinal. Therefore it seems to be hardly possible that the chromophore-containing region of rhodopsin is structurally involved in pore formation. Rather, an opening of a pore is a consequence of a light-induced functioning of rhodopsin. Taking into account the striking similarity in the responses of bacteriorhodopsin (which is a light-driven H^+ pump) and animal rhodopsin we would like to postulate that the latter is also functioning as a photoelectric generator. If this is so, the disc membrane may be regarded as an electro-excitable membrane possessing pores which open as a result of the membrane potential formation by rhodopsin.

To test this possibility, we performed a series of experiments trying to substitute an external battery-produced electric field for the light, to decrease the decay time of the photoresponse. Photoreceptor discs were incorporated into a fluoropore filter impregnated with a decane solution of asolectin. The fluoropore is much more resistant to imposed electric potentials than is the collodion film used previously. The properties of this system were probed with laser flashes of



so low an energy that the probing did not cause any measurable changes in the kinetics of photoelectric response. Pretreatment of the system with the electric field of the same direction as generated in the light (fig.4), produced a strong decrease in both the rise and decay times of the photoresponse to a weak laser flash produced after dissipation of the imposed field. The amplitude of the electric response decreased only slightly (fig.4A). In this respect, the field effect clearly differs from the effect of the strong flash that greatly decreased not only the decay time but also the amplitude of the photoresponse (fig.3A,B). Such relationships seem quite reasonable if the field does not cause rhodopsin bleaching.

An electric field of opposite direction is found to be without effect (fig.4B,C). The rightly-directed electric field imposed after treatment with the wrongly-directed field, proves still to be effective (fig.4C). Qualitatively similar relationships were revealed when collodion film instead of fluoropore was used. The only requirement was that the disc membrane resistance should be sufficiently high ($t_{1/2}$ of decay >1 s). In this case the voltage of the external battery could be 500–800 mV, below the level of the collodion film damage (not shown).

No effects of the electric field pretreatment were observed in the bacteriorhodopsin system (not shown).

Thus, the action of light on the kinetics of photoresponse, indicating increase in the disc membrane conductance, can be reproduced in the dark by an electric field of the proper direction.

The last problem we studied was the mechanism of a reversal of the light-induced conductivity increase (i.e., closing of pores). Collodion film was treated with photoreceptor discs as in the above experiments.

Fig.4. Effect of electric field on laser flash-induced photoresponse in a 'photoreceptor disc–fluoropore filter' system. (A): (1) before electric field treatment; (2) an electric potential difference of 15 V (rhodopsin-free side of the filter positive) was imposed across the filter in the dark for 4.5 min. Then the electric battery was switched off and, after relaxation of the imposed potential difference to zero, the filter was illuminated with a laser flash of the same energy as before field treatment (50 μJ). (B). (1,2) two flashes before the field treatment; (3) after 4.5 min treatment with a field of the same direction as produced by light (rhodopsin-free filter side positive); (4) after treatment with an oppositely directed electric field. (C): (1) before the field application. Then the following consecutive treatments were used: 2 min of the field of the right direction (2); 2 min of the opposite field (3) and 2 more min of the right field (4).

Then the film was exposed to several potent laser flashes bleaching the main portion of rhodopsin, so that an ~10-fold decrease in the photoelectric response amplitude and in the decay time took place. When such a system was stored in the dark, a many-fold rise of the decay time was observed. The effect developed in the min time scale (not shown).

4. Discussion

The above data are in agreement with our tentative concept [18,19,33] about the similarity of the electrogenic functions of bacterial and animal rhodopsins. One can speculate that the yet unknown role of animal rhodopsin in vision consists in charging the photoreceptor disc membrane at the expense of light energy just as bacteriorhodopsin charges the cytoplasmic membrane of *Halobacteria*. In terms of this concept, the difference between bacterial and animal systems is only the fate of the electric potential. In bacteria it is utilized to support ATP synthesis and other $\Delta\bar{\mu}_{H^+}$-driven processes of membrane-linked work. In photoreceptor discs this potential is used to open pores or channels permeable to a chemical transmitter of the photoreceptor process, pre-accumulated in the intradisc space. If so, the disc membrane falls into the category of electro-excitable membranes responding with an opening of a channel to a change in the membrane potential.

If Ca^{2+} is the chemical transmitter, the chain of light-induced events in the retinal rods should be the following:

Light ⟶ rhodopsin-mediated charging of disc membrane (disc interior positive) ⟶ opening of pore in disc membrane ⟶ Ca^{2+} release into cytoplasm ⟶ closing of Na^+ channels in the cell membrane and its hyperpolarization.

In cones, a similar process can be initiated with opening of Ca^{2+}-permeable pores in the cell membrane.

A feature that distinguishes the pores in photoreceptor membrane from those in other electro-excitable membranes is the time courses of pore opening and closing. A disc membrane pore opens very fast and remains open for some time after dissipation of the membrane potential that caused its formation. This should increase the sensitivity of the receptor system to the signal, so that a single photon can, in principle, induce release of all the Ca^{2+} accumulated in the dark inside a disc.

It should be remembered that both inter- and intra-disc-spaces are very narrow. Therefore the lateral electric resistance along the disc membrane can be rather high, slowing down delocalization of the electric field. It is hardly probable that a delocalized field can perform any work to open a pore since absorption of 1 photon should produce, according to our calculation, $\leqslant$0.03 mV membrane potential if it is delocalized over the entire disc. Apparently, the local field is involved in the opening of the pore which is arranged near rhodopsin or integrated into the rhodopsin molecule.

An unsolved problem is the nature of the charge transported by animal rhodopsin. The analogy with bacteriorhodopsin suggests that it should be H^+. In connection with indications of the existence, besides bacteriorhodopsin, of a Na^+-transporting retinal-dependent protein in *Halobacteria* [34,35], we investigated the possible Na^+ requirements for the animal rhodopsin photoeffect and found none. In [36], considering several bodies of indirect evidence, it was concluded that illumination causes H^+ movement from the outside to the inside of the disc.

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