EXPERIMENTAL STUDY

Glucose triggers protein kinase A-dependent insulin secretion in mouse pancreatic islets through activation of the K_{ATP}^+ channel-dependent pathway

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Abstract

Objective: To assess the significance of protein kinase A (PKA) in glucose triggering of ATP-sensitive K^+ (K_{ATP}^+) channel-dependent insulin secretion and in glucose amplification of K_{ATP}^+ channel-independent insulin secretion.

Methods: Insulin release from cultured perfused mouse pancreatic islets was determined by radioimmunoassay.

Results: In islets cultured at 5.5 mmol/l glucose, and then perfused in physiological Krebs–Ringer medium, the PKA inhibitors, H89 (10 μ mol/l) and PKI 6–22 amide (30 μ mol/l) did not inhibit glucose (16.7 mmol/l)-induced insulin secretion, but inhibited stimulation by the adenylyl cyclase activator, forskolin (10 μ mol/l). In the presence of 60 mmol/l K^+ and 250 μ mol/l diazoxide, which stimulates maximum Ca^{2+} influx independently of K_{ATP}^+ channels, H89 (10 μ mol/l) inhibited Ca^{2+} -evoked insulin secretion, but failed to prevent glucose amplification of K_{ATP}^+ channel-independent insulin secretion. In the presence of 1 mmol/l ouabain and 250 μ mol/l diazoxide, which cause modest Ca^{2+} influx, glucose amplification of K_{ATP}^+ channel-independent insulin secretion was observed without concomitant Ca^{2+} stimulation of PKA activity. In islets cultured at 16.7 mmol/l glucose, glucose (16.7 mmol/l)-induced insulin secretion in physiological Krebs–Ringer medium was augmented and now inhibited by H89 (10 μ mol/l), implicating that culture at 16.7 mmol/l glucose may increase Ca^{2+} -sensitive adenylyl cyclase activity and hence PKA activity. In accordance, Ca^{2+} -evoked insulin secretion at 60 mmol/l K^+ and 250 μ mol/l diazoxide was improved, whereas glucose amplification of K_{ATP}^+ channel-independent insulin secretion was unaffected.

Conclusions: Glucose may activate PKA through triggering of the K_{ATP}^+ channel-dependent pathway. Glucose amplification of K_{ATP}^+ channel-independent insulin secretion, on the other hand, occurs by PKA-independent mechanisms.

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Introduction

It is generally accepted that two signalling pathways may cooperate in glucose stimulation of insulin secretion from pancreatic β -cells. These are the ATP-sensitive K^+ (K_{ATP}^+) channel-dependent and the K_{ATP}^+ channel-independent pathway. The K_{ATP}^+ channel-dependent pathway is mediated through an increase in the ATP:ADP ratio, closure of the cell surface K_{ATP}^+ channels, cell depolarization and opening of the voltage-sensitive Ca^{2+} channels and a rise in intracellular Ca^{2+} , which then triggers glucose-induced insulin secretion. In addition, glucose also amplifies Ca^{2+} -stimulated insulin secretion. This second pathway is now being recognized as the K_{ATP}^+ channel-independent but Ca^{2+} -dependent pathway, because of its requirement for increased intracellular Ca^{2+} . Whereas the

second messengers involved in the K_{ATP}^+ channel-dependent pathway are well characterized, the underlying messengers involved in the K_{ATP}^+ channel-independent pathway are not yet known (1, 2).

At least five hypotheses regarding second messengers in K_{ATP}^+ channel-independent insulin secretion are currently considered: (i) Glucose may stimulate production of glutamate, which may sensitize the secretory machinery to Ca^{2+} (3). This mechanism is however disputed (4–6), and apparently a tenfold rise in glutamate does not stimulate insulin secretion (4). (ii) Glucose may induce an increase in cytosolic malonyl-CoA and consequently an increase in long-chain acyl-CoA in the cytosol, which may potentiate insulin release through, for example, activation of protein kinase C (PKC) (7). Lowering of malonyl-CoA with increased oxidation of long-chain acyl-CoA does not, however,

affect glucose-induced insulin secretion (8, 9) and apparently long-chain acyl-CoA and glucose amplify secretion by differential mechanisms (10). (iii) Activation of protein acylation may be involved, since cerelunin, an inhibitor of acylation inhibits K_{ATP}^+ channel-independent insulin secretion (11, 12). Cerelunin, yet, may have non-specific effects and inhibits glucose metabolism (13). (iv) Glucose may activate hormone-sensitive lipase, which may increase long-chain acyl-CoA and diacylglycerol (14–17). In other studies, still, glucose-induced insulin secretion was unaffected by inhibition of hormone-sensitive lipase (18–20). (v) Changes in ATP concentrations may facilitate the K_{ATP}^+ channel-independent pathway (21), e.g. by activation of protein kinase A (PKA), which may constitute an ATP sensor in exocytosis (22, 23). In this way, cAMP and ATP may synergize in exocytosis, and indeed cAMP stimulation of K_{ATP}^+ channel-independent insulin secretion has been described as being dependent on glucose (24).

PKA modulates the activity of several cation channels in the β -cell plasma membrane, leading to a transient increase in glucose-stimulated Ca^{2+} influx and elevation of cytosolic free Ca^{2+} concentration (25). In addition cAMP may modulate cytosolic Ca^{2+} through stimulation of Ca^{2+} mobilization from intracellular stores by PKA-dependent and PKA-independent mechanisms (26), the latter involving cAMP-regulated guanine nucleotide exchange factor II (cAMPGEFII) also termed exchange protein 2 activated by cAMP (Epac2) (27). More importantly, the cAMP/PKA pathway enhances glucose-stimulated insulin secretion at a distal site, beyond the elevation of cytosolic Ca^{2+} . This distal site of cAMP action is thought to be responsible for most of the effect on secretion (28). Since these actions of cAMP/PKA are known to be glucose-dependent, cAMP appears to be a modulator of the pancreatic β -cell signalling system, which in synergy with glucose may act to regulate ion channel activity and to sensitize the exocytotic machinery to Ca^{2+} .

Apparently therefore, PKA could take a centre stage in glucose triggering and amplification of insulin secretion, since PKA both may stimulate K_{ATP}^+ channel-dependent insulin secretion by increasing Ca^{2+} influx/mobilization (25) and may stimulate K_{ATP}^+ channel-independent insulin secretion through activation of hormone-sensitive lipase (17), stimulation of Ca^{2+} uptake and ATP formation in mitochondria (26) and mediate distal effects of ATP in exocytosis (22, 23).

In contrast to the involvement of cAMP in the insulin response to G-protein-coupled receptor agonists like glucagon-like peptide-1 (GLP-1), the role of the cAMP/PKA pathway in glucose-stimulated insulin release has not been established. A function is supported by studies showing that cAMP levels are increased in β -cells stimulated with glucose (29), and that overexpression of cAMP phosphodiesterase 3B reduces glucose-induced insulin secretion (30). Other

studies, however, indicate that glucose-stimulated insulin release occurs efficiently regardless of β -cell PKA activity (31, 32), and it has been suggested that even though cAMP can facilitate insulin release, it may not be necessary for glucose-stimulated insulin release (31, 32). Therefore, the role of cAMP in glucose-stimulated insulin secretion remains to be established.

In the present study, we have re-examined the role of the cAMP/PKA pathway in glucose stimulation of K_{ATP}^+ channel-dependent and K_{ATP}^+ channel-independent insulin secretion. It is demonstrated that glucose has a capacity to stimulate PKA in mouse islets. This effect is mediated by Ca^{2+} during triggering of the K_{ATP}^+ channel-dependent pathway. Glucose amplification by the K_{ATP}^+ channel-independent pathway, however, is not dependent on PKA and may therefore not represent amplification of PKA-stimulated exocytosis.

Materials and methods

Crude bacterial collagenase was obtained from Boehringer Mannheim. ^{125}I -labelled insulin and guinea-pig anti-insulin serum were from Novo Nordisk A/S (Bagsværd, Denmark). BSA, forskolin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), diazoxide and ouabain were from Sigma. H89 (N-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinoline-sulphonamide), protein kinase inhibitor (PKI) 6–22 amide and calphostin C were from Calbiochem (San Diego, CA, USA). 8-CPT-cAMP (8-(4-chlorophenylthio)-adenosine-3', 5'-cyclic monophosphate) and 8-CPT-2'-*O*-Me-cAMP (8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3', 5'-cyclic monophosphate) were from Biolog (Bremen, Germany). All other chemicals were of analytical grade.

Isolation and culture of islets

Islets were prepared by collagenase digestion of the pancreas of male albino mice (NMRI) (approximately 18–22 g body weight) fed *ad libitum* on a standard laboratory diet. Principles of laboratory animal care were followed. Islets were kept in tissue culture for 22–24 h in TCM 199 medium (1.26 mmol/l Ca^{2+} , 5.5 mmol/l glucose) or when stated (0.26 mmol/l Ca^{2+} , 5.5 or 16.7 mmol/l glucose) supplemented with 10% (v/v) newborn calf serum (Gibco), 20 mmol/l HEPES, 5 mmol/l $NaHCO_3$, 100 units penicillin/ml and 100 μ g streptomycin/ml. PKI 6–22 amide was introduced during the 22–24 h culture period.

Insulin release

Insulin release from islets was determined by perfusion in a noncirculating system with beads of 0.25 ml Bio-Gel P2 (BioRad) as a supporting medium, as described previously (10). Twenty-five islets per

chamber were perfused at 37°C at a flow rate of 0.26 ml/min. The perfusion medium was Krebs–Ringer medium supplemented with 20 mmol/l HEPES, 5 mmol/l NaHCO₃, 2 mg BSA/ml and 3.3 mmol/l glucose. Islets were perfused for 45 min to obtain a basal release rate and then challenged with different insulin secretagogues and test agents for 60 min periods as indicated. The effluent medium was collected for periods of 5 or 10 min. Insulin was determined by radioimmunoassay. The rate of insulin release was expressed in nanograms per minute per 100 islets.

Miscellaneous

TPA, forskolin, diazoxide, H89 and calphostin C were added in a small volume of dimethyl sulphoxide (DMSO), final concentration 0.01–0.1%. Results are given as means ± S.D. for $n = 3–8$ experiments in each condition as specified. Statistical evaluation of the data was made by ANOVA, followed by the Newman–Keuls test for multiple comparisons; not significant, $P > 0.05$. For statistical comparisons of the insulin data, the total release during the 60 min of stimulation was calculated.

Results

Role of PKA in glucose-induced insulin secretion

In physiological Krebs–Ringer medium, the PKA inhibitor H89 (10 µmol/l) did not inhibit glucose (16.7 mmol/l)-induced insulin secretion in islets cultured at 5.5 mmol/l glucose, but appeared to inhibit amplification by forskolin (10 µmol/l) by approximately 27% ($P < 0.05$, $n = 6$) (Fig. 1a). Likewise, the PKA inhibitor PKI 6–22 amide (30 µmol/l) failed to affect glucose (16.7 mmol/l)-induced insulin secretion, but inhibited amplification by forskolin (10 µmol/l) by 39% ($P < 0.05$, $n = 4$) (Fig. 1b), suggesting that glucose *per se* does not activate PKA.

Forskolin potentiation of insulin secretion was mimicked by the non-selective cAMP analogue 8-CPT-cAMP (250 µmol/l) ($n = 4$) but not by the cAMPGEF (Epac) selective cAMP analogue 8-CPT-2'-O-Me-cAMP (250 µmol/l) ($n = 6$) (Fig. 2), suggesting that forskolin stimulated insulin secretion by cAMP activation of PKA and not by activation of GEFs.

Role of PKA in glucose stimulation of K_{ATP}⁺ channel-independent insulin secretion

In the presence of depolarizing K⁺ concentrations and diazoxide, which stimulates Ca²⁺ influx through voltage-sensitive Ca²⁺ channels and opens K_{ATP}⁺ channels respectively, glucose amplification of insulin secretion is confined to an amplification of Ca²⁺-induced insulin secretion through the K_{ATP}⁺ channel-independent

Figure 1 Effects of H89 and PKI 6–22 amide on glucose- and forskolin-induced insulin secretion. (a) Islets were perfused with (○) or without (●) 10 µmol/l H89 (H89₁₀) and with 16.7 mmol/l glucose (Gl_{16.7}) and 10 µmol/l forskolin (Forsk₁₀) as indicated. (b) Islets were cultured for 24 h with (○) or without (●) 30 µmol/l PKI 6–22 amide (PKI 6–22 amide₃₀) and then perfused with 16.7 mmol/l glucose (Gl_{16.7}) and 10 µmol/l forskolin (Forsk₁₀) as indicated. Results are means ± S.D. ($n = 4–6$).

Figure 2 Effects of 8-CPT-cAMP and 8-CPT-2'-O-Me-cAMP on glucose-induced insulin secretion. Islets were perfused at 16.7 mmol/l glucose (Gl_{16.7}) and 250 µmol/l 8-CPT-cAMP (CPT-cAMP₂₅₀) or 250 µmol/l 8-CPT-2'-O-Me-cAMP (CPT-MecAMP₂₅₀) as indicated. ○, experiments in the absence of cAMP analogues (in panel b). Results are means ± S.D. ($n = 4–8$).

pathway. At 60 mmol/l K^+ and 250 μ mol/l diazoxide, an increase in glucose from 3.3 to 16.7 mmol/l stimulated insulin secretion 2.94 ± 0.88 ($n = 6$)-fold in the absence and 2.63 ± 0.56 ($n = 6$)-fold in the presence of forskolin (10 μ mol/l) (Fig. 3a). At 3.3 mmol/l glucose, H89 (10 μ mol/l) led to an almost total abrogation of insulin secretion in the presence of 60 mmol/l K^+ and 250 μ mol/l diazoxide ($P < 0.001$, $n = 4$). This inhibition did not, however, prevent amplification of insulin secretion by glucose (16.7 mmol/l), which in the presence of H89 (10 μ mol/l) still stimulated insulin secretion 2.25 ± 0.83 ($n = 6$)-fold, suggesting that glucose stimulation of K_{ATP}^+ channel-independent insulin secretion may occur independently of PKA (Fig. 3b).

Role of PKA in glucose stimulation of ouabain-induced K_{ATP}^+ channel-independent insulin secretion

So far these data suggest that glucose may stimulate PKA activity through the K_{ATP}^+ channel-dependent pathway and that glucose may amplify K_{ATP}^+ channel-independent insulin secretion by a PKA-independent mechanism. To evaluate further whether glucose stimulation of K_{ATP}^+ channel-independent insulin secretion also may proceed in the absence of concomitant activation of PKA by Ca^{2+} , we looked

for alternatives distinct from K^+ depolarization for stimulation of the K_{ATP}^+ channel-independent pathway. In the presence of the Na^+/K^+ -ATPase inhibitor, ouabain (1 mmol/l) and diazoxide (250 μ mol/l), which cause modest membrane depolarization and Ca^{2+} influx (33, 34), glucose (16.7 mmol/l) increased insulin secretion (Fig. 4). H89 (10 μ mol/l), however, failed to inhibit insulin secretion in this setting ($n = 4$) (Fig. 4), suggesting that glucose amplification of K_{ATP}^+ channel-independent insulin secretion may also occur independently of concomitant activation of the cAMP/PKA pathway.

Role of PKC in K^+ -induced insulin secretion

As outlined above, K^+ depolarization *per se* appeared to activate the cAMP pathway (Fig. 3). As observed previously (10), maximum depolarization with 60 mmol/l K^+ in the presence of 250 μ mol/l diazoxide obliterates the stimulatory effect of the PKC activator TPA on insulin secretion (Fig. 5a and b). K^+ (60 mmol/l) may not, however, stimulate adenylyl cyclase through activation of PKC, since the broad specific PKC inhibitor calphostin C (1 μ mol/l), which inhibits TPA-stimulated insulin secretion (10), failed to inhibit insulin secretion at 3.3 or 16.7 mmol/l glucose ($n = 3$) (Fig. 5a and b). It is most likely, therefore, that K^+ depolarization stimulates adenylyl cyclase activity through stimulation of Ca^{2+} influx.

Figure 3 Effects of H89 on K_{ATP}^+ channel-independent insulin secretion. Islets were perfused in the absence (a) or in the presence (b) of 10 μ mol/l H89 (H89₁₀) and at 60 mmol/l K^+ + 250 μ mol/l diazoxide ($K_{60}^+ + Dz_{250}$) with 3.3 mmol/l glucose (Gl_{3.3}, ○) or 16.7 mmol/l glucose (Gl_{16.7}, ●) and 10 μ mol/l forskolin (Forsk₁₀) as indicated. Results are means $\pm$ S.D. ($n = 4-6$).

Figure 4 Effects of glucose and H89 on ouabain-induced K_{ATP}^+ channel-independent insulin secretion. Islets were perfused at 1 mmol/l ouabain + 250 μ mol/l diazoxide (Ouabain₁ + Dz₂₅₀) and with 3.3 mmol/l glucose (Gl_{3.3}, ■), 16.7 mmol/l glucose (Gl_{16.7}, ●) or 16.7 mmol/l glucose + 10 μ mol/l H89 (H89₁₀, ○) as indicated. Results are means $\pm$ S.D. ($n = 4$).

Figure 5 Effects of calphostin C on K^+_{ATP} channel-independent insulin secretion. Islets were perfused with ($\circ$) or without ($\bullet$) $1 \mu\text{mol/l}$ calphostin C (Calph₁) and at 60 mmol/l K^+ + $250 \mu\text{mol/l}$ diazoxide ($K^+_{60} + Dz_{250}$) with (a) 3.3 mmol/l glucose ($Gl_{3.3}$) or (b) 16.7 mmol/l glucose ($Gl_{16.7}$) and $0.16 \mu\text{mol/l}$ TPA ($TPA_{0.16}$) as indicated. Results are means $\pm$ S.D. ($n = 3$).

Role of PKA in glucose-induced insulin secretion in islets cultured at 16.7 mmol/l glucose

Culture of islets at stimulatory glucose concentrations has previously been shown to sensitize glucose-induced insulin secretion (35) and to increase adenylyl cyclase activity (36). In accordance, an increase from 5.5 to 16.7 mmol/l glucose during 24 h of pre-culture in TCM 199 medium (0.26 mmol/l Ca^{2+}) amplified glucose-induced secretion in a way that was obliterated by H89 ($10 \mu\text{mol/l}$) ($n = 6$) (Fig. 6), demonstrating that glucose may sensitize insulin secretion through activation of adenylyl cyclase. Similar results were obtained after culture at 11 mmol/l glucose (results not shown).

In accordance, glucose sensitization amplified insulin secretion in the presence of 60 mmol/l K^+ and $250 \mu\text{mol/l}$ diazoxide (Fig. 7), suggesting that glucose may increase Ca^{2+} -sensitive adenylyl cyclase activity. Glucose sensitization did not, however, affect the efficacy of glucose (16.7 mmol/l) in stimulation of K^+_{ATP} channel-independent insulin secretion (Fig. 7), which now amounted to a 2.08 ± 0.69 ($n = 3$)-fold increase in islets cultured at 16.7 mmol/l glucose for 24 h .

Discussion

According to the present experiments glucose has the capacity to stimulate PKA activity in mouse islets.

Figure 6 Effects of H89 on glucose-induced insulin secretion in islets cultured at 16.7 mmol/l glucose. Islets were cultured for 24 h in TCM 199 medium (0.26 mmol/l Ca^{2+}) supplemented with 5.5 mmol/l glucose ($\circ$) or 16.7 ($\bullet$) mmol/l glucose and then perfused with 16.7 mmol/l glucose ($Gl_{16.7}$) and $10 \mu\text{mol/l}$ forskolin ($Forsk_{10}$) in (a) the absence or (b) the presence of $10 \mu\text{mol/l}$ H89 ($H89_{10}$). Results are means $\pm$ S.D. ($n = 4-6$).

Figure 7 Effects of glucose on K^+_{ATP} channel-independent insulin secretion in islets cultured at 16.7 mmol/l glucose. Islets were cultured for 24 h in TCM 199 medium (0.26 mmol/l Ca^{2+}) supplemented with (a) 5.5 mmol/l glucose ($Gl_{5.5}$) or (b) 16.7 mmol/l glucose ($Gl_{16.7}$) and then perfused with 60 mmol/l K^+ + $250 \mu\text{mol/l}$ diazoxide ($K^+_{60} + Dz_{250}$) with 3.3 mmol/l glucose ($Gl_{3.3}$, $\circ$) or 16.7 mmol/l glucose ($Gl_{16.7}$, $\bullet$) and with $10 \mu\text{mol/l}$ forskolin ($Forsk_{10}$) as indicated. Results are means $\pm$ S.D. ($n = 3$).

This stimulation is only observed after culture at stimulatory glucose concentrations, which previously has been reported to induce adenylyl cyclase activity in islets (36); this dependency on culture conditions may explain the somewhat diverging results in different studies, where glucose has been suggested either to increase cAMP (29, 30) or to be without effect on cAMP (31, 32). The mechanism by which glucose increases adenylyl cyclase activity during culture is at present unknown, since it may not involve protein synthesis (36). Apparently however, glucose may increase Ca^{2+} -sensitive adenylyl cyclase activity, which previously has been shown to be diminutive in freshly isolated mouse islets (37). Thus culture at high glucose concentrations increased Ca^{2+} -stimulated insulin secretion, which appeared to proceed without activation of PKC, another candidate for stimulation of adenylyl cyclase activity in mouse islets (38).

Recent studies have suggested that cAMP not only activates PKA but also stimulates insulin secretion in islets by stimulation of cAMPGEFII (Epac2) (39, 40). Indeed cAMPGEFII has been shown to stimulate Ca^{2+} release from endoplasmic reticulum (40) and to cause a direct stimulation of exocytosis in β -cells (41). According to the present experiments, however, cAMP-GEFs may only make a minor contribution to the overall effect of cAMP on exocytosis, since the membrane-permeable cAMPGEF-selective analogue 8-CPT-2'-O-Me-cAMP failed to affect the insulin secretion rate. Most probably, therefore, glucose stimulation of adenylyl cyclase activity stimulates insulin secretion mainly by virtue of the ability of cAMP to increase PKA activity.

In accordance with a previous study in mouse islets, where the cAMP antagonist adenosine-3', 5'-cyclic monophosphorothioate (Rp-cAMPS) was used (21), K^{+} depolarization *per se* appeared to activate the cAMP/PKA pathway. Thus glucose appears to stimulate PKA through triggering of the $\text{K}_{\text{ATP}}^{+}$ channel-dependent pathway of stimulated insulin release. In this way PKA may stimulate insulin secretion by modulation of Ca^{2+} handling (25) and through direct effects on exocytosis (28). In addition, PKA may activate hormone-sensitive lipase (17), leading to release of long-chain acyl-CoA, which may stimulate insulin secretion by means of protein acylation (11, 12) or by PKC activation (7), although the latter as demonstrated previously (10) and herein seems less likely.

Recently it has been suggested that PKA could constitute the ATP sensor in exocytosis of β -cell granula (22, 23). PKA has previously been demonstrated to cause a direct stimulation of exocytosis in pancreatic β -cells (28) and since ATP is considered a possible second messenger in glucose stimulation of $\text{K}_{\text{ATP}}^{+}$ channel-independent secretion (21), glucose might stimulate PKA activity and $\text{K}_{\text{ATP}}^{+}$ channel-independent secretion due to its ability to increase ATP in islet cells. Indeed one study demonstrated that forskolin

stimulation of $\text{K}_{\text{ATP}}^{+}$ channel-independent insulin secretion was dependent on glucose and was obliterated in the absence of glucose (24), suggesting a possible significant interplay of glucose and PKA in amplification of $\text{K}_{\text{ATP}}^{+}$ channel-independent exocytosis. According to the present study, however, glucose stimulation of $\text{K}_{\text{ATP}}^{+}$ channel-independent insulin secretion may not involve PKA activation and may even proceed without sizeable PKA activity. Thus glucose also stimulated insulin secretion via the $\text{K}_{\text{ATP}}^{+}$ channel-independent pathway in the presence of ouabain, which did not activate PKA.

In conclusion, therefore, the present study clearly demonstrates that glucose amplification $\text{K}_{\text{ATP}}^{+}$ channel-independent insulin secretion arises independently of PKA. Glucose has the capacity to stimulate PKA in islets. This activation, however, is accomplished through glucose triggering of $\text{K}_{\text{ATP}}^{+}$ channel-dependent insulin secretion.

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