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Dual effect of NO on K^+_{ATP} current of mouse pancreatic B-cells: stimulation by deenergizing mitochondria and inhibition by direct interaction with the channel

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Abstract

Nitric oxide (NO) is assumed to contribute to the impairment of B-cell function in type 1 diabetes mellitus (IDDM). In the present paper we show that in mouse B-cells with intact metabolism authentic NO (20 μM) led to a biphasic effect on the K^+_{ATP} current, namely a transient increase and a consecutive almost complete inhibition. This resembles closely the effect that we have observed previously with the NO donor *S*-nitrosocysteine (SNOC, 1 mM) suggesting that merely NO caused both phases of this effect. We now demonstrate that the rise in the current amplitude was accompanied by a depolarization of the mitochondrial membrane potential $\Delta\Psi$ and a concomitant reduction in the ATP/ADP ratio. Thus, it seems likely that the increase in current amplitude is due to the interference of NO with cell metabolism. The subsequent inhibition of the K^+_{ATP} current is assumed to be caused by a direct effect on the channel since K^+_{ATP} single channel current activity measured in excised patches was strongly reduced by authentic NO and SNOC. Our data reveal new insights into the mechanisms underlying the biphasic action of NO on K^+_{ATP} channels in pancreatic B-cells.

Keywords: Pancreatic B-cell; K^+_{ATP} current; Nitric oxide; Mitochondrial membrane potential

1. Introduction

Nitric oxide (NO) participates in autoimmune reactions leading to severe disease states like type 1 diabetes mellitus [1]. It has been suggested that NO produced by activated macrophages in vivo plays an important role in the impairment of B-cell function and finally destruction [2]. According to its action in the organisms NO is either synthesized in low concentrations by constitutive Ca^{2+}/calmodulin-dependent isoforms of the NO synthase or in high concentrations by an inducible, not Ca^{2+}-regulated isoform of the enzyme which is activated in response to cytokines or endotoxins [3].

In a previous paper we have shown that the NO donor *S*-nitrosocysteine (SNOC) evokes rapid changes in the activity of ion channels and it was concluded that this may lead to rapid changes in B-cell function [4]. With low concentrations of SNOC (10 μM and 100 μM) the whole-cell K^+_{ATP} current measured in the perforated-patch mode increased. However, with 1 mM of the NO donor the effect on the K^+_{ATP} current became biphasic. The initial increase was followed by a marked reduction of

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the current suggesting that at higher concentrations SNOC displays two different effects. We speculated that the rise in the K^+_{ATP} current may be due to a decrease in the intracellular ATP/ADP ratio while the current inhibition could be caused by a direct interaction of the NO donor with the channel or tightly associated proteins. An increase in the K^+_{ATP} channel activity by two other NO donors, sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) was observed in the cell-attached mode by Tsuura et al. [5]. They also assumed that a fall in the ATP/ADP ratio could be the reason for this observation. It was one aim of this study to prove (or reject) this hypothesis. Doubts have been cast whether NO donors [6–9] evoke identical effects as authentic NO [10,11] on B-cell function. Therefore, the effects of authentic NO gas on the K^+_{ATP} current and the mitochondrial membrane potential $\Delta\Psi$ as an indicator for ATP production were investigated and compared to the effects of the NO donor SNOC.

Parts of the study have been published earlier in abstract form [12].

2. Material and methods

2.1. Cell and islet preparation

The experiments were performed on islets or single cells of fed female NMRI mice (25–30 g), killed by cervical dislocation. Islets were isolated by collagenase digestion of the pancreas. For patch-clamp experiments and measurements of $\Delta\Psi$ islet cells were dispersed in Ca^{2+}-free medium and cultured up to 3 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin [13]. ATP/ADP ratio was determined with freshly prepared islets.

2.2. Solutions and chemicals

To record currents through single K^+_{ATP} channels at 32°C, seals were obtained using a pipette solution composed of (in mM): 130 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, pH 7.4 adjusted with KOH. The bath solution used for perifusion of the cells (solution 1) was composed of (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 0.5 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. Before patch excision, the bath solution was replaced by a Mg^{2+}-free solution (solution 2) with the following composition (in mM): 130 KCl, 4.6 $CaCl_2$, 10 EDTA, 20 HEPES, pH 7.20 adjusted with KOH. The lack of Mg^{2+} and ATP in this solution prevents phosphorylation and reduced K^+_{ATP} channel rundown probably by this mechanism [14,15]. Whole-cell K^+_{ATP} current recordings were performed at 32°C with nystatin in the pipette solution (150–250 μM). The pipette solution also contained (in mM): 10 KCl, 10 NaCl, 70 K_2SO_4, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, pH 7.15 adjusted with KOH. The bath solution was the same as for the measurements of single K^+_{ATP} channels used before patch excision (solution 1). This bath solution was also used for the determination of $\Delta\Psi$ at 37°C. ATP/ADP ratio was determined in a steady-state incubation at 37°C with a bath solution (solution 3) of the following composition (in mM): 122 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, 15 glucose, supplemented with 0.5% BSA. Conventional whole-cell recordings were performed as described earlier [16].

SNOC was freshly synthesized immediately before each use as follows: an equimolar solution of 100 mM sodium nitrite and cysteine in H_2O was prepared, acidified with HCl for *S*-nitrosylation ($pH < 2$) and neutralized with NaOH [17]. For the preparation of the NO solution an air-tight bottle containing 10 ml H_2O was bubbled with nitrogen for 30 min at 4°C. Afterwards, this solution was bubbled with NO gas for 15 min at 4°C, yielding a saturated solution of approx. 1.9 mM NO at 25°C [18]. Rhodamine 123 was obtained from Molecular Probes (Eugene, OR, USA), NO gas and nitrogen from Messer Griesheim (Reutlingen, Germany). ATP and ADP were from Boehringer (Mannheim, Germany), the BioOrbit 1243-102 ATP-monitoring kit from Merlin Diagnostika (Bornheim-Fersel, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany) in the purest form available.

2.3. Recording methods

Whole-cell K^+_{ATP} currents were measured at 32°C in the perforated-patch configuration at a holding

potential of −70 mV and during 300 ms pulses to −80 mV and −60 mV at 15 s intervals. Under these experimental conditions the current is almost entirely K^+_{ATP} current blockable by tolbutamide [19]. Single K^+_{ATP} channel currents were recorded in inside-out patches at 32°C at a membrane potential of −50 mV. Open probabilities (P_o) were calculated for a recording time of 30 s. Patch-clamp recordings were performed and evaluated with an EPC-9 patch-clamp amplifier and software 'Pulse' (HEKA, Lambrecht, Germany). For off-line analysis, data were stored on video tape, played back by means of a MacLab4S interface with software 'Chart' (WissTech, Spechbach, Germany) and evaluated with 'Igor' (WaveMetrics, Lake Oswego, OR, USA).

ATP/ADP ratio was determined by measuring ATP and ADP in the same batch of eight islets. At the end of the incubation period extracellular fluid was sucked off and islets were disintegrated with NaOH/cysteine solution (40 mM/0.5 mM) and stored at −20°C. For luminescence measurements aliquots of each sample were dissolved with a buffer containing (in mM): 20 creatine phosphate, 100 glycine, 1 $MgSO_4$ at pH 9.0 with or without creatine kinase (20 pg/ml). Aliquots were either neutralized with HCl to pH 7.65 immediately or after an incubation with creatine kinase for 10 min at room temperature to convert all ADP to ATP. ATP concentration was measured in a luciferin/luciferase assay using the ATP-monitoring kit with the luminescence biometer 1253 (Bio-orbit, Merlin).

The mitochondrial membrane potential ($\Delta\Psi$) was measured by determining the rhodamine 123 fluorescence on an Axiovert 100 microscope (Zeiss, Stuttgart, Germany) with an equipment and software delivered by TILL photonics (Planegg, Germany). Excitation light wavelength (480 nm) was adjusted by means of a diffractive grating and then directed through the objective (PlanNeofluar 40×, Zeiss) by means of a glass fibre light guide and a dichroic mirror. The emitted light was filtered (LP 515 nm) and measured by a CCD camera. Single cells or small clusters of cells were loaded with rhodamine 123 (10 μg/ml) for 10 min at 37°C. An increase of Rh 123 fluorescence corresponds to a decrease of mitochondrial membrane potential [20].

Where applicable, experimental data are expressed as arithmetic means ± S.E.M. Statistical significance was accepted at $P \leq 0.05$.

3. Results

3.1. Effects of NO on K^+_{ATP} whole-cell currents of mouse pancreatic B-cells

We have tested authentic NO gas on the K^+_{ATP} whole-cell current measured in the perforated-patch mode. At a concentration of 20 μM a biphasic effect was observed in all cells tested. The initial increase in the K^+_{ATP} current was followed by a marked, irreversible suppression of the current (Fig. 1). On average,

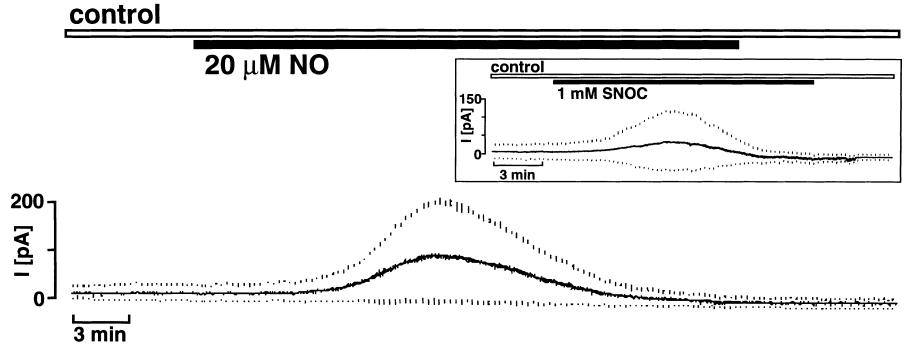

Fig. 1. Effect of NO (20 μM) on K^+_{ATP} whole-cell current of mouse pancreatic B-cells. The K^+_{ATP} whole-cell currents were measured with the perforated-patch technique. Bath solution under control conditions was solution 1. NO was added to the bath solution during the time indicated by the solid horizontal bar. The current is shown at the holding potential of −70 mV (solid line) and during 300 ms steps to −80 and −60 mV (lower dashed trace and upper dashed trace, respectively). This record is representative of six experiments with similar results. The inset shows for comparison the effect of the NO donor *S*-nitrosocysteine (SNOC, 1 mM) on the perforated-patch K^+_{ATP} whole-cell current as published earlier [4].

the current under control conditions elicited by a 10 mV depolarizing step was 25 ± 6 pA ($n = 6$). After addition of 20 μM NO the K^+_{ATP} current first increased to 121 ± 24 pA and afterwards decreased to 18 ± 6 pA ($n = 6$). For comparison the inset shows the effect of the NO donor SNOC at a concentration of 1 mM on the K^+_{ATP} current that has been published earlier [4]. With 2 μM NO the current increased in eight out of 11 experiments from 18 ± 9 pA to 66 ± 27 pA (not shown). In four of these experiments the rise induced by 2 μM NO was transient and the current decreased to 3 ± 1 pA. In three out of the 11 experiments no alteration of the current by 2 μM NO was observed.

3.2. *Effects of SNOC and NO on the ATP/ADP ratio and the mitochondrial membrane potential in mouse pancreatic B-cells*

It seemed likely that the increase in the K^+_{ATP} whole-cell current observed in the perforated-patch configuration is due to a metabolic factor since no increase in the K^+_{ATP} current amplitude was observed even with 10 mM SNOC in the standard whole-cell configuration in which the cell interior is dialysed by the pipette solution ($n = 13$, not shown). To elucidate whether this metabolic factor is ATP we have measured the ATP/ADP ratio (Fig. 2A). The left column shows the ATP/ADP ratio in the presence of 15 mM glucose before the addition of SNOC which mounted up to 3.03 ± 0.21 ($n = 5$). Addition of 0.1 mM and 1 mM SNOC (right columns) for 10 min significantly reduced the ATP/ADP ratio to 2.06 ± 0.21 ($n = 5$, $P < 0.05$) and 1.45 ± 0.06 ($n = 5$, $P < 0.05$), respectively. If only control solution is added the ATP/ADP ratio remained unchanged during this period of 10 min (2.92 ± 0.11, $n = 5$, right column). The ATP/ADP ratio is determined in a steady-state incubation and does not allow to estimate the time course of changes. Thus, for an on-line registration we have monitored changes in rhodamine fluorescence reflecting alterations in the mitochondrial membrane potential ΔΨ which are assumed to be indicative of changes in ATP synthesis [21]. SNOC induced a rapid, dose-dependent depolarization of ΔΨ (Fig. 2B). On average, the change in arbitrary units (a.u.) was 1043 ± 115 and 1688 ± 74 after addition of 0.1 mM and 1 mM SNOC, respectively

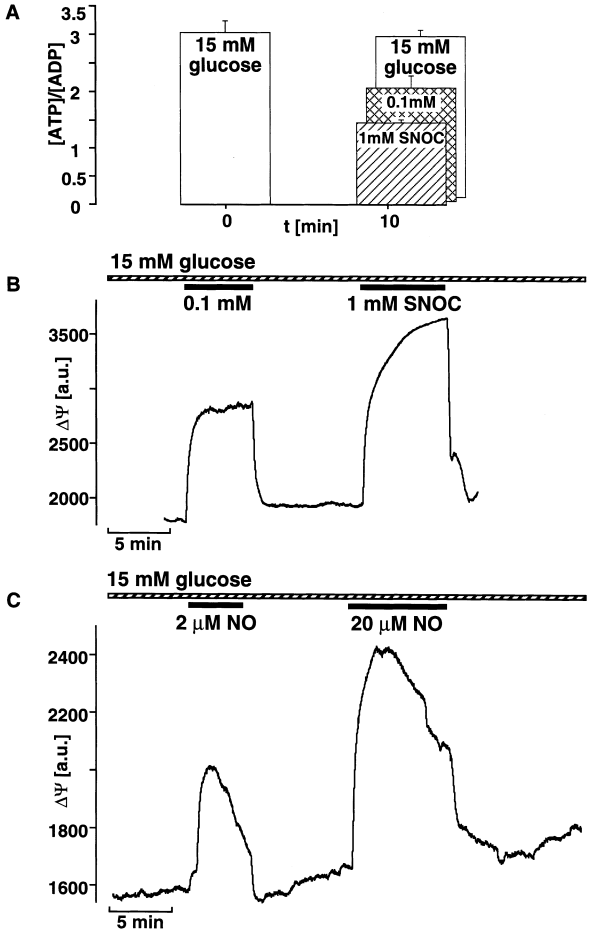

Fig. 2. Effect of NO and SNOC on the ATP/ADP ratio and the mitochondrial membrane potential (ΔΨ) of mouse pancreatic B-cells. (A) Mean changes in the ATP/ADP ratio were monitored 10 min after addition of 0.1 mM and 1 mM SNOC to the incubation medium (solution 3). The left column represents the ATP/ADP ratio under control conditions with bath solution 3, containing 15 mM glucose. Values are means ± S.E.M. of five experiments. (B) The mitochondrial membrane potential ΔΨ has been monitored continuously. 0.1 mM and 1 mM SNOC have been added in the presence of 15 mM glucose at the time indicated by the horizontal bars. This recording is representative of four experiments with similar results. (C) Continuous registration of ΔΨ. NO (2 μM and 20 μM) was added during the time indicated by the horizontal bars at 15 mM glucose. The recording represents 8–10 experiments for the individual manoeuvres.

($n = 4$). The addition of 2 μM ($n = 8$) or 20 μM ($n = 10$) NO in the presence of 15 mM glucose also strongly depolarized ΔΨ (Fig. 2C). On average, the change induced by 2 μM and 20 μM NO gas was 368 ± 53 a.u. and 673 ± 98 a.u., respectively. In contrast to SNOC which continuously releases NO, the effects with NO gas were at least partly transient

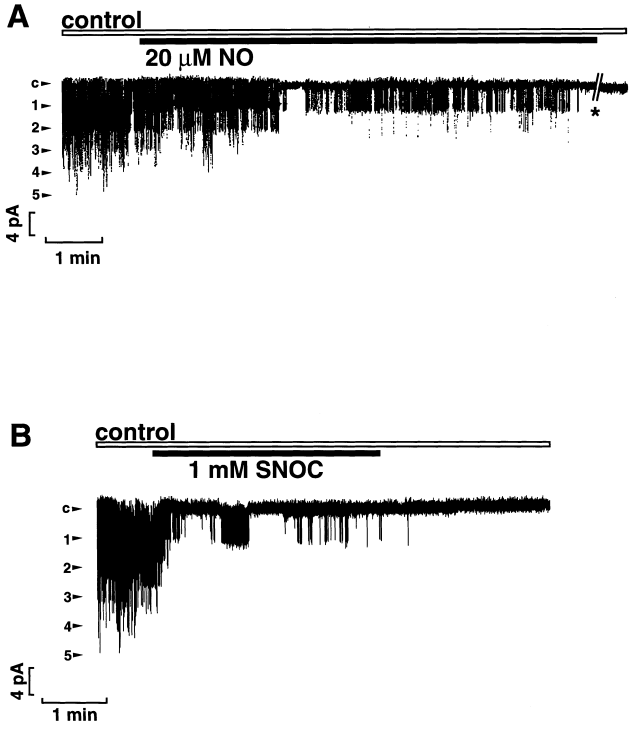

Fig. 3. Effects of NO (20 μM) and SNOC (1 mM) on single K^+_{ATP} channel currents of mouse pancreatic B-cells. The registrations have been performed in the inside-out patch configuration at a membrane potential of −50 mV. Bath solution under control conditions was solution 2. c represents the closed state. (A) NO was added to the bath solution during the time indicated by the horizontal bar. This record is representative of five experiments with similar results. * indicates an interruption of 2 min. (B) SNOC was added to the bath solution during the time indicated by the horizontal bar. The recording is representative of seven experiments with similar results.

probably due to the rapid decomposition of the molecule.

3.3. *Effects of NO and SNOC on single K^+_{ATP} channels of mouse pancreatic B-cells*

We have suggested that the inhibitory effect of NO and SNOC on the K^+_{ATP} whole-cell current is caused by a direct interaction with the K^+_{ATP} current by affecting the channel itself or proteins closely related to it. To prove this assumption we have tested the effect of the compounds on single channel current activity in inside-out patches. As shown in Fig. 3A 20 μM NO almost completely and irreversibly inhibited the single channel current activity. Under control conditions up to five channels were simultaneously open. On average, P_o was reduced from 0.67 ± 0.14 under control conditions to 0.02 ± 0.02 after addition of NO ($n = 5$). Fig. 3B demonstrates that the K^+_{ATP} single channel activity is also markedly and irreversibly reduced after addition of 1 mM of the NO donor SNOC to the bath solution. Under control conditions up to five channels opened simultaneously. On average, 1 mM SNOC reduced P_o from 0.20 ± 0.03 ($n = 7$) under control conditions to 0.01 ± 0.01 ($n = 7$). In time-matched controls no reduction of the single channel current activity was observed ($n = 9$, not shown).

4. Discussion

Overwhelming evidence has accumulated that NO contributes to the impairment of B-cell function and to the eventual destruction of B-cells during the development of IDDM. NO might be produced by activated macrophages [22], activated islet endothelial cells [23] or by the B-cells themselves in response to cytokines [24]. NO has been shown to interfere with enzymes of the glycolysis [25] and the citric acid cycle [26] and to mediate DNA damage accompanied by NAD^+ depletion [27,28]. Moreover, the use of NO donors led to the suggestion that NO also induces rapid changes of the membrane potential and the ion channel activity in B-cells [4,5]. Tsuura et al. [5] have shown that the NO donors SNP and SNAP open K^+_{ATP} channels of mouse pancreatic B-cells with intact cell metabolism. The use of several insulin secretagogues and intermediates of glycolysis led them to suggest that inhibition of phosphofructokinase is responsible for the impairment of glucose metabolism by NO. We have demonstrated that the NO donor SNOC hyperpolarizes the B-cell membrane measured with intracellular microelectrodes in intact islets and increases the K^+_{ATP} whole-cell current registered in the perforated-patch mode with intact cell metabolism at concentrations between 0.01 and 1 mM [4]. However, at the high concentration of the NO donor the augmentation of the current was followed by a strong inhibition pointing to two different modes of action of the compound. The effect of authentic NO gas at a concentration of 20 μM on whole-cell K^+_{ATP} current measured in the perforated-patch mode was also biphasic and almost iden-

tical to the effect of 1 mM SNOC suggesting that the stimulatory and the inhibitory effect of SNOC on the K^+_{ATP} current is caused by NO. At a concentration of 2 μM NO had either no effect on the K^+_{ATP} current or steadily increased it (similar to low concentrations of SNOC [4]) or evoked the biphasic effect also observed with the higher concentration. The difference in the concentration dependence of the two effects by roughly two orders of magnitude [4] may also explain why the activating effect of NO occurs much earlier in the experiments than the inhibition of the K^+_{ATP} current. In this context it is interesting that Brorson and coworkers [29] reported that a SNOC concentration of 1 mM resulted in a mean NO concentration of approx. 10 μM during a period of 20 min at 22°C.

The present paper discloses correlations between the action of NO and SNOC on the K^+_{ATP} current and B-cell metabolism. The increase in the current amplitude can be attributed to a diminution of the ATP/ADP ratio by SNOC and NO. An increase in the glucose concentration is accompanied by a hyperpolarization of $\Delta\Psi$ reflecting a rise in ATP synthesis [21]. This is confirmed by our own observations (data not shown). SNOC depolarized $\Delta\Psi$ in a concentration-dependent manner. Thus, we assume that $\Delta\Psi$ indeed changes in parallel with the ATP/ADP ratio and that alterations in $\Delta\Psi$ can be taken as a measure for changes in ATP synthesis in our cell system. NO also depolarized $\Delta\Psi$ suggesting an inhibition of ATP production. Consistently, it has been reported recently that NO released from NO donors produced a rapid, reversible depolarization of the mitochondrial membrane potential and depletion of cellular ATP in cultured hippocampal neurones [29]. Additionally, it has been shown that NO donors activate the poly(ADP-ribose) polymerase (PARP) in islet cells [28], an effect which by the consumption of reduction equivalents will of course contribute to the ATP diminution of the cells in response to NO. Nevertheless, to our knowledge direct effects of NO or NO donors on the ATP content in pancreatic islet cells have not been shown before. However, the ATP/ADP ratio is diminished after exposure of islets to the cytokine interleukin-1β [30] which is known to induce the formation of NO in islets [31,25,32] and islet B-cells [26].

Since NO and SNOC inhibited the single channel current activity in excised patches it seems justified to speculate that the inhibitory action can be explained by a direct interaction of NO with the channel or closely related proteins. NO has been shown to react with cysteine-free SH groups of serum and plasma membrane associated proteins [33–36]. Thus, it is conceivable that NO and SNOC act on SH groups essential for the regulation of K^+_{ATP} channels in B-cells. It has been shown previously that SH group reagents directly inhibit B-cell K^+_{ATP} currents [37–39] and that the inhibition is more pronounced when the reagent is membrane permeant so that it can act at the cytosolic side [39]. Furthermore, it was found for B-cell K^+_{ATP} channels and mutations expressed in oocytes that the relevant cysteine residue for channel inhibition by SH group reagents is at position 42 of the cytosolic NH_2-terminal part of Kir6.2 [40].

In conclusion, the data presented in this study reveal that NO as well as the NO donor SNOC evoke a complex pattern of interaction with pancreatic K^+_{ATP} channels: the K^+_{ATP} current is stimulated by influencing B-cell metabolism via a reduction in the ATP/ADP ratio and on the other hand inhibited by direct interference with the channel or a regulatory protein in its close vicinity.

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