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Effects of quinine on the intracellular calcium level and membrane potential of PC 12 cultures

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Abstract

The mechanism for the perception of bitterness appears to be quite complicated, even for quinine, which is a model bitter substance, and thus has yet to be completely elucidated. To investigate the possibility of being able to predict the bitterness of quinine solutions, we examined the effects of quinine on intracellular calcium ion concentration ($[Ca^{2+}]i$) and membrane potentials in PC 12 cultures. $[Ca^{2+}]i$ and membrane potentials were analysed by fluorescence confocal microscopic imaging using the Ca^{2+}-sensitive probe Calcium Green 1/AM and the membrane potential-sensitive probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$). Quinine elicited an increase in the membrane potential along with a concentration-dependent increase in $[Ca^{2+}]i$. These increases were inhibited by extracellular Ca^{2+}-free conditions, thapsigargin, which is a Ca^{2+}-pump inhibitor, and U73122, which is a phospholipase C inhibitor. The quinine-induced increase in $[Ca^{2+}]i$ levels was inhibited by nifedipine, an L-type Ca^{2+}-channel blocker, ω-conotoxin, a T-type Ca^{2+}-channel blocker, and BMI-40, which is a bitterness-masking substance. These results suggest that responses in PC 12 cultures may be used as a simple model of bitterness perception.

Introduction

Quinine is an alkaloid isolated from the bark of the cinchona tree; it is widely used as an antimalarial drug. It is also used as a bitter flavouring agent and has been used as a standard substance in the study of bitterness perception. However, the mechanism of bitterness perception remains unclear.

Quinine has been reported to stimulate inositol-1,4,5-trisphosphate (IP_3) production, decrease intracellular cAMP and to directly block potassium ion channels. However, whether these mechanisms coexist within the same taste cells has not yet been determined (Spielman et al 1996; Ming et al 1998). In addition, quinine has been reported to induce an inward current in bullfrog taste receptor cells via the opening of a non-selective cation channel (Tsunenari & Kaneko 2001; Tsunenari et al 1996; 1999). Kumazawa et al (1985) reported membrane depolarization in the N-18 clone of neuroblastoma cells, and found that when testing several different substances known to be bitter, this depolarization was predictive of the bitterness. We found that quinine induced an increase in $[Ca^{2+}]i$ in cultured neuro-2a cells via the activation of the phospholipase C (PLC)–IP_3 pathway and the opening of N-type Ca^{2+} channels (Nakamura et al 2003).

In the present study, we used the membrane-potential-sensitive probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) and Calcium Green 1/AM to examine the effects of quinine on the membrane potential and $[Ca^{2+}]i$ in PC 12 cultures, which are now widely used in neuroscience research. We also examined the possibility of using these findings to develop a bitter-taste evaluation system.

Materials and Methods

Materials

Quinine hydrochloride, thapsigargin, U73122 and nifedipine were obtained from Sigma Chemical Co. (St Louis, MO, USA). ω-Conotoxin-GVIA was purchased from Peptide Institute,

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Inc. (Osaka, Japan). Calcium Green 1/AM and $DiBAC_4(3)$ were purchased from Molecular Probes (Eugene, OR, USA). All drugs except for quinine and the fluorescent probes were dissolved in DMSO (final concentration < 0.1%). Drug concentrations are expressed as the final molar concentration in the physiological saline solution (PSS) on the plates. PSS had the composition (in mM): NaCl 140; KCl 4.0; $CaCl_2$ 2.0; $MgCl_2$ 2.0; HEPES 10; glucose 10; pH 7.4 (adjusted with NaOH).

Cell culture

PC 12 cells were obtained from Dainippon Sumitomo Pharma Co. (Osaka, Japan) and were cultured in Dulbecco's Modified Eagle's Medium (Nacalai Tesque Co., Kyoto, Japan) supplemented with 10% fetal calf serum, 50 units mL^{-1} penicillin and 50 $\mu g\,mL^{-1}$ streptomycin in 5% CO_2/95% air at 37°C. The cells were passaged every 3 days in fresh medium for up to 20 passages. They were seeded onto collagen-coated glass coverslips (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) at a density of 10%. At 24 h after cultivation, they were differentiated for 10 days in differentiation medium (Neurobasal medium supplemented with 1% fetal calf serum, 0.5 mM L-glutamine, 100 ng mL^{-1} neurone growth factor, 1% nitrogen supplement and 0.025 mM 2-mercaptoethanol).

Measurement of $[Ca^{2+}]i$ in PC 12 cells

After 10 days' differentiation, the PC 12 cultures were loaded with 5 μM Calcium Green 1/AM for 30 min at 37°C. Cells were then rinsed twice with PSS and incubated in PSS for an additional 15 min at 37°C to allow for completion of the hydrolysis of any intact ester linkages in intracellular Calcium Green 1/AM. After loading, the coverslips were placed under an inverted phase-contrast microscope coupled to a Nipkow disk confocal scanner (CSU10; Yokogawa Electric Corp., Tokyo, Japan). An excitation wavelength of 488 nm was provided by an argon–krypton laser (Omnichrome, Chino, CA, USA) and a 515 nm long-pass emission filter through the planfluor objective (×20; numerical aperture = 0.5). Fluorescence images were acquired from 30 × 30 pixels and were collected every 5 s. The response of each cell can be seen in the 30 × 30 pixels. Quinine was added directly to the cultured cells; inhibitors or the vehicle (PSS, controls) were added 15 min before addition of quinine. $[Ca^{2+}]i$ values were determined from the peak fluorescence intensity (F) with quinine divided by the fluorescence intensity value (F_0) just before quinine administration (F/F_0).

Measurement of membrane potential in PC 12 cells

The effect of quinine on the membrane potential in PC 12 cells was measured with $DiBAC_4(3)$ in a fluorescent confocal microscopic imaging system. After 10 days' differentiation, the PC 12 cultures were rinsed twice with 500 μL PSS containing 5 μM $DiBAC_4(3)$ and incubated with 1.8 mL PSS in a cell incubator for 30 min to ensure dye distribution across the cell membrane. Assays were carried out at 37°C and were initiated by the addition of 180 μL of a 10× concentration of the test compound prepared in PSS containing $DiBAC_4(3)$. Drugs were applied in the same way as described above. The coverslips were analysed using the method described above and membrane potential determined from the peak fluorescence intensity (F) with quinine divided by the fluorescence intensity value (F_0) just before quinine administration (F/F_0).

Gustatory sensation tests in human volunteers

Healthy volunteers (9 women, aged 20–23 years) took part in the gustatory sensation tests. The volunteers were first asked to keep standard quinine hydrochloride solutions (0.01, 0.03, 0.1, 0.3 and 1 mM) in their mouths for 15 s. The participants were given information on the concentrations and the bitterness scores for each of the solutions (Nakamura et al 2002). Subsequently, they were asked to taste a 5 mL sample of a 0.3 mM test drug solution, and then asked to give the sample a bitterness score. The samples were also kept in the mouth for 15 s. After tasting each sample, subjects gargled well and waited for at least 20 min before tasting the next sample.

Statistical analysis

All data are presented as the mean ± s.e. m for individual groups (n = 4–11). Statistical analysis of the data was done using a Student's t-test and analysis of variance, followed by Fisher's post-hoc protected least significant difference (PLSD) test, where appropriate. A 0.05 level of probability was regarded as significant. Statistical analyses were performed using computer software (Stat View 4.5, Abacus Concepts, Berkeley, CA, USA).

Results

Effect of quinine on $[Ca^{2+}]i$ and the membrane potential

Figure 1 shows fluorescent confocal microscopic images from PC 12 cultures before and after treatment with 1 mM quinine. The fluorescent intensity inside the cell was enhanced by treatment with quinine. Figure 1c shows the time course for change in $[Ca^{2+}]i$ after treatment with the quinine solution. $[Ca^{2+}]i$ increased within 15 s of quinine stimulation, and then gradually returned to a basal level over 1 min.

Figure 2 shows the effect of quinine on the membrane potential in the PC 12 cultures as fluorescent confocal microscopic images for PC 12 cells before and after treatment with 1 mM quinine. The fluorescent intensity inside the cells was enhanced by treatment with quinine. Figure 2c shows that membrane potential was increased by treatment with quinine, increasing within 5 s, followed by a subsequent return to basal levels within 20 s of quinine stimulation. The quinine-induced change in membrane potential was more rapid than the change in $[Ca^{2+}]i$ in PC 12 cultures.

Concentration–response curves for the effect of quinine on $[Ca^{2+}]i$

Figure 3 shows the concentration–response curve for $[Ca^{2+}]i$ in PC 12 cultures. The points indicate the peak $[Ca^{2+}]i$ values after treatment with the quinine solution. Quinine increased the $[Ca^{2+}]i$ in a concentration-dependent manner.

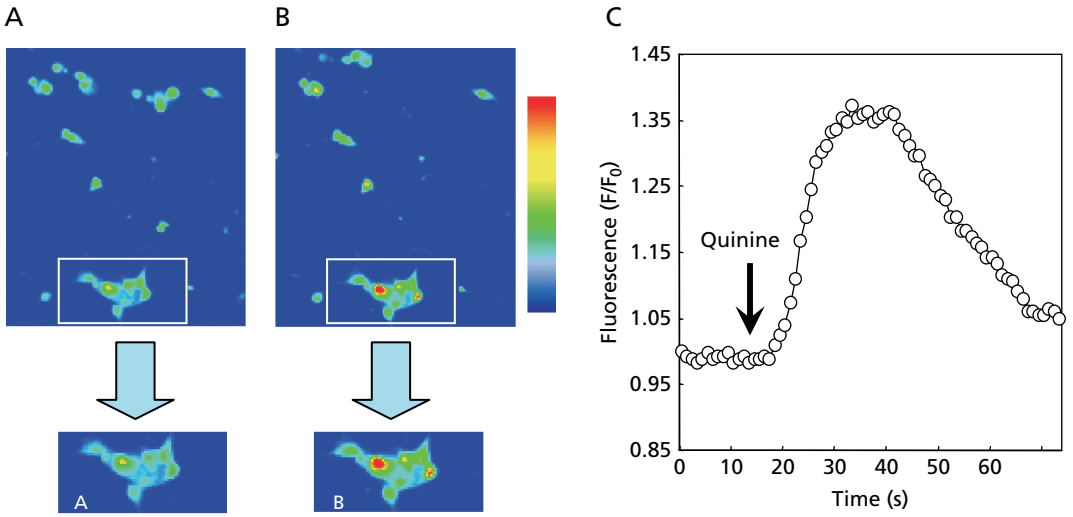

Figure 1 Effect of 1 mM quinine on intracellular calcium ion concentration ($[Ca^{2+}]i$) in PC 12 cultures: fluorescent confocal microscopic images of the $[Ca^{2+}]i$ levels of the PC 12 cultures before (A) and after (B) treatment with quinine for 18 s. The bar represents fluorescent intensity. The upper red zone is high fluorescent intensity and the lower blue zone is low intensity. The graph (C) shows the time course for the changes in the $[Ca^{2+}]i$ levels in the PC 12 cultures treated with quinine (determined as peak fluorescence intensity (F) with quinine divided by the fluorescence intensity value (F_0) just before quinine administration).

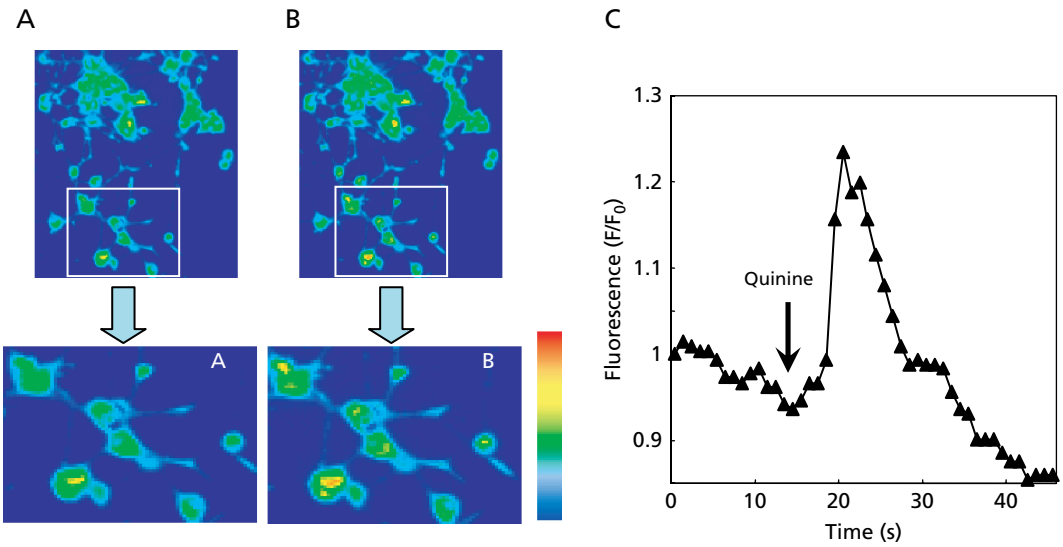

Figure 2 Effect of 1 mM quinine on the membrane potential in PC 12 cultures: fluorescent confocal microscopic images of the membrane potential in PC 12 cultures before (A) and after (B) treatment with quinine for 6 s. The bar represents fluorescent intensity. The upper red zone is high fluorescent intensity and the lower blue zone is low intensity. The graph (C) shows the time course for the change in the membrane potential in the PC 12 cultures treated with quinine (determined as peak fluorescence intensity (F) with quinine divided by the fluorescence intensity value (F_0) just before quinine administration).

Participation of external and internal Ca^{2+} in the quinine-induced $[Ca^{2+}]i$ responses

Figure 4 shows the effect of calcium-free medium, 10 μM nifedipine (an L-type Ca^{2+}-channel blocker) and 1 μM ω-conotoxin (a T-type Ca^{2+}-channel blocker) on the increase in $[Ca^{2+}]i$ induced by quinine in PC 12 cultures. Quinine-induced increases in $[Ca^{2+}]i$ were inhibited by extracellular Ca^{2+}-free conditions, nifedipine and ω-conotoxin (Figure 4a). Figure 4b shows that 3 μM thapsigargin (a Ca^{2+}-pump inhibitor) and 10 μM U73122 (a PLC inhibitor) also inhibited the quinine-induced increase in $[Ca^{2+}]i$ in PC 12 cultures. U73343 (10 μM), an inactive analogue of U73122, did not inhibit the quinine-induced increase in $[Ca^{2+}]i$.

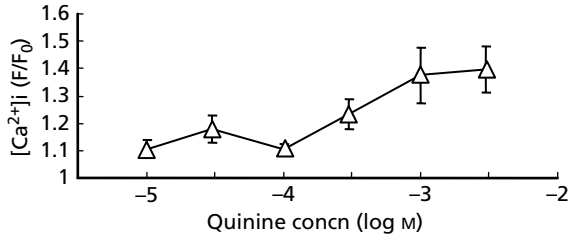

Figure 3 Relationship between the concentration of quinine and intracellular calcium concentration ([Ca^{2+}]i; determined as peak fluorescence intensity (F) with quinine divided by the fluorescence intensity value (F_0) just before quinine administration) in PC 12 cultures.

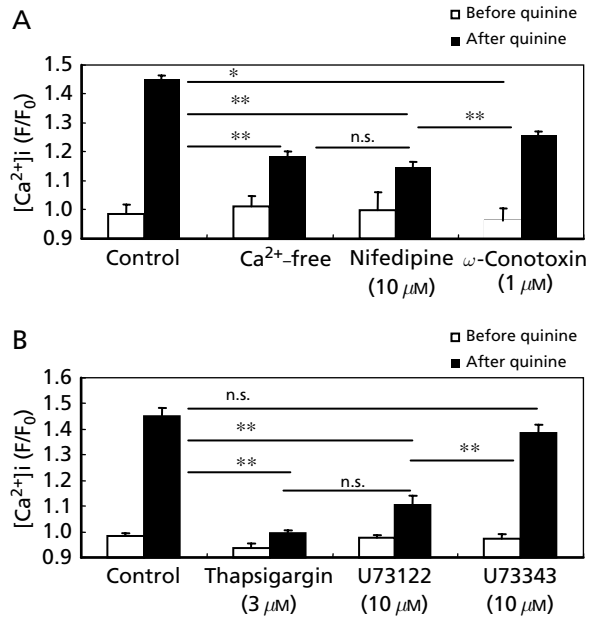

Figure 4 Effects of calcium-free medium, nifedipine, ω-conotoxin, thapsigargin, U73122 and U73343 on the increase in the intracellular calcium ion concentration ([Ca^{2+}]i; determined as peak fluorescence intensity (F) with quinine divided by the fluorescence intensity value (F_0) just before quinine administration) induced by 1 mM quinine in PC 12 cultures. *$P < 0.05$; **$P < 0.01$ vs control. In control experiment, vehicle was added to culture cells 15 min before quinine.

Effects of BMI-40 on the quinine-induced membrane potential and [Ca^{2+}]i responses

Figure 5 shows that 0.025% BMI-40, a bitterness-masking substance, inhibited both the increase in [Ca^{2+}]i and increase in membrane potential induced by quinine in PC 12 cultures.

Relationship between the human gustatory bitterness score and the added concentration of quinine

Figure 6 shows a dose-dependent increase in bitterness scores for the quinine solution in human volunteers. The correlation coefficient (r^2) for the multiple regression analysis was 0.8949 ($P = 0.0009$**).

Discussion

There have been a number of reports on the activation of G proteins that participate in the bitter transduction mechanism for quinine. This involves a G-protein-mediated activation of PLC, which results in increased intracellular levels of IP_3. Additionally, Ca^{2+} released from intracellular stores by IP_3 seems to lead directly to neurotransmitter release (Akabas et al 1988; Hwang et al 1990; Spielman et al 1994; Ogura et al 1997). It has also been reported that several other bitter compounds do not require G-protein-coupled receptors for transduction (Ogura & Kinnamon 1999). In the mudpuppy, *Necturus maculosus*, quinine directly blocks voltage-gated potassium channels located on the apical membrane of taste cells, which leads to membrane depolarization and transmitter release (Kinnamon 1988; Bigiani & Roper 1991; Cummings & Kinnamon 1992). Tsunenari et al (1999) have also shown that in the frog taste cell, quinine directly activates a cationic channel and induces an inward current from near the resting potential. Furthermore, quinine activates a Ca^{2+} channel and elicits an increase in [Ca^{2+}]i of the taste cells (Tsunenari et al 1999; Caicedo & Roper 2001; Tsunenari & Kaneko 2001).

In the present study, quinine elicited an increase in the membrane potential and in [Ca^{2+}]i. The peak in membrane potential occurred more quickly than the peak in [Ca^{2+}]i. These observations suggest that quinine may induce membrane depolarization, leading to the activation of L-type and/or T-type Ca^{2+} channels. While the exact trigger for the depolarization by quinine is not clear, inhibition of a potassium ion channel or activation of a cation channel may contribute to the depolarization (Kinnamon & Roper 1988; Bigiani & Roper 1991; Cummings & Kinnamon 1992; Tsunenari et al 1999; Caicedo & Roper 2001; Tsunenari & Kaneko 2001). Under extracellular Ca^{2+}-free conditions, the quinine-induced increase in [Ca^{2+}]i was significantly, but not completely, inhibited. Thus, it is possible that the quinine-induced increase in [Ca^{2+}]i levels is due to both extracellular and intracellular calcium components. Indeed, both thapsigargin and U73122 inhibited the quinine-induced increase in [Ca^{2+}]i. Therefore, Ca^{2+} released from calcium stores by stimulation of the IP_3 receptor contributes to the increase in [Ca^{2+}]i induced by quinine.

Taken together, these findings suggests that quinine-induced increases in [Ca^{2+}]i comprise two main mechanisms: one that is extracellular in nature and occurs via the Ca^{2+} channels, and a second one that is derived from intracellular Ca^{2+} stores via the PLC–IP_3 pathway. Overall, there seem to be many common points between the intracellular signals of the PC 12 cultures that are activated by quinine and the bitter taste transmitter release mechanism that is found within the taste cells. Both of the responses to quinine (i.e. the depolarization and the increased calcium levels) were prevented by BMI-40, which is a bitterness-masking substance. We have also found significant correlation between the results for the human gustatory sensation test with quinine and quinine-induced [Ca^{2+}]i increases in PC 12 cultures. These results are

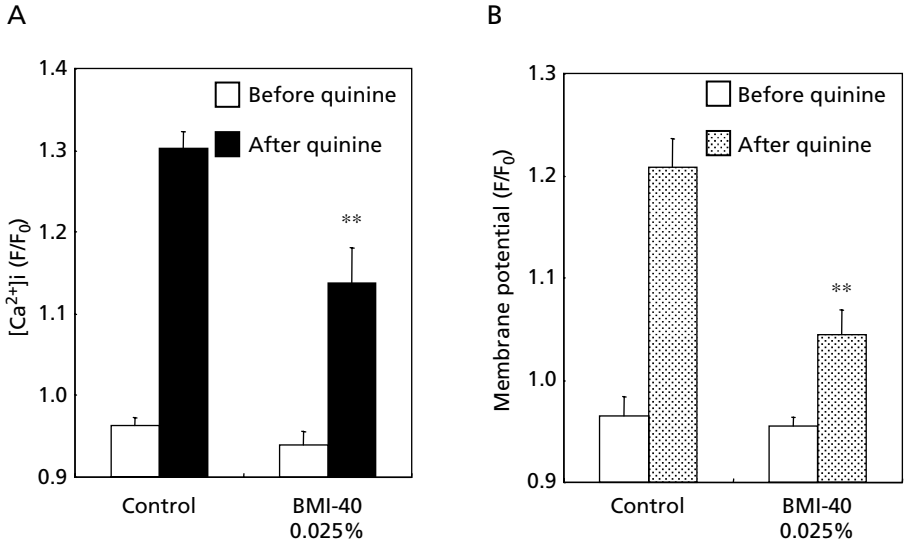

Figure 5 Effect of BMI-40 on the increase in intracellular calcium ion concentration ($[Ca^{2+}]i$ (A) and membrane potential (B) (both determined as peak fluorescence intensity (F) with quinine divided by the fluorescence intensity value (F_0) just before quinine administration) induced by 1 mM quinine in PC 12 cultures. $^{**}P < 0.01$ vs control.

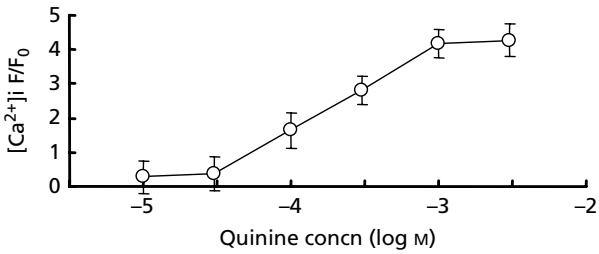

Figure 6 Relationship between the concentration of quinine and human gustatory bitterness scores.

the same as has been reported for the neuro2a cells (Nakamura et al 2003).

Caceido and colleagues have found that when using Ca^{2+} imaging, bitter taste stimuli are detected in the taste buds, and using this method it was possible to record changes in Ca^{2+} concentration in intact taste buds. This suggests that when taste cells are exposed to bitter stimuli, a Ca^{2+} imaging assay could be used to help discriminate between the possible mechanisms (Caicedo et al 2000; Caicedo & Roper 2001).

In addition, the $[Ca^{2+}]i$ response in PC 12 cultures may be useful as a convenient screening model for the evaluation of bitterness. However, before this procedure can be established as a screening method, we need to determine whether the reactions of the PC 12 cultures to quinine are indeed providing a specific signal for bitter tastes.

Conclusion

Quinine elicited an increase in the membrane potential, along with a concentration-dependent increase in $[Ca^{2+}]i$. These increases were inhibited by extracellular Ca^{2+}-free conditions, thapsigargin, which is a Ca^{2+}-pump inhibitor, and U73122, which is a PLC inhibitor. The quinine-induced increase in $[Ca^{2+}]i$ was also inhibited by nifedipine, an L-type Ca^{2+}-channel blocker, ω-conotoxin, a T-type Ca^{2+}-channel blocker, and BMI-40, which is a bitterness-masking substance. The results led us to speculate that responses to the PC 12 cultures may be useful as simple models for bitterness perception.

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