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RESEARCH ARTICLE

An optical dynamic mass redistribution assay reveals biased signaling of dualsteric GPCR activators

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Abstract

Increasing attention is paid in basic science and in drug discovery to pathway selective intracellular signaling as a novel approach to achieve precise control of cell function via G protein-coupled receptors (GPCRs). With respect to signaling, GPCRs are often promiscuous in that more than one intracellular biochemical pathway is activated upon receptor stimulation by the endogenous transmitter or by exogenous drugs. We studied signaling by a novel class of GPCR activators that were designed to bind simultaneously to the orthosteric transmitter-binding site and the allosteric site of muscarinic acetylcholine receptors. An optical biosensor technique was applied to measure activation-induced dynamic mass redistribution (DMR) in CHO cells stably expressing the muscarinic receptor subtype of interest. The use of tools to modulate signaling and measuring G protein activation directly proved that DMR is a valid and comfortable approach to gain real-time insight into intracellular signaling pathway activation and to identify signaling pathway-selective drugs.

Keywords: *Dynamic mass redistribution; functional selectivity; dualsteric ligands; GPCR; muscarinic receptor*

Abbreviations: DMR, dynamic mass redistribution; GPCR, G protein-coupled receptors; [^{35}S]GTPγS, Guanosine 5′-(gamma-thio)triphosphate; PTX, pertussis toxin.

Introduction

The superfamily of G protein-coupled receptors (GPCRs) has major importance for drug therapy and drug development. It is highly desirable to focus drug action on diseased body functions to avoid unwanted side effects. Under conditions of systemic drug therapy, selectivity is commonly achieved on the level of drug binding. In the last years increasing evidence has emerged that selectivity of drug action may also be achieved on the level of intracellular signaling pathway activation (1,2). There is ample evidence that GPCR activation may normally translate into activation of more than one signaling pathway. Such promiscuous signaling may travel along both G protein-dependent and G protein-independent routes (3). It is likely that the pattern of activation of intracellular signaling pathways is governed by the array of conformations that a ligand-bound receptor is allowed to adopt. Consequently, ligands that imprint a specific conformation on the receptor protein with high fidelity should achieve selective signaling pathway activation, also referred to as biased signaling.

Recently, we have exploited this concept in muscarinic acetylcholine receptors (4). These receptors contain the transmitter-binding site in the depth of their ligand-binding pocket. In addition to this orthosteric site the receptor harbours an allosteric site that is located in the extracellular entrance of the binding pocket (5). The allosteric site is far less conserved among the five subtypes of muscarinic receptors than the orthosteric site. We designed allosteric/orthosteric hybrid compounds (Figure 1) to gain simultaneous binding to both the orthosteric and the allosteric receptor region (4,6). This concept allows to link receptor activation mediated

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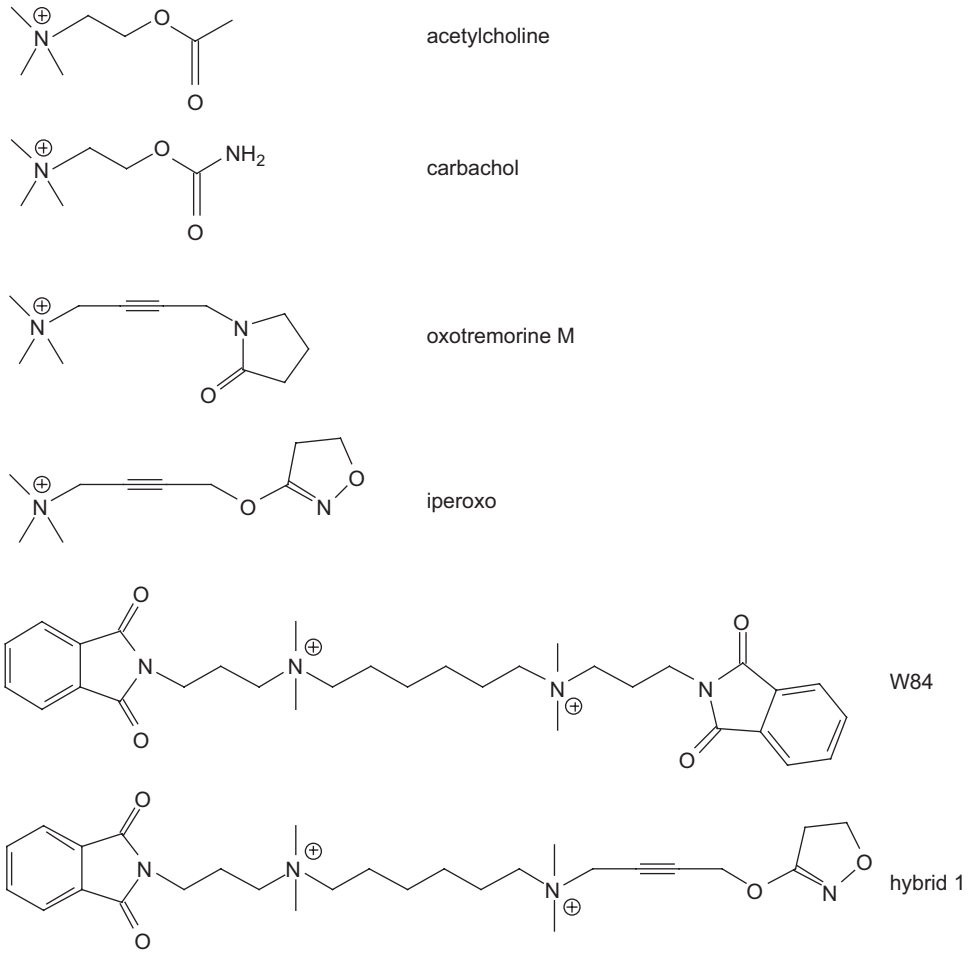

Figure 1. Chemical structures of the test compounds. Acetylcholine, carbachol, oxotremorine M and iperoxo are orthosteric agonists, W84 is an allosteric modulator, and hybrid 1 is a dualsteric (allosteric/orthosteric) agonist.

by the orthosteric site with subtype-selective binding governed by the allosteric site (4). Since such dualsteric ligands extend the interface of ligand/receptor-interaction, it was aimed at gaining insight into signaling pathway activation by this novel type of receptor activators. Measurement of activation-related dynamic mass redistribution (DMR) in intact cells allows a real-time insight into intracellular signaling (7,8). Findings obtained with the Epic® optical biosensor technique for DMR measurement suggest selective signaling by dualsteric ligands (4). In the present study we carried out a thorough validation of our approach and provide the proof of concept for pathway selective, biased signaling by dualsteric agonists.

Material and methods

Test compounds

Acetylcholine iodide, carbachol chloride, oxotremorine M iodide (Figure 1) and atropine sulfate were obtained from Sigma-Aldrich (Steinheim, Germany). Iperoxo iodide and the related dualsteric agonist "hybrid 1" dibromide (Figure 1) have been introduced elsewhere (4,9) and were generously provided by Prof. Dr. Ulrike Holzgrabe, Würzburg, Germany, and Prof. Dr. Marco De Amici, Milan, Italy.

Dynamic mass redistribution (DMR) assay (Corning Epic® Biosensor measurements)

Principle of the technique

We used Chinese hamster ovary (CHO) cells that were untransfected or stably transfected with the human M_2 or M_5 receptor gene. Cells were grown to confluence on the bottom of microplate wells that are individually equipped with optical biosensors [RWG (resonant waveguide grating)]. Broadband laser light is sent into the biosensor, which selects light of a given wavelength and guides this light to travel parallel to the bottom of the microwell (Figure 2) (7,8,10,11). The electromagnetic field of the light extends for a depth of 150 nm ("detectable DMR") into the adjacent cells. Depending on the

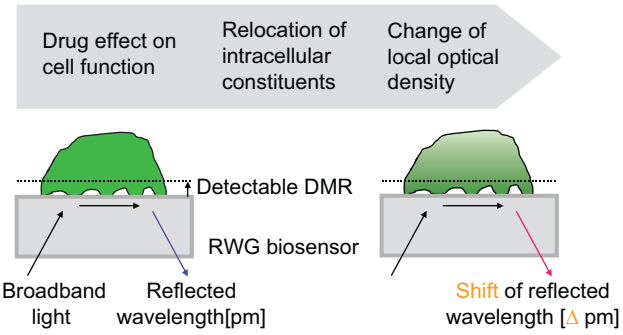

Figure 2. Principle of the Epic® technique for real-time measurement of dynamic mass redistribution. Modified from Ref. 10, for details see text.

optical density of the cell layer in this area, the travelling light loses energy. The light beam is reflected for wavelength measurement. In response to an intervention that affects cell function, intracellular constituents such as proteins, vesicles, or organelles are relocated, which leads to a change of local optical density. A shift of reflected wavelength reflects dynamic mass redistribution and is recorded in real time.

The beta version of the Corning Epic® System was used. It consists of a temperature-control unit, an optical detection unit, and an integrated robotic liquid handling device. CHO cells, untransfected or stably transfected with the hM_2 or hM_5 receptor gene, were seeded in a density of 12,500 cells per well in 384-well Epic® microplates with 40 µL of growth medium and cultured at 37°C in an atmosphere of 5% CO_2 for about 20 hr with or without pertussis toxin (100 ng/mL) to achieve confluent cell layers. Each well of a 384-well Epic® microplate contains a resonant waveguide grating biosensor. After removing cell culture medium and washing the cells twice with 50 µL of assay buffer per well (HBSS with 20 mM HEPES, pH 7.0), cells were allowed to rest in 30 µL of assay buffer for 2 hr in the Epic® reader at a constant temperature of 28°C. After addition of 10 µL test compound dissolved in assay buffer DMR responses were monitored for at least 1 hr. In prestimulation experiments, forskolin is added to the assay buffer in the appropriate concentration after the washing procedure. A stock solution (40 mM) of forskolin is prepared in DMSO. In forskolin 10 µM, this results in a final DMSO concentration of 0.025% in the assay buffer; this concentration has no detectable effect on the cells. The shown optical recordings are baseline corrected [i.e., drug-induced wavelength shifts were corrected for signals obtained by addition of a drug-free solvent control ("blank")]. Blank signals range between 0 and 30 pm.

Microplates for Epic® were obtained from Corning (NY, USA). Flp-In™-CHO cells, Hanks' Balanced Salt Solution (HBSS), HEPES buffer solution, and pertussis toxin were obtained from Invitrogen Corp. (Carlsbad, CA). Forskolin was obtained from Applichem (Darmstadt, Germany).

[^{35}S]GTPγS assay

Membrane preparation from CHO cells was carried out as described before (12). For inactivating the $G_{i/o}$ pathway, cells were pretreated overnight with pertussis toxin (100 ng/mL) before membrane preparation. [^{35}S]GTPγS-binding experiments were carried out as described before (13) in a buffer composed of (final concentrations): 10 mM HEPES, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.4, containing 10 µM GDP and the test compounds at the indicated concentrations. After adding 0.07 nM [^{35}S]GTPγS (final concentration), the mixture was incubated for 60 min at 30°C before separating membrane-bound radioactivity by vacuum filtration.

Results and discussion

DMR signatures reflect differential signaling of muscarinic receptor subtypes

The five muscarinic receptor subtypes can be subdivided into two groups depending on the profile of intracellular signaling pathway activation (14). The even-numbered subtypes M_2 and M_4 preferentially couple to $G_{i/o}$, whereas the odd-numbered subtypes M_1, M_3, and M_5 prefer coupling to G_q. With respect to the binding of allosteric agents, M_2 often has highest and M_5 lowest affinity (15–18). CHO cells stably expressing M_2 or M_5 receptors respond to the physiological transmitter in the Epic® device with a positive wavelength-shift (Figure 3A and B), which reflects an increase of optical density. The chosen concentration of acetylcholine is sufficient for full receptor binding and activation according to radioligand binding and G protein-activation experiments (4). The DMR signal declines with time after the initial steep peek in the case of M_2-mediated cell activation, whereas the M_5-induced signal is rather stable over the observation period of 45 min. To check for the expected differential signaling pathway activation, pertussis toxin (PTX) was applied. PTX selectively inactivates $G_{i/o}$ by catalyzing ADP-ribosylation of its α-subunit. As a consequence, $G_{i/o}$-mediated inhibition of adenylyl cyclase is prevented (19). Pretreatment with PTX eliminated the upward deflection of the DMR signal in M_2-CHO cells, thus demonstrating the crucial role of the $G_{i/o}$ pathway for M_2 receptor-mediated cell activation under control conditions. In contrast, PTX had no effect on the M_5-mediated DMR signal, indicating that $G_{i/o}$ is not involved in the cellular response to M_5 receptor activation.

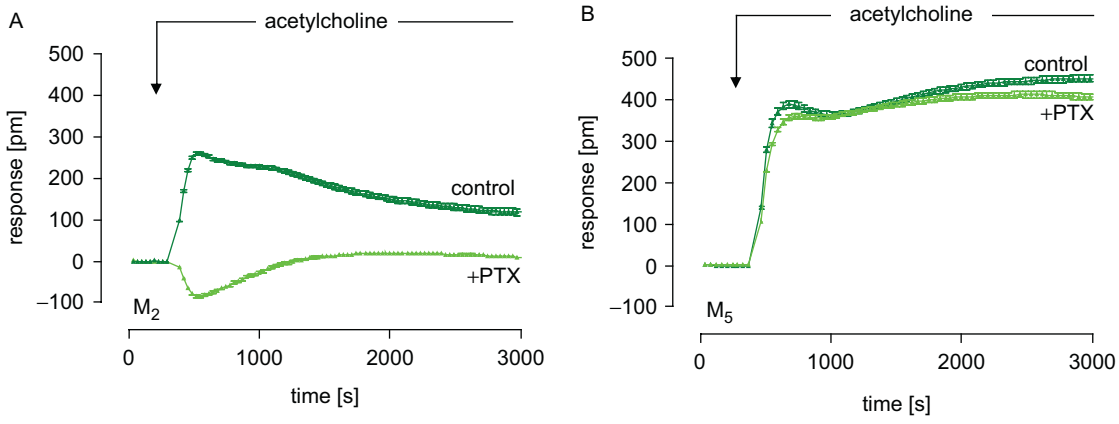

Figure 3. DMR signatures of acetylcholine-induced muscarinic receptor activation reveal subtype-specific signaling. (A, B) CHO cells expressing the indicated muscarinic receptor subtype were challenged with acetylcholine, 100 μM. Plotted is the picometer [pm] shift of reflected light wavelength against the time [s]. (A) hM_2 is a $G_{i/o}$-coupled (even numbered) M-receptor; $G_{i/o}$ protein-signaling is sensitive to pertussis toxin (PTX). (B) hM_5 is a G_q-coupled (odd-numbered) M-receptor; G_q protein-signaling is insensitive to PTX. Shown are mean values ± SEM derived from a representative experiment carried out with four separate determinations. Similar findings were obtained in two additional experiments.

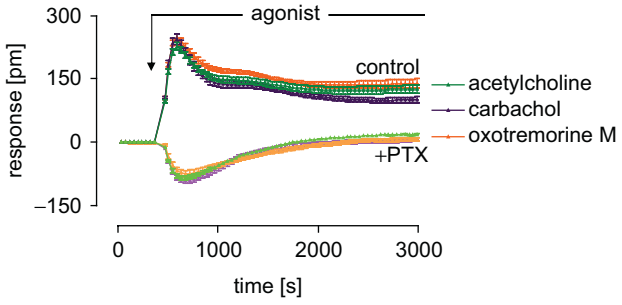

Figure 4. DMR signatures reveal promiscuous hM_2 signaling by acetylcholine-type agonists. DMR signatures as induced by conventional orthosteric full agonists were recorded in hM_2-CHO cells; indicated is the pm-shift of reflected light wavelength against the time [s]. Agonists were applied in equieffective supramaximal concentrations (acetylcholine 100 μM, carbachol 1000 μM, oxotremorine M 10 μM). In a uniform pattern, agonist-induced $G_{i/o}$ pathway activation (control) was eliminated by pretreatment of cells with pertussis toxin (PTX), thus unveiling putative G_s pathway activation. Shown are mean values ± SEM from a representative experiment carried out with eight separate determinations. Similar findings were obtained in two to three additional experiments.

DMR signals reveal promiscuous M_2 signaling by conventional orthosteric agonists

As shown in Figure 3A, PTX pretreatment did not only extinguish the acetylcholine-induced $G_{i/o}$ signal in M_2-CHO cells but also revealed mass redistribution into the opposite direction. Further measurements confirmed that this finding is representative for conventional muscarinic receptor activating drugs, because the effect of high concentrations of acetylcholine and the classical muscarinic agonists carbachol and oxotremorine M were virtually identical (Figure 4). Taken together, these findings show that conventional muscarinic agonists are promiscuous M_2-receptor activators in living CHO cells as more than one signaling pathway is stimulated. This observation is in line with biochemical measurements that show additional G_s pathway activation by muscarinic agonists (20–22). G_s proteins stimulate adenylylcyclase, which leads to increased cAMP formation.

We suspected that the downward-deflected DMR signal reflects G_s activation. To test this hypothesis, forskolin, which is a cell membrane-penetrating direct activator of adenylylcyclase, was applied and DMR recordings were obtained. Indeed, forskolin also induced a downward deflected DMR signature (Figure 5A), which is independent of the M_2 receptor (Figure 5B) and reminiscent of the downward-deflected signal induced by orthosteric agonists in PTX-pretreated M_2-CHO cells (compare with Figure 4). Hence, the downward-deflected DMR signals are concluded to represent G_s pathway activation.

Proof of biased signaling of a dualsteric muscarinic agonist

The design of the dualsteric agonist from an allosteric and an orthosteric building block is illustrated in Figure 1. The orthosteric building block iperoxo is a highly potent agonist devoid of subtype selectivity (9). The allosteric building block W84 is an allosteric inverse agonist with M_2-selectivity (13,23–25). The resulting hybrid proved to be a muscarinic agonist with M_2-selectivity in living tissue preparations with a true dualsteric binding mode: the agonistic activity is mediated via the orthosteric site; the binding subtype-selectivity depends on the allosteric site (4). Remarkably, a maximally active concentration of the dualsteric compound did not induce the downward deflected DMR signal (Figure 6) (4), which is typical for conventional orthosteric full agonists. This finding suggests selective, biased signaling pathway activation.

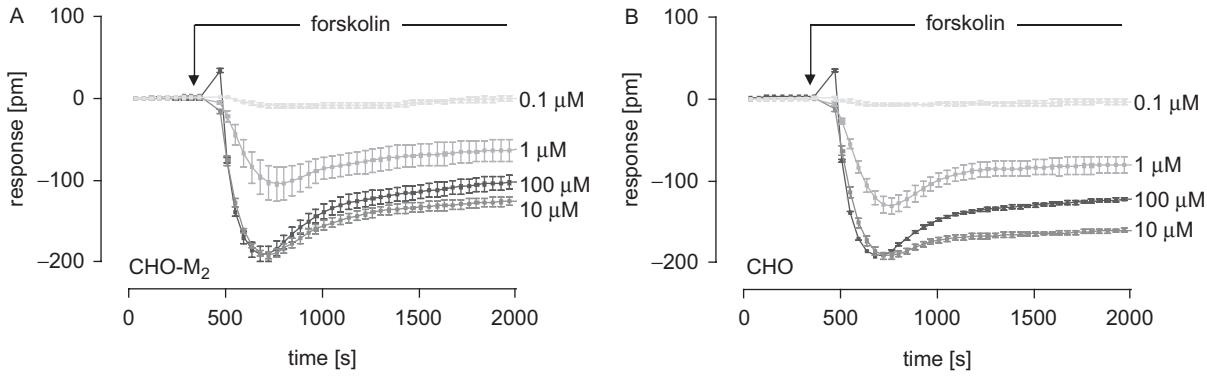

Figure 5. Adenylylcyclase activation by forskolin circumvents the hM_2-receptor/G_s-pathway but yields the same DMR signature. Forskolin was applied at the indicated concentrations to activate adenylylcyclase directly in hM_2-expressing CHO cells (A) and in non-transfected CHO cells (B). Shown are mean values ± SEM of a representative experiment carried out with four separate determinations. Similar findings were obtained in two additional experiments.

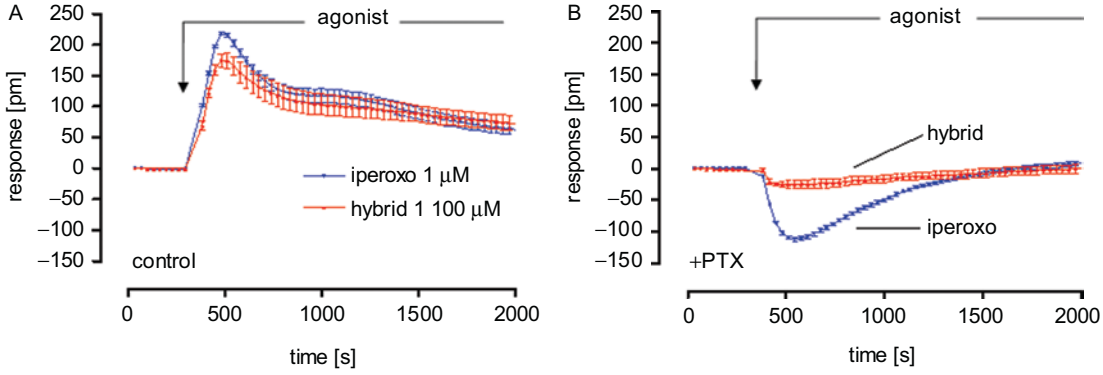

Figure 6. Signaling induced by a dualsteric agonist lacks the G_s-component. (A) DMR signatures were recorded in hM_2-CHO cells for the dualsteric agonist "hybrid 1" and its agonist building block iperoxo. (B) Pretreatment of cells with pertussis toxin (PTX, 100 ng/mL) abrogates signaling by the dualsteric agonist, whereas the orthosteric agonist iperoxo induces a signature into the downward-deflected "G_s-direction." Shown are mean values ± SEM of a representative experiment carried out with eight separate determinations. Similar findings were obtained in three additional experiments. (Modified from Ref. 4)

To validate this conclusion on the level of the receptor/G protein interaction, we measured G protein activation directly by means of [^{35}S]GTPγS binding in membranes from PTX-pretreated M_2-CHO cells. Compared with cell membranes from control M_2-CHO cells, pretreatment with PTX diminishes receptor-mediated [^{35}S]GTPγS binding by about 90% (data not shown). Nevertheless, Figure 7 clearly shows stimulation of [^{35}S]GTPγS binding by the conventional agonists acetylcholine and iperoxo, whereas the dualsteric agonist is devoid of any effect after PTX pretreatment. The level of [^{35}S]GTPγS binding in the presence of the dualsteric agonist does not differ from [^{35}S]GTPγS-binding in the presence of atropine, which stabilizes an inactive receptor conformation. Obviously, dualsteric agonist-induced signaling is restricted to the $G_{i/o}$ pathway.

In conclusion, the current study demonstrates that the Epic® optical biosensor technique for measuring dynamic mass redistribution is a convenient and powerful

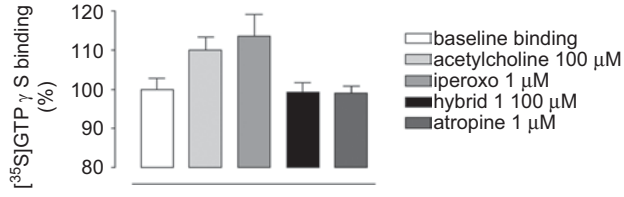

Figure 7. The dualsteric agonist lacks the PTX-insensitive component of G protein activation. Binding of [^{35}S]GTPγS was measured in membranes from hM_2-CHO cells having been pretreated overnight with pertussis toxin (PTX) before membrane preparation. Baseline binding represents the drug-free solvent control; receptor activators (acetylcholine, iperoxo, hybrid 1) were added at concentrations that were equieffective and supramaximal in membranes from control (PTX-untreated) hM_2-CHO cells; the inverse agonist atropine prevents receptor-mediated G protein activation. Indicated are mean values ± SEM from a representative experiment with eight separate determinations. Similar findings were obtained in one additional experiment.

label-free approach for the identification of drugs that modulate GPCR signaling in a pathway-selective fashion.

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***Declaration of interest*:** The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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