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Purified lens junctional protein forms channels in planar lipid films

(gap junctions/reconstitution/cell–cell communication)

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ABSTRACT **Junctions isolated from bovine lenses were solubilized with the detergent octyl glucoside, and their protein(s) was reconstituted in unilamellar vesicles. The protein(s) appears as annular-shaped intramembrane particles ≈10 nm in diameter on the vesicles' fracture faces. The addition of the vesicle-containing junctional protein(s) to both sides of preformed lipid films induced voltage-dependent channels. The channels have a conductance of 200 pS in 0.1 M salt solutions and are thus large enough to account for the electrical coupling observed between intact lens fibers; they turn off when the magnitude of the voltage is increased and in the presence of octanol. Although the identity of the reconstituted channels as the communicating pathway between lens fibers remains to be proven, it is most likely that the reconstituted channels are formed by MIP-26, the major protein component of the isolated lens junctions.**

Mammalian lens is comprised of elongated cells called fibers. Fibers are connected to each other through an elaborate system of intimate plasma membrane contacts that cover large portions of their surfaces (1). It has been suggested that these specialized junctions contain an intrinsic channel that is responsible for the communication observed between lens fibers. Consequently, lens fiber junctions have been isolated and studied structurally (2, 3), chemically (2, 4–6), and immunologically (7, 8). These studies have reported that the lens junctions are composed of a main intrinsic protein called MIP-26 (6–8). Recently, the complete amino acid sequence of the protein has been obtained and, from analysis of the sequence, a model has been proposed that is compatible with a channel-forming structure (9).

We report here that purified protein(s) from lens fiber junctions, presumably MIP-26, produces large voltage-dependent channels when incorporated into planar lipid films.

MATERIALS AND METHODS

Isolation and Reconstitution. The junctions used in this study were isolated from bovine lenses without treating the membrane fractions with detergents or exogenous proteases (2). $NaDodSO_4$ gel electrophoresis demonstrated a major band of 26–27 kDa and two smaller variable bands of 22 kDa and 16 kDa, respectively, in gels loaded with 60–80 μg of protein. (Coomassie blue staining patterns of the junctions isolated from this study were similar to those shown in figure 1 of ref. 2.) Electron microscopy of the isolated fraction showed mostly undulating junctions, ≈13 nm in overall thickness, containing extensive square arrays of protein units (2).

Aliquots of the isolated lens junctions [200 μl of 2–3 mg of protein per ml in 4 mM Tris·HCl/200 mM NaCl/20% (vol/vol) glycerol/1 mM $CaCl_2$/5 mM EGTA, pH 8.0] were solubilized by adding crystals of the detergent octyl glucoside until the solution cleared (≈25 mg of detergent per mg of junctional protein). The clear solution was centrifuged at 100,000 × *g* for 90 min. The supernatant contained 65–75% of the starting protein and was used for reconstitution.

The solubilized junctional protein was reconstituted into unilamellar liposomes by the procedure of Mimms *et al.* (10). In all 10 reconstitutions reported in this study, the following procedure was used: 10 mg of egg lecithin was dried under a stream of nitrogen. The dried lipid was suspended in 0.4 ml of buffer {200 mM NaCl/25 mM *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES), pH 7.0} containing 45 mg of octylglucoside. The clear lipid/octyl glucoside solution was mixed with a controlled amount of solubilized protein (i.e., 0.017 mg, 0.085 mg, 0.17 mg) and the final volume was adjusted to 500 μl. Dialysis of this clear solution against 4 liters (two changes) of 25 mM TES (pH 7.0) with 200 mM NaCl yielded a whitish suspension. The different preparations were characterized by freeze-fracture electron microscopy according to methods described (2).

Bilayers. Planar bilayers were formed as described by Hall (11) from bacterial phosphatidylethanolamine on 0.015-inch-thick Teflon septa with 0.3-mm-diameter holes punched in them. The region of the hole was precoated with ≈0.5 μl of squalane. Lipid (10 μl of lipid at 5 mg/ml in pentane) was spread on the surface of the aqueous phase on either side of the septum and the level of the liquid was raised over the hole to form the membrane. Voltage was applied by either a computer-controlled digital-to-analogue converter or a battery-driven potentiometer. Current was measured by using an operational amplifier with a 100-MΩ feedback resistor. Current–voltage curves were recorded on a Hewlett-Packard 7034A X-Y plotter and single-channel currents at constant applied voltage were stored on magnetic tape using an FM tape recorder at a bandwidth of 5 kHz. Current traces shown were reproduced on a Gould 2200 fast pen recorder by playing back the taped data at real time rates.

RESULTS

Freeze-Fracture. We found the liposomes to be unilamellar vesicles with an average diameter of 0.35 ± 0.1 μm by freeze-fracture methods (Fig. 1). The junctional protein(s) used in reconstitution showed up as distinct intramembrane particles on the vesicle fracture faces. The density of particles increased as the amount of protein used in reconstitution increased. The vesicles prepared with the ratio of 1:25,000 (mol/mol) resulted in the best conditions to study both single-channel events and multichannel membranes. Therefore, we analyzed the particle distribution of this preparation in greater detail. We found an average of 2 particles per vesicle (98 vesicles from two different reconstitutions); ≈80% of the vesicles contained particles. (The Poisson distribution predicts a mean of 1.6 particles per vesicle if 80% of the vesicles contain particles.)

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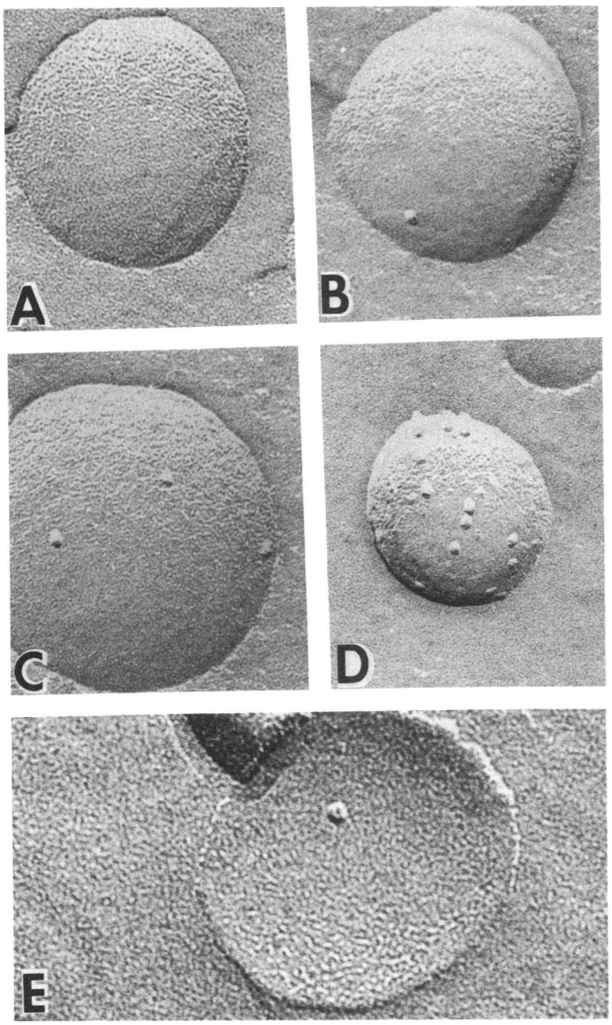

FIG. 1. Freeze-fracture electron micrographs of single-walled vesicles containing lens junction protein. (*A*) Liposome from a control preparation containing no protein. (*B–D*) Selected liposomes from preparations containing increasing amounts of lens protein as listed above. Note the appearance of distinct particles on the fracture faces of the liposomes in *B–D*. (*E*) A higher magnification view of a particle having an annular shape surrounding a small central deposit of metal. (Ref. 2 shows a $NaDodSO_4$ gel of the staining material.) (*A–D*, ×162,750; *E*, ×232,500.)

Some of the intramembrane particles, especially those on concave fracture faces, displayed annular shapes ≈10 nm in diameter with a small central deposit of metal (Fig. 1*E*). The size of these particles and their annular nature suggests that at least some of the particles are oligomers, because a single copy of a polypeptide chain weighing ≈27 kDa (close to the molecular size of the major bands of the gels) would not be large enough to make the annular structure seen in Fig. 1*E*.

Conditions to Incorporate Channels. Voltage-dependent channels appeared within 5–10 min after bilateral addition of vesicle-containing lens junction protein(s) to a preformed lipid bilayer. Channels sometimes appeared spontaneously, but the probability of channel appearance increased if the holding potential was 200–300 mV. Best results were obtained by adding 2 μl (≈10^{12} vesicles) of a preparation reconstituted at 1:25,000 (mol/mol). Thus far, we have produced channels in >35 membranes. In five experiments, we attempted to produce channels by unilateral addition of the protein-containing vesicles. We failed to see the channels even after adding up to 20 μl of the vesicle suspension and then waiting for 30–40 min. In two of these experiments 30 min after unilateral addition, vesicles were added to the side opposite the first addition. Four to 5 min later, voltage-dependent channels appeared. In the others, the membrane broke before bilateral addition was completed.

The pH of the solution in the bilayer chamber is also important. Best incorporation was obtained at pH 5.6–5.7. At pH 6.2–6.5, incorporation also occurred but larger amounts of vesicles (10 μl instead of 2 μl) were necessary. At pH 7.2, we failed to incorporate channels into the membrane. However, channels incorporated at pH 5.7 were still observed when the pH was increased to pH 7.2 subsequent to incorporation. At pH 7.2, the channels were labile and often closed permanently, leaving the membrane conductance at the level of an unmodified membrane.

Electrical Characteristics. Fig. 2 shows a typical current–voltage curve of a membrane containing only a few channels in 1 M KCl. The positive branch of the I-V curve shows two channel closures of ≈1800 pS each, one at 85 mV and one at 120 mV. The noise of the positive trace increases as the voltage is increased from 0 to 85 mV, reflecting partial closures to an unstable substate. The negative branch of the I-V curve shows a single closure of ≈2000 pS at −80 mV. This is followed by a brief poorly resolved opening and a second closure of ≈1800 pS at just less than 100 mV. Unmodified membrane conductance was $<10^{-10}$ S (1.4 μS/cm^2).

The single-channel characteristics are shown at different positive and negative voltages in Fig. 3*A*. At absolute voltages <40 mV (not shown), the channels are fully open and exhibit little noise. When the absolute value of the voltage is raised above ≈50 mV, the channel begins to flicker noisily to a short-lived lower conductance state. This change is illustrated in the +50 mV and −56 mV traces in Fig. 3*A*. As the voltage is increased still further, the channel sometimes closes to the fully off state, and occasionally a new level just above the off state is seen. This is illustrated in the +78 mV and −88 mV traces in Fig. 3A.

Three sections of the −88 mV trace of Fig. 3*A* are shown with expanded current and time scales in Fig. 3*B*. They show that the channel has four distinct conductance states. The opened state (state of maximum conductance) and the closed state (state of minimum conductance) are more stable than the two intermediate states, which have shorter lifetimes and more noisy conductance levels. These intermediate states can be considered metastable transitions between the fully opened and the fully closed states.

This type of channel was seen in 34 of 35 membranes analyzed in this study. The conductance of the channel is proportional to salt concentration (1500–2000 pS in 1 M salt and 200 pS in 0.1 M salt) and the channel is not selective for

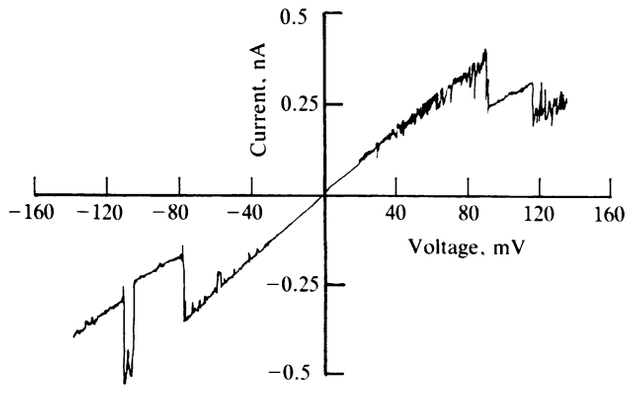

FIG. 2. Current–voltage curve of a membrane containing a small number of voltage-dependent channels inserted in the planar film by the addition of vesicles containing lens junction protein to both sides of the preformed bilayer. The aqueous solutions were 1.0 M KCl/25 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 5.7. Voltage was swept away from 0 at 5 mV/sec for each branch of the I-V curve.

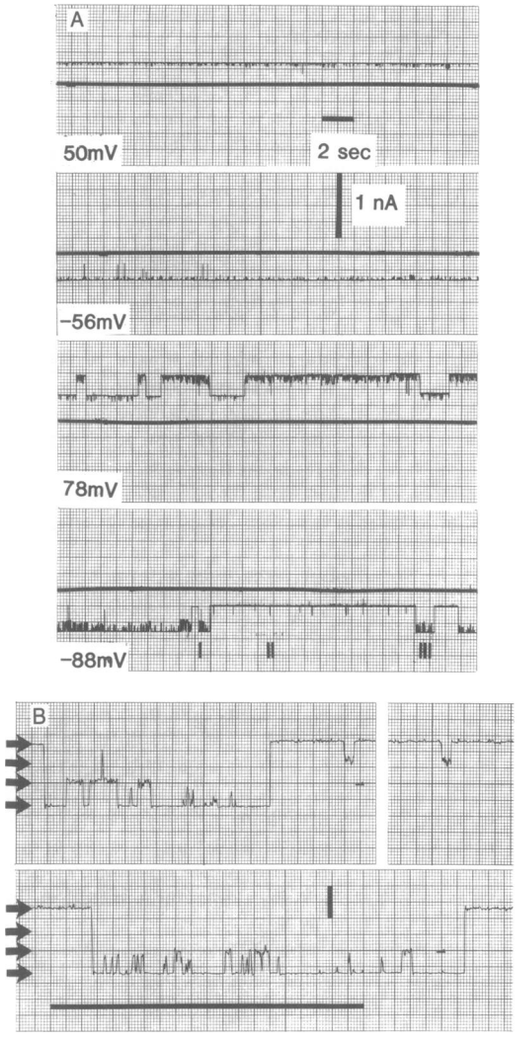

FIG. 3. Single-channel properties. (*A*) Single-channel current records at two positive and two negative voltages. The calibration bars for time and current apply to all four traces and the dark horizontal line marks the zero-current level for each trace. (*B*) Expanded time scale and current scale abstracts of three marked (lower case roman numerals) regions of the −88 mV trace of *A*. The zero-current level is off-scale above each strip. Current increases downward. Arrows at the left of each strip mark the currents corresponding to the four conductance states of the channel. Calibration bars are 1.0 sec (horizontal) and 0.1 nA long.

sodium over potassium. These single-channel characteristics can explain adequately the voltage dependence of membranes containing many channels. Moreover, the addition of Ca^{2+} up to millimolar concentration did not close the channels.

Although the channel is characterized by four conductance states, it spends nearly all of its time in either the opened state or the closed state. Therefore, the voltage dependence can be described with a two-state Boltzmann distribution of the form:

$$G = \frac{(G_{on} - G_{off}) \exp[-ne(V - V_o)/kT]}{1 + \exp[-ne(V - V_o)/kT]} + G_{off},$$

where G_{on} is the conductance with all the channels fully on, G_{off} is the conductance with all the channels fully off, V_o is the voltage at which half the channels are open, n is the apparent equivalent gating charge, k is Boltzmann's constant, and T is the temperature (kelvin). This two-state model gives values of V_o from 60 mV to 100 mV and values of n between 1 and 2. The uncertainty in the values obtained for n and V_o may reflect genuine variation in channel properties, or they may arise from failure to achieve steady state.

Lens fiber junctions as well as gap junctions from other tissues can be uncoupled by octanol (12, 13). Therefore, we explored the effect of this alcohol on the behavior of the reconstituted channel. Fig. 4*A* shows a current–voltage curve in 0.1 M KCl before the addition of octanol. Fig. 4*B* shows a current–voltage curve of the same membrane 2 hr 25 min after the addition of octanol to a final concentration of 0.22 vol % on both sides of the membrane. The voltage in Fig. 4*B* was first swept negative at 10 mV/sec. The conductance at low voltage is nearly that of the unmodified membrane. At about −95 mV, the conductance increases abruptly and then drops back to the unmodified membrane level in a ragged manner as the voltage sweeps back toward 0 V. No further increased conductance is seen during the reverse sweep to 0 V or the sweep to the positive side.

Additional Controls. The ability of the protein-containing vesicles to produce channels is lost after heating the fraction by microwave irradiation. This demonstrates that the channels are not products of denatured protein. Also, the solution containing octyl glucoside-solubilized lens junctions added directly to both sides of a preformed membrane (5 μl of 0.15 mg·ml^{-1}; chamber, 2.3 ml each side) produced a different type

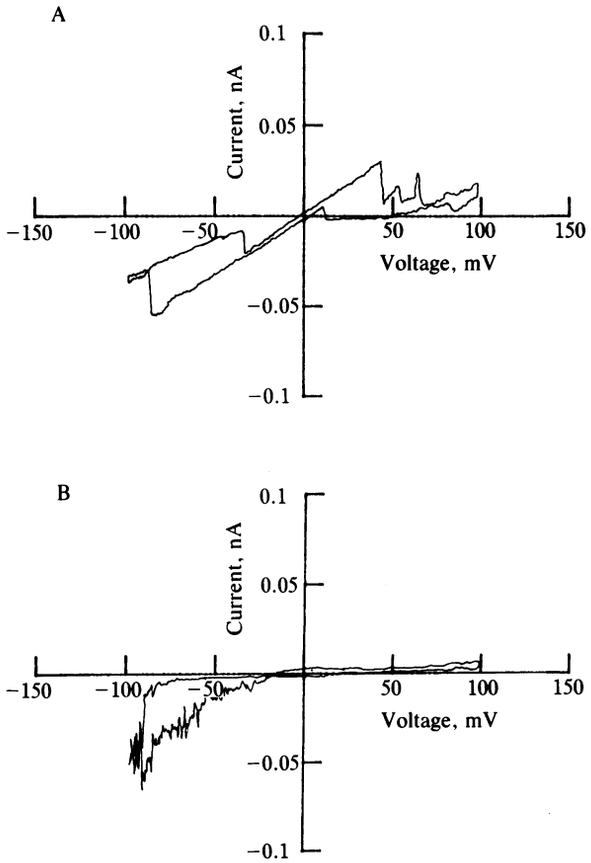

FIG. 4. The effect of octanol on channel properties. (*A*). Current–voltage curve obtained in 0.1 M KCl/25 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 5.7, before the addition of octanol. Voltage was swept at 20 mV/sec and the separation of the traces and the apparently negative current at positive voltages is due to the capacitative charging current. (*B*) Current–voltage curve of the same membrane after adding octanol. Note that the membrane conductance at 0 mV has decreased to bare membrane level. At about −95 mV, the conductance turns on abruptly, and as the voltage sweep direction is reversed, the conductance decreases to 0 in a ragged uneven manner. Arrows on the trace indicate the direction of voltage sweep.

of channel. With this type of incorporation, the conductance at 0 V is nearly that of an unmodified bilayer and increases with increasing voltage in a noisy manner. This conductance is due to protein and not detergent because addition of octyl glucoside 100 times as great as the amount used to solubilize the protein has no effect on the membrane conductance. This experiment suggests that the lens junction protein(s) must be in membrane-bound form to produce the channel.

We also incorporated lens junction protein(s) that was digested with trypsin prior to solubilization. Gel electrophoresis of the digested lens junctions showed that the prominent 26-kDa band almost disappeared and that most of the protein appeared at a band of 16 kDa. Vesicles reconstituted under identical conditions to those used previously to produce channels (Figs. 2 and 3) but prepared with detergent-solubilized junctions treated previously with the protease did not induce channel formation when incorporated in planar lipid films.

DISCUSSION

This paper reports that purified lens junction protein(s) incorporated into planar lipid films produce characteristic and reproducible channels. Previous reconstitutions of these protein(s) into liposomes have studied their permeability (14, 15) and how calcium and calmodulin interact to gate the channels (15). However, these studies have not included an electrical characterization of these channels.

The reconstituted channel has a single-channel conductance similar to one of the large channels described in patch-clamped lens epithelium (400 pS in 0.15 M salt; ref. 16). The apparent equivalent gating charge calculated with a two-state model (n = 1–2) is comparable to that reported for gap junctions of killifish blastomeres (n = 2) but less than that estimated for amphibian embryos (n = 8). The reconstituted channel seems to be permanently open, and it is not closed by the addition of Ca^{2+} to millimolar concentrations. This observation is in good agreement with the report of Girsch and Peracchia (15) in studying the permeability of lens junction protein(s) containing liposomes. The authors showed by an indirect method that Ca^{2+}, in the absence of calmodulin, did not close the channels to the flux of water. The voltage dependence of the reconstituted channel resembles, at least superficially, the dependence reported in gap junctions of amphibian and killifish blastomeres (17) and *Chironomus* salivary gland (18). However, the channels studied *in vivo* start closing at lower voltages and, hence, have a larger voltage dependence. The reconstituted channel has a different pH dependence than that seen in most cells. The channel stays open at acid pH and tends to close permanently to the conductance of an unmodified bilayer at neutral pH. This is the opposite behavior observed in gap junctions from several tissues (17) in which acidification produces uncoupling. A possible explanation for these differences could be that the pH and voltage gates are independent, as studies of amphibian embryo gap junctions seem to suggest (17), and that the reconstitution process used in this study recovers only the voltage-dependent gate.

An important question that arises from our study is to determine what protein in the isolated junctional preparation is forming the channels. We can eliminate the possibility that the channels are due to water-soluble contaminants because detergent-solubilized lens junction protein(s) added to the bilayer chamber at a concentration 50 times higher than that used to reconstitute channels from vesicles does not form channels. The possibility of contamination arising from lens fiber plasma membrane protein(s) is more difficult to eliminate because only minute amounts of protein are required to produce the number of channels observed per membrane. However, the data collected here allow us to put some constraints on the amount of protein(s) that must be present in the vesicles to account for the channels: the probability, p, that any given vesicle contains the channel-forming protein can be estimated from the number of successful experiments, m (34 in this study), compared to the number of unsuccessful ones, n (1 in this study), by the equation $p = 1 - [n/(m + n)]^{1/\nu}$, where ν is the number of vesicles that adhere to the planar membrane. The probability that no vesicle that adheres to the membrane contains the channel-forming protein is equal to $(1 - p)^{\nu}$. The probability of observing the channel can be estimated experimentally by $m/(m + n)$. Therefore, $(1 - p)^{\nu} \leq n/(m + n)$ or $p \geq 1 - [n/(m + n)]^{1/\nu}$. Observations of direct adhesion of vesicles filled with fluorescent dyes indicate that only 20–50 vesicles will adhere to the planar bilayers under the conditions we used for these experiments (unpublished results). If $\nu = 20$, then p must be at least 0.17 (i.e., 17 vesicles of 100 should contain the channel). On the other hand, the freeze-fracture data indicate that only 80% of the vesicles contain intramembrane particles and that these channels are most likely oligomers constructed of several polypeptides. Therefore, 21% of these particles (worst case) are made of channel-forming protein. Consequently, we argue that a contaminant present in <20% of the total protein (which should show up in gels loaded with 80 μg of proteins used to characterize the isolated junction fractions) cannot be responsible for the channel. Therefore, the channel should be comprised by either MIP-26 or one of the other two bands in the gels running at 22 and 16 kDa, respectively.

Quantum jumps of conductance between cells of *Xenopus laevis* embryos that have been micromanipulated into contact were first recorded by Loewenstein *et al.* (19). Moreover, these jumps were of equal magnitude and were sensitive to calcium. The range of conductances allowed by the method used to record these jumps includes the value of channel conductance observed in this reconstitution. In addition, single channels with similar electrical characteristics have been observed by incorporating liver gap junction protein(s) into planar bilayers (20). That the same plasma membrane contaminant is responsible for the presence of 200-pS channels in junctions isolated from bovine lens and mouse liver seems unlikely.

Note Added in Proof. Neyton and Trautmann (21) have patch-clamped pairs of coupled rat lacrimal gland cells. They found step changes in junctional conductance in the range 70–180 pS in 140 mM salt solutions. The average step conductance charge was 120 pS.

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