Calmodulin and Ca^{2+} -Controlled Cytoplasmic Streaming in Characean Cells

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ABSTRACT. The presence of calmodulin (CaM) was demonstrated in the homogenate of *Chara* internodal cells by the radioimmunoassay method. It amounted to 400 ng per ml homogenate.

The possible involvement of CaM in cytoplasmic streaming was examined by applying CaM-binding drugs to intact cells and plasmalemma-permeabilized cells. Thirty μM of trifluoperazine (TFP) applied to intact *Chara* cells did not interfere with the cessation of streaming accompanying membrane excitation, which causes a transient increase in cytoplasmic Ca^{2+} concentration. After a long exposure to TFP, the action potential resulted in an irreversible depolarization and inhibited the recovery of streaming due to loss of the semipermeability of the plasma membrane.

In permeabilized cells, neither the motile system nor the Ca^{2+} -induced cessation was inhibited by TFP up to 100 μM , by fluphenazine (FPH) at 1 μM , or by N-(6-aminoethyl)-5-chloro-1-naphthalene sulfonamide (W-7) at 10 μM . However, the recovery of streaming induced by washing away the Ca^{2+} was completely inhibited by these drugs. On the other hand, these drugs did not affect the streaming of tonoplast-free cells, irrespective of the presence or absence of 10 μM Ca^{2+} . Thus cytoplasmic streaming is controlled by two Ca^{2+} -sensitizing components, one related to the streaming cessation and the other, probably CaM, related to recovery.

FPH at 50 μM or W-7 at 100 μM , even in the absence of Ca^{2+} , stopped the cytoplasmic streaming of permeabilized cells. Washing away the drugs did not lead to streaming recovery after W-7 treatment, but sometimes did after FPH treatment.

In Characean cells cessation of the cytoplasmic streaming accompanying an action potential has been thought to be due to a sudden increase in the cytoplasmic free Ca^{2+} concentration (8, 9). The cytoplasmic streaming in plasmalemma-permeabilized cells is stopped by Ca^{2+} at concentrations higher than 10^{-6} M and restored by washing away the Ca^{2+} , which closely mimics the excitation-cessation coupling

Abbreviations used: ATP, adenosine-5'-triphosphoric acid; APW, artificial pond water; EGTA, ethyleneglycol-bis-(β -aminoethyl ether)N,N'-tetraacetic acid; CaM, calmodulin; CaM-BDs, calmodulin-binding drugs; TFP, trifluoperazine; FPH, fluphenazine; W-7, N-(6-aminoethyl)-5-chloro-1-naphthalene sulfonamide.

(37). Direct evidence that the cytoplasmic Ca^{2+} increases with the action potential was presented by Williamson and Ashley (39), who observed light emission of a Ca^{2+} -sensitive photoprotein, aequorin, which was injected into the cytoplasm of intact Characean cells. Kikuyama and Tazawa (15) introduced aequorin by intracellular perfusion into tonoplast-free *Chara* cells and observed the increase of light emission by action potential. Now is evoked the question of how Ca^{2+} regulates the cytoplasmic streaming.

After the discovery of an activation of cyclic nucleotide phosphodiesterase by Cheung (5) and its Ca^{2+} -dependence by Kakiuchi and Yamazaki (11), a number of Ca^{2+} -dependent enzymatic regulations have been attributed to CaM. In order to study the possible participation of Ca^{2+} -CaM in Ca^{2+} -dependent cellular events, a phenothiazine, trifluoperazine (TFP), has been used as a pharmacological probe. To see whether Ca^{2+} -CaM is involved in the regulation of cytoplasmic streaming in Characean cells, we also investigated the effects of three CaM-BDs, TFP, FPH, and W-7 (a naphthalene sulfonamide) on the Ca^{2+} -induced cessation and recovery of streaming. It was found that the CaM-BDs did not inhibit the Ca^{2+} -induced cessation but inhibited the recovery, suggesting the presence of two different Ca^{2+} -sensitizing components regulating the Characean cytoplasmic streaming. Localization of both components in the endoplasm was suggested.

MATERIALS AND METHODS

Nitella axilliformis Imahori and *Chara australis* R. Brown (= *C. corallina* Klein ex Wild., em. R.D.W.) were used throughout the experiment. Both algae were cultured in the manner described before (36, 37). Internodal cells were isolated from neighboring cells and stored in artificial pond water (APW) containing 0.1 mM each of NaCl, KCl, and CaCl_2 .

To study the effects of CaM-BDs on the coupling of membrane excitation with the cessation of the cytoplasmic streaming, both the streaming rate and the membrane potential were simultaneously measured. The membrane potential was measured by the K^+ -anesthesia method (29). As shown in Fig. 1, an internodal cell was set in a plexiglass chamber par-

Fig. 1. The apparatus to see the effects of TFP and FPH on membrane properties and streaming of intact Characean cells (C). M: microscope, A and B: two pools partitioned with vaseline sealing (slanted lines) at the partition wall, W: Ag-AgCl wire for the application of electric current pulses. Pool A was filled with 55 mM KCl and pool B with APW plus 100 mM sorbitol, which is isotonic with 55 mM KCl.

tioned into two pools, A and B, with vaseline sealing at the partition wall. Pool A was filled with 55 mM KCl and pool B with APW supplemented with 100 mM sorbitol to make it isotonic with 55 mM KCl. The pH of each medium was adjusted to 7.5 with 5 mM HEPES-Tris buffer. The potential difference of pool A against pool B (E_{AB}), which was nearly equal to the membrane potential of the cell in B, was measured by extracellular electrodes. To stimulate the cell fragment in B, electric current pulses were applied between the two pools through Ag-AgCl wire (W). For measurement of the membrane resistance, trains of small inward current pulses were applied. The resulting ohmic change of potential record represents the electrical resistance between the two chambers (R_{AB}). R_{AB} is composed of three components,

$$R_{AB} = R_A + R_B + R_s$$

where R_A and R_B are the membrane resistances of the cell parts in A and B, respectively. R_s is the sum of the electrical resistance of the external medium and that of the cell interior. R_{AB} was approximately equal to the sum of R_B and R_s . R_s was almost constant during measurement. The cytoplasmic streaming of the cell in B was observed by the microscope (M). The streaming rate was measured with an eyepiece micrometer and a stopwatch.

To directly apply CaM-BDs into the cytoplasm, two sorts of demembranated cell models were prepared, the plasmalemma-permeabilized cell model and the tonoplast-free cell model. The plasmalemma-permeabilized cell models were prepared after Shimmen and Tazawa (30). In this case, the Mg medium listed in Table 1 was used for demembranation. Solutions including CaM-BDs were applied to the cell from the outside by superfusing the cell with Mg or Mg-ATP medium (Table 1) containing the drugs. The Mg-ATP medium containing $10 \mu\text{M}$ Ca^{2+} was prepared by adding 1 mM CaCl_2 solution while measuring the concentration with a calcium-sensitive electrode (F2112Ca, Radiometer, Copenhagen). The superfusion was done after Shimmen and Tazawa (30).

Tonoplast-free cells were prepared after Tazawa *et al.* (35) by perfusing vacuoles with Mg or Mg-ATP medium (Table 1) containing the CaM-BDs to be tested and ligating them with threads at both ends. Since the tonoplast disintegrated within 30 min, the effect of the drugs on streaming was checked 30 min after perfusion. In some cases the open-vacuole method (34, 36) was used, where both ends of the tonoplast-free cells were kept open and intracellularly perfused with solutions containing CaM-BDs while continuously observing the streaming.

TFP, FPH, and W-7 were dissolved into pure water as stock solutions. They were diluted with Mg-ATP medium or Mg-ATP medium containing Ca^{2+} (Table 1).

Calmodulin in *Chara australis* was analyzed by the radioimmunoassay (RIA) after Muto and Miyachi (24). CaM was purified from spinach as described before (23). Antibodies to

TABLE 1. COMPOSITIONS OF PERFUSION MEDIA (mM)

	Mg	Mg-ATP
MgCl ₂	6	6
EGTA	5	5
PIPES	30	30
ATP	0	1
Sorbitol	105-200	105-200
K ⁺	64-71	64-71
pH	7.1	7.1

the spinach CaM were produced from a rabbit. Also a part of the CaM was radioiodinated according to Bolton and Hunter (3). *Chara* internodal cells were extracted with mortar and pestle. The homogenate was centrifuged at $10,000 \times g$ for 30 min. The resultant supernatant fluid was mixed with fixed amounts of the anti-CaM and the ^{125}I -labeled spinach CaM for competitive RIA. The radioactivity of the final pellet produced by adding goat anti-rabbit I_gG was determined with a gamma counter to quantify the amount of *Chara* CaM.

Experiments were done at room temperature ($20\text{--}25^\circ\text{C}$).

RESULTS

Effects of TFP and FPH on electrical properties and cytoplasmic streaming of intact cells. TFP dissolved in APW at various concentrations was applied extracellularly to internodal cells of *Chara australis* and *Nitella axilliformis* to determine its effects on the electrical properties of the plasmamembrane and cytoplasmic streaming. In Fig. 2 an intact *Chara* cell had the resting membrane potential of -200 mV and the streaming rate of $73\text{ }\mu\text{m}\cdot\text{s}^{-1}$. On application of a depolarizing current pulse larger than the threshold, an action potential was elicited; the streaming completely stopped but soon recovered its original rate.

When $30\text{ }\mu\text{M}$ TFP was applied to the cell (to pools A and B), the membrane potential depolarized by about 100 mV and the membrane resistance decreased, but the streaming was not affected. The membrane potential and resistance recovered gradually. When the cell was stimulated electrically, a normal action potential was generated, accompanied by reversible cessation of the streaming. The amount of overshoot at the peak of the action potential increased. This could be repeated. However, the action potential after long exposure to TFP caused irreversible depolarization and inhibition of streaming (the third action potential in Fig. 2). The responses of the potential to inward current pulses after the third action potential are due to R_s (cf. Eqn.). This means that the plasma membrane became very permeable to ions. When cells were kept in $20\text{--}50\text{ }\mu\text{M}$ TFP for more than 40 min without electrical stimulation, spontaneous action potentials accompanied by depolarization and cessation of the streaming were induced. Neither the membrane potential nor the streaming could be recovered by washing away the TFP. In such cells the form of chloroplasts changed from ellipsoidal to spherical due to swelling, which is evidence that the plasma membrane had lost its semipermeability. The incubation times with CaM-BDs to make the depolarization and inhibition of streaming irreversible by

Fig. 2. Effects of TFP on electrical properties and cytoplasmic streaming of an intact *Chara* cell. For further explanation see the text.

TABLE 2. INCUBATION TIME OF INTACT *CHARA* AND *NITELLA* CELLS WITH CaM-BDs MAKING THE DEPOLARIZATION AND STREAMING CESSATION IRREVERSIBLE BY SPONTANEOUS OR ELECTRICALLY INDUCED ACTION POTENTIAL

CaM-BDs	μM	min
TFP	20	$54 \pm 28(3)^{1)}$
	30	44 (2)
	50	$12 \pm 1(11)$
FPH	50	$13 \pm 2(3)$

¹⁾ The average time is shown with SE (number of cells).

TABLE 3. EFFECTS OF CaM-BDs ON CYTOPLASMIC STREAMING OF NORMAL CELLS, PLASMALEMMA-PERMEABILIZED CELLS, AND TONOPLAST-FREE CELLS; THOSE ON $10 \mu\text{M}$ Ca^{2+} -INDUCED STREAMING CESSATION AND ITS RECOVERY IN PERMEABILIZED CELLS

CaM-BDs	Normal cells		Permeabilized cells + Ca^{2+}			Tonoplast-free cells - Ca^{2+} + Ca^{2+} or - Ca^{2+}	
	Inhibition of streaming	Inhibition of Ca^{2+} -induced cessation	Inhibition of recovery	Inhibition of streaming	Inhibition of streaming	Inhibition of streaming	Inhibition of streaming
TFP (μM)	- + (10) (20-50)	- (100)	- $\pm$ + (1) (10) (25, 100)	- (100)	- (100)	- (100)	- (100)
FPH (μM)	+ (50)	- (1)	+ (1)	+ (50)	- (100)	- (100)	- (100)
W-7 (μM)		- (10)	- + (1) (10)	$\pm$ + (10) (100)	- (10)	- (10)	- (10)

Normal cells and tonoplast-free cells were prepared from *Nitella* and *Chara* cells; permeabilized cells were from *Nitella* cells.

spontaneous or electrically induced action potentials is shown in Table 2. No difference was observed between *Chara* and *Nitella* (data not shown). The same phenomena were also observed with FPH (Tables 2 and 3).

Effects of CaM-BDs on cytoplasmic streaming of permeabilized cells. CaM-BDs were extracellularly applied to permeabilized cells of *Nitella*. In both Figs. 3 and 4 the Mg medium was changed to Mg-ATP medium at time zero. The streaming was recovered by addition of ATP. When the streaming attained the maximum rate, $25 \mu\text{M}$ TFP (Fig. 3A) or $1 \mu\text{M}$ FPH (Fig. 3B) was added to the Mg-ATP medium before $10 \mu\text{M}$ Ca^{2+} was applied. In both cases the drugs neither inhibited the cytoplasmic streaming nor affected the Ca^{2+} -induced streaming cessation, but completely inhibited the restoration of streaming washing away the Ca^{2+} . TFP did not affect the Ca^{2+} action even when it was applied with Ca^{2+} (Fig. 4A). Removal of both Ca^{2+} and TFP sometimes caused a partial transient recovery of streaming. If TFP was applied after Ca^{2+} inhibition, the streaming sometimes recovered only transiently (Fig. 4B) similar to the transient recovery shown in Fig. 4A. But the streaming was induced again by subsequent removal of TFP. In summary, TFP always inhibited the

streaming recovery whether it was given before Ca^{2+} treatment, with Ca^{2+} , or after Ca^{2+} application. TFP was most effective when it was applied before Ca^{2+} (Fig. 3A).

The effects of the CaM-BDs on streaming are summarized in Table 3. As shown in Table 3 and Fig. 5, 50 μM FPH and 100 μM W-7 completely inhibited the streaming

Fig. 3. Effect of TFP on the recovery of streaming after Ca^{2+} treatment of *N. axilliformis*. A permeabilized cell bathed in Mg medium was superfused with Mg-ATP at time zero. When the streaming recovered, either 25 μM TFP (A) or 1 μM FPH (B) was added to the superfusion medium. Subsequently, the TFP or FPH was removed and 10 μM Ca^{2+} was added, which resulted in cessation of streaming. Washing away Ca^{2+} with Mg-ATP did not reactivate the streaming in both A and B.

Fig. 4. Effect of TFP on the streaming recovery after treatment of *N. axilliformis* with 10 μM Ca^{2+} . The streaming of permeabilized cells was reactivated by perfusing with Mg-ATP at time zero. The streaming was inhibited by 10 μM Ca^{2+} . The Ca^{2+} inhibition was not affected by addition of 25 μM TFP, but the recovery by removing both Ca^{2+} and TFP was only transient (A). When Ca^{2+} was washed away with the Mg-ATP medium containing 25 μM TFP, the streaming recovery was also only transient and removal of TFP restored the streaming completely (B).

Fig. 5. Effect of 50 μ M FPH (A) and 100 μ M W-7 (B) on the streaming of *N. axilliformis*. The streaming of permeabilized cells was reactivated by perfusing with Mg-ATP at time zero. High concentrations of drugs inhibited the streaming in the absence of exogenous Ca^{2+} . The once-inhibited streaming was sometimes restored by removing FPH (A), but not by removing W-7 (B).

in the absence of exogenous Ca^{2+} . After the drugs were washed away, the streaming sometimes recovered after FPH (Fig. 5A), but did not recover after W-7 (Fig. 5B).

Effects of CaM-BDs on cytoplasmic streaming of tonoplast-free cells. The cytoplasmic streaming of tonoplast-free cells was almost insensitive to Ca^{2+} . The streaming was inhibited by Ca^{2+} at 500 μ M. At 10 μ M Ca^{2+} , no inhibition was observed (36). None of the CaM-BDs added to Mg-ATP medium without or with 10 μ M Ca^{2+} affected the streaming (Table 3). It is self-evident that in tonoplast-free cells no data about the action of CaM-BDs on the streaming recovery was obtained.

Presence of CaM in *Chara* internodal cells. CaM was found to exist at about 400 ng per ml homogenate of whole *Chara* internodal cells by radioimmunoassay.

DISCUSSION

CaM was demonstrated to exist in *Chara* cells. The concentration was found to be 0.4 μ g/ml cell homogenate, which is distinctly lower than those (10–40 μ g/g fresh weight) found for different tissues of green pea plants (24). Assuming that the cytoplasm volume amounts to 5% of the cell volume in *Chara* (25) and that all the CaM localizes in the cytoplasm, the concentration of CaM in the cytoplasm is estimated to be 8 μ g/ml cytoplasm. Since the molecular weight of CaM is 16,700 (19), the concentration of CaM in *Chara* cytoplasm is estimated to be about 0.5 μ M, which is about five times higher than the free Ca^{2+} concentration in the Characean cytoplasm at the resting state (37, 39). The concentration of CaM in the cytosol should be higher than 0.5 μ M if CaM localizes in the cytosol as reported by Muto (22). Recently, Muto (unpublished data) found that the cross-reactivity of *Chlamydomonas* CaM with the anti-spinach CaM is conspicuously lower (about 1/10) than that of spinach CaM. If *Chara* CaM behaves like *Chlamydomonas* CaM, the CaM level in *Chara* would be 5 μ M or more, which is in the same order of magnitude as that in higher plants.

CaM is assumed not to be involved in the Ca^{2+} -induced cessation of cytoplasmic streaming, since the CaM-BDs TFP, FPH, and W-7 did not affect the Ca^{2+} action on the cytoplasmic streaming in permeabilized cells. However, CaM is assumed to be involved in the recovery of streaming which had been stopped by Ca^{2+} . This in turn means that there exists another Ca^{2+} -sensitizing component which mediates the streaming cessation on membrane excitation, although we know nothing about the chemical nature of this component. Interestingly, high concentrations of FPH and W-7, even in the absence of Ca^{2+} , stopped the streaming of plasmalemma-permeabilized cells but did not inhibit the streaming of tonoplast-free cells, which was almost insensitive to Ca^{2+} . Kauss (12) reported that FPH and other phenothiazine drugs caused activation of a key enzyme participating in volume regulation of *Poteroiochromonas* in the absence of Ca^{2+} . It seems likely that high concentrations of these drugs, even in the absence of Ca^{2+} , may activate the Ca^{2+} -sensitizing component which is present in the plasmalemma-permeabilized cells.

In intact cells, 30 μM TFP depolarized the membrane potential only slightly, and the excitability of the membrane remained quite intact during the first 40 min (Fig. 2, Table 2). The cytoplasmic streaming was not affected by TFP in so far as the electrical behavior of the cell was normal (1). After longer exposure to TFP, both the repolarization after the action potential and the recovery of the cytoplasmic streaming were inhibited. This may mean that the membrane becomes permeable to ions due to the deterioration caused by TFP, already observed by Beilby and MacRobbie (1) in *Chara corallina*. This was confirmed by the fact that chloroplasts became spherical because of swelling, probably caused by leakage of ions.

It is well known that phenothiazines such as TFP affect living membranes (6, 12, 17). The cytoplasmic streaming in cultured cells of tomato is inhibited by the phenothiazines TFP and chlorpromazine at 10 μM (40) but not by W-7 and R24571, which are assumed to have no serious effect on membrane properties (2, 7, 10, 13). Thus, involvement of CaM in the streaming was suspected by Woods *et al.* (40), though there is another report that even R24571 affects CaM-independent membrane processes (18). In the permeabilized cell model, however, effects of CaM-BDs on the streaming via the membrane are excluded. Apart from the problem of side effects on the membrane, there is still the question of the specificity of phenothiazines (20, 21, 27). We found that in permeabilized cells the recovery of streaming which has been stopped once by Ca^{2+} is inhibited by relatively low concentrations of the phenothiazines TFP and FPH as well as by W-7, which is structurally unrelated to the phenothiazines. Also, our finding that drugs applied before Ca^{2+} are more effective than those applied simultaneously or after Ca^{2+} is similar to the observation made by Louis *et al.* (18) that the concentrations of drugs for 50% inhibition were not significantly dependent on the concentration of CaM if the drugs were pre-incubated with CaM and its target 10 min before initiating their reaction. All these findings are consistent with the possibility that TFP, FPH, and W-7 exert their inhibitory effect on streaming by interfering with either CaM or a Ca^{2+} -sensitizing component which is at least pharmacologically closely related to CaM.

The action potential-induced cessation of cytoplasmic streaming in *Characeae* cells is believed to be caused by a transient increase in Ca^{2+} concentration (8, 9, 14, 15, 39). The time needed for cessation after an action potential is less than 1 sec in intact Characean cells (33). In the plasmalemma-permeabilized cell model, the exogenous Ca^{2+} caused cessation of streaming within several minutes (37). This

might mean that either the diffusion of exogenous Ca^{2+} into the permeabilized cell or the washing out of preexisting EGTA (5 mM) from the cytoplasm is limiting. In both cases increase in external Ca^{2+} concentration should shorten the time necessary for Ca^{2+} -induced cessation and lengthen the time for full recovery of the streaming caused by removing Ca^{2+} . We applied Ca^{2+} at two quite different concentrations, 10 μM and 1 mM, to permeabilized cells. The stopping time was 4.9 ± 0.6 min (10 cells) at 10 μM and 3.5 ± 0.9 min (4 cells) at 1 mM. The recovery time was 2.9 ± 0.4 min (10 cells) at 10 μM and 3.6 ± 0.7 min (4 cells) at 1 mM. There were no significant differences in either stopping or recovery time for the hundredfold increase in Ca^{2+} concentration. Even at 1 μM $[\text{Ca}^{2+}]_0$, both the stopping and recovery times are only slightly different from those at 10 μM (37). Thus it is assumed that the mechanism of Ca^{2+} -induced cessation is more or less affected by permeabilization of the plasmamembrane. Although the integrity of the cytoplasm is maintained after permeabilization, the Ca^{2+} -sensitizing component or some soluble low molecular weight component which intensifies the effect of the Ca^{2+} -sensitizing component may be lost or affected by the treatment with EGTA via an unknown mechanism.

The Ca^{2+} -sensitizing component is assumed to be present in the streaming endoplasm because the Ca^{2+} -sensitivity was drastically reduced in the tonoplast-free cells, in which most of the endoplasm is dispersed in the vacuolar space (36, 37, 38). Therefore it is assumed that Ca^{2+} -action is linked not to actin but to putative myosin present in the endoplasm, either directly or indirectly via the Ca^{2+} -sensitizing component. This assumption can be supported by the following facts. The Mg-ATPase of synthetic actomyosin from *Physarum* plasmodium was inhibited to half with micromolar Ca^{2+} , probably via action on myosin (16). Latex beads coated with *Physarum* myosin can move along actin bundles of tonoplast-free *Nitella* cells faster in the absence of Ca^{2+} than in its presence (28). On the other hand, the movement of beads coated with scallop myosin, whose interaction with actin is stimulated by Ca^{2+} (4), occurred only in the presence of Ca^{2+} (28). Movement of latex beads coated with skeletal muscle myosin which lacked Ca^{2+} -sensitivity was not affected by Ca^{2+} at all (32). These results clearly show that actin bundles of *Characeae* cannot be the site giving Ca^{2+} -sensitivity to cytoplasmic streaming.

Differing from the cessation time, the recovery time in permeabilized cells (3–4 min) is about the same as that in intact cells, which is 5 min or more (31, 39). The rate of initial recovery is assumed to be controlled by the rate of removal of Ca^{2+} bound to some component of the motile system, but the time needed for complete recovery is not affected by the rate of Ca^{2+} removal (31). After the action potential, the cytoplasmic Ca^{2+} concentration returned to its resting level in 40–60 sec, while the streaming took 75–350 sec to recover (39). Thus the removal of Ca^{2+} from the probable complex of Ca^{2+} and the Ca^{2+} -sensitizing component is not a determinant of the rate of recovery of streaming. This suggests that the recovery process, which is assumed to be related to CaM, is time-dependent, as Scharff (26) reviewed that the transitions between non-activated and activated enzyme states exhibit a time-lag when the CaM concentration is low, which matches our finding that the concentration of *Chara* CaM is low so long as it is not *Chlamydomonas*-type CaM but spinach-type, as mentioned above. At present we are ignorant about the action mechanism of CaM in the streaming recovery; however, we can at least say that the Ca^{2+} -sensitizing component, which is hypothesized to be CaM, does not directly act on actomyosin

because the CaM-BDs of appropriate concentrations in the absence of Ca^{2+} do not interfere with the streaming.

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