AKAP-Mediated Targeting of Protein Kinase A Regulates Contractility in Cardiac Myocytes

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Abstract—Compartmentalization of cAMP-dependent protein kinase A (PKA) by A-kinase anchoring proteins (AKAPs) targets PKA to distinct subcellular locations in many cell types. However, the question of whether AKAP-mediated PKA anchoring in the heart regulates cardiac contractile function has not been addressed. We disrupted AKAP-mediated PKA anchoring in cardiac myocytes by introducing, via adenovirus-mediated gene transfer, Ht31, a peptide that binds the PKA regulatory subunit type II (RII) with high affinity. This peptide competes with endogenous AKAPs for RII binding. Ht31P (a proline-substituted derivative), which does not bind RII, was used as a negative control. We then investigated the effects of Ht31 expression on RII distribution, Ca²⁺ cycling, cell shortening, and PKA-dependent substrate phosphorylation. By confocal microscopy, we showed redistribution of RII from the perinuclear region and from periodic transverse striations in Ht31P-expressing cells to a diffuse cytosolic localization in Ht31-expressing cells. In the presence of 10 nmol/L isoproterenol, Ht31-expressing myocytes displayed an increased rate and amplitude of cell shortening and relaxation compared with control cells (uninfected and Ht31P-expressing myocytes); with isoproterenol stimulation we observed decreased time to 90% decline in Ca²⁺ but no significant difference between Ht31-expressing and control cells in the rate of Ca²⁺ cycling or amplitude of the Ca²⁺ transient. The increase in PKA-dependent phosphorylation of troponin I and myosin binding protein C on isoproterenol stimulation was significantly reduced in Ht31-expressing cells compared with controls. Our results demonstrate that, in response to β-adrenergic stimulation, cardiomyocyte function and substrate phosphorylation by PKA is regulated by targeting of PKA by AKAPs. (Circ Res. 2001;88:291-297.)

Key Words: A-kinase anchoring proteins | protein kinase A | cardiac myocyte | β-adrenergic receptor | contractility

Stimulation of the β-adrenergic signaling pathway in cardiac myocytes results in activation of cAMP-dependent protein kinase A (PKA) and phosphorylation of several PKA substrates. These include the sarcolemmal L-type Ca²⁺ channel, the ryanodine receptor (RyR) and phospholamban (PLB) of the sarcoplasmic reticulum (SR), and the myofibrillar proteins troponin I (TnI) and myosin binding protein C (MBP-C).¹⁻⁵ Phosphorylation of these substrates acts in concert to generate both enhanced contractility and accelerated relaxation in response to β-adrenergic stimulation. Although PKA has broad substrate specificity, it can be highly selective in a physiological setting, because different stimuli are capable of eliciting phosphorylation of specific subsets of targets.⁶ The high level of specificity may be explained, in part, by targeting of PKA to distinct subcellular locations via interaction with A-kinase anchoring proteins (AKAPs).⁷ Intracellular gradients of cAMP within the cytosol may also contribute to PKA specificity.⁸ PKA holoenzyme contains 2 regulatory (R) and 2 catalytic (C) subunits. The 2 isoforms of PKA, types I and II, are distinguished by the R isoform. Most RII is targeted to specific subcellular sites by interaction with AKAPs.⁷⁻⁹ Targeting of RI by AKAPs has also been recently reported.¹⁰ Members of the AKAP family are functionally similar; they bind RII dimers and, by means of a targeting domain, anchor PKA II holoenzyme to specific subcellular locations. However, the subcellular targeting domain of each AKAP is unique; regions of similarity among AKAPs are restricted to domains of limited homology within the conserved amphipathic helix that binds RII dimers.¹¹

More than 50 AKAPs have been identified in a number of organisms.⁷ In particular, 2 AKAPs have been reported in the rat and human heart, mAKAP (formerly AKAP100)¹² and AKAP18.¹³ The latter was independently identified as AKAP15 in skeletal muscle.¹⁴ RII and mAKAP colocalize to the junctional SR (jSR)/transverse tubule⁹ and the perinuclear...
PKA is targeted by mAKAP to the RyR and may be involved in PKA regulation of the RyR. AKAP18 is a lipid-anchored AKAP that is reported to be necessary for PKA-dependent modulation of the skeletal and cardiac L-type Ca\(^{2+}\) channel under \(\beta\)-adrenergic stimulation. AKAP15 also interacts directly with the \(\alpha_1\) subunit of the skeletal calcium channel.

The goals of these studies were (1) to determine the physiological role of AKAP-mediated PKA anchoring in the regulation of cardiomyocyte contractility and (2) to identify protein substrates phosphorylated by anchored PKA in cardiac myocytes. We used the adenovirus gene transfer method to introduce Ht31, the RII-binding peptide derived from a human thyroid AKAP, into adult rat ventricular myocytes. Ht31 binds RII with the same nanomolar affinity as the full-length AKAP. Consequently, Ht31 peptide, which does not contain a targeting domain, competes with full-length AKAPs for binding of RII and PKA type II holoenzyme. Ht31P (a proline-substituted derivative), used as a negative control, contains 2 prolines substituted for 2 hydrophobic residues within the RII binding domain. This disrupts the secondary structure of the amphipathic helix necessary for RII binding and therefore does not block AKAP/RII interaction. We determined the effect of disruption of PKA targeting by endogenous AKAPs. This was achieved by assessing the effect of Ht31 expression in cardiac myocytes on PKA distribution, \(\mathrm{Ca}^{2+}\) cycling, myocyte cell shortening, and PKA-dependent substrate phosphorylation.

Materials and Methods

Adenovirus Construction

Recombinant adenovirus directing the expression of either hemagglutinin (HA) epitope–tagged Ht31 peptide (Ad-HA-Ht31) or HA epitope–tagged Ht31P peptide (Ad-HA-Ht31P) was generated using standard procedures. cDNAs, encoding either Ht31 (residues 418 to 718) or Ht31P (Ala499Pro and Ile507Pro), kindly supplied by Dr John Scott (Vollum Institute, Oregon Health Sciences University, Portland, Oreg), were cloned into pXCI2. The reporter construct Ad-GFP was obtained from Quantum Biotechnologies.

Isolation, Culture, and Adenoviral Infection of Cardiac Myocytes

Sprague-Dawley male rats (200 to 250 g) were housed and cared for by the Cleveland Clinic Foundation Animal Facility, which is approved by the American Association for Accreditation of Laboratory Animal Care and is under the full-time supervision of a veterinarian. All methods used are approved by the Cleveland Clinic Foundation Animal Care and Use Committee and are consistent with the recommendations of the American Veterinary Medical Association.

Adult rat ventricular myocytes were isolated as previously described with modifications, as necessary, for primary culture of the cells. Myocytes were plated (1 \(\times\) 10\(^6\) rod-shaped cells per milliliter) on laminin (4 \(\mu\)g/cm\(^2\)). Viability was routinely between 75% and 95% rod-shaped myocytes with no granulations or blebs. Adenovirus was diluted to 1 \(\times\) 10\(^6\) plaque-forming units per milliliter, and cells were infected for 1 hour.

Indirect Immunofluorescence

Myocytes were immunostained as described. Anti-RII antibody was diluted 1:100, and FITC-conjugated donkey anti-goat secondary antibody was diluted 1:500. The cells were examined using a Leica TCS-NT confocal laser scanning microscope with a \(\times 63\) objective lens as described.

Measurements of Intracellular Ca\(^{2+}\) Transients and Myocyte Cell Shortening

Simultaneous measurements of intracellular \(\mathrm{Ca}^{2+}\) and contraction were performed under field stimulation (0.5 Hz) as described. After a 4-minute equilibration period in Krebs-Henseleit buffer ([in mmol/L] NaCl 118, KCl 4.8, MgCl\(_2\) 1.2, CaCl\(_2\) 1.2, K\(_2\)HPO\(_4\) 1.2, NaHCO\(_3\) 1.65, glucose 16.5, and pyruvate 7.5, and 50 \(\mu\)M penicillin and 50 \(\mu\)g/mL streptomycin, pH 7.35) (KHB) with no agonist, the superfusate was switched to KHB containing 10 nmol/L isoproterenol (Iso). Cell shortening and intracellular \(\mathrm{Ca}^{2+}\) were then measured for each cell under baseline conditions (no agonist) and in the presence of Iso. Dose-response experiments verified that 10 nmol/L Iso elicited a maximal response in acutely isolated, uninfected adult rat ventricular cardiomyocytes (data not shown).

For both the \(\mathrm{Ca}^{2+}\)-transient and cell shortening, the following parameters were measured: time to peak, time to 90% decline (T90r), change in amplitude from resting level, maximum rate of rise, and maximum rate of decline. Data for these parameters were collected for each cell (averaged over 10 contractions) after the amplitude of the cell shortening (achieved at 3 to 5 min) and the back-phosphorylation experiments were carried out under (1) field stimulation at 0.5 Hz with no agonist present (baseline) and (2) field stimulation in the presence of 10 nmol/L Iso (stimulated). Results were analyzed with Laboratory View software. Percentage cell shortening (%CS) was calculated as follows: (change in amplitude from resting cell length–resting cell length) \(\times\) 100.

Statistical Analysis of \(\mathrm{Ca}^{2+}\) Cycling and Cell Shortening Data

Before running any analyses, the \(\mathrm{Ca}^{2+}\) cycling and cell shortening data were visually inspected to assess the assumption of normality. Because logarithmically transformed data were distributed normally, analyses were performed on the transformed data.

An ANOVA was used to test for group differences under baseline conditions. Hypothesis testing was conducted using a 2-sided alternative and a significance level of 0.01. The more stringent standard for significance was set because experiments were carried out on individual cells and the level of peptide expression in both groups varied from cell to cell, as determined by immunofluorescence. Because there were no statistically significant differences between the means of the Ht31P and uninfected groups, a control group was created by combining these 2 groups. The remainder of the analyses were conducted on the basis of a 2-group instead of a 3-group design. An ANCOVA between groups (control versus Ht31-expressing cells), controlling for the baseline measurements (in the absence of Iso stimulation), was carried out to determine whether there was a difference between the means of the 2 groups in \(\mathrm{Ca}^{2+}\) cycling and cell shortening parameters. A 2-sided alternative hypothesis and a significance level of 0.01 were used.

To test for differences within groups (ie, baseline versus stimulated), a paired Student \(t\) test was used. Note that because there is no contribution of between-group variability, smaller absolute differences can be found to be statistically significant by paired \(t\) test than by ANCOVA. This is evident in the differences in resting cell length between groups (\(-/\) vs +Iso) versus between groups. The Statistical Analysis System (SAS) was used to generate data summaries and statistical tests.

Back-Phosphorylation

Back-phosphorylation experiments were carried out on cultured myocytes as described. Bands were visualized using a StormImager and quantified using ImageQuant software. Unlike the \(\mathrm{Ca}^{2+}\) cycling and cell shortening measurements, back-phosphorylation experiments were carried out on populations of cells, and the data were normally distributed. Therefore, comparisons between groups were made by 2-tailed Student \(t\) test with a \(P\) value <0.05 considered significant.
Results

Characterization of Ht31 and Ht31P Expression in Cardiomyocytes

One advantage of the adenoviral gene transfer system is that it gives us the ability to obtain nearly 100% efficiency of infection with the use of optimal virus concentration. Under the conditions used in these experiments, infection efficiency was >98% as assessed using fluorescent microscopy on cells infected with the reporter construct Ad-GFP (n=3; data not shown). All experiments were carried out 48 hours after infection.

There was no difference in the percentage of rod-shaped cells between uninfected and infected cells under these conditions. Furthermore, there was no significant difference in RII distribution, intracellular Ca²⁺ cycling, cell shortening, or PKA substrate phosphorylation between uninfected and Ht31P-expressing myocytes either in the presence or absence of Iso. These results indicate that infection with recombinant adenovirus itself did not affect the parameters measured and that there were no discernible nonspecific peptide effects. Because statistical analysis showed no difference between Ht31P-expressing and uninfected cells in Ca²⁺ cycling or cell length parameters, all functional data were expressed as Ht31-expressing myocytes versus control (Ht31P-expressing cells + uninfected cells).

Distribution of RII in Ht31-Expressing and Control Myocytes

We anticipated that expression of Ht31 in cardiac myocytes would result in redistribution of RII from subcellular locations, where the enzyme was targeted by endogenous AKAPs, to a more diffuse cytosolic localization, whereas Ht31P expression would have no discernible effect on RII distribution. In agreement with our previous observations in freshly isolated rat ventricular myocytes, RII localized to the perinuclear region and to periodic transverse striations in uninfected (data not shown) and Ht31P-expressing myocytes (Figure 1A). In contrast, in Ht31-expressing myocytes, targeting of RII to the cross-striations or perinuclear area was reduced to nondetectable levels. Instead, RII was located diffusely throughout the cytosol (Figure 1B). Similar results were obtained by Lester et al in a pancreatic cell line expressing Ht31.

Effects of Ht31 on Intracellular Ca²⁺ Transients and Cell Shortening

Figure 2 shows representative traces of Ca²⁺ transients and cell shortening under baseline and stimulated conditions for an uninfected and a Ht31-expressing myocyte. Note that in both cells, as previously reported, the resting level of cytosolic Ca²⁺ increased and cell length decreased on treatment with 10 nmol/L Iso. Consistent with the expected effects of β-adrenergic stimulation on Ca²⁺ cycling and cell shortening, in response to Iso stimulation, we observed a significant decrease in T90r of the Ca²⁺ transient (Figure 3A) and significantly faster maximal rate of Ca²⁺ reuptake (Figure 3G) in both groups of myocytes. There was also a significant increase in amplitude of the Ca²⁺ transient in control cells (Figure 3C). This increase is somewhat less than that observed in freshly isolated myocytes, most likely as a result of changes in cultured myocytes such as a decreased T-tubule network or adaptation to a 2-dimensional environment. We also observed a significantly faster maximal rate of Ca²⁺ release (Figure 3E) in control myocytes. On Iso stimulation, Ht31-expressing myocytes displayed a similar trend in these 2 parameters, but this did not achieve statistical significance. There was also a significantly decreased T90r cell relength-
different between the 2 groups treated with 10 nmol/L Iso was T90r of the Ca\(^{2+}\) transient, which was significantly shorter in Ht31-expressing cells (Figure 2A). In contrast, several cell shortening parameters were altered with Ht31 treatment. The increase in %CS (Figure 3D), faster rate of shortening (Figure 3F), and faster rate of relaxation (Figure 3H) in response to \(\beta\)-adrenergic stimulation were significantly greater in Ht31-expressing cells.

### Phosphorylation of PKA Substrates in Ht31- and Ht31P-Expressing Myocytes

Back-phosphorylation measures the extent of unfilled PKA sites on protein substrates by back-phosphorylating empty (unphosphorylated) sites with radiolabeled phosphate from [\(\gamma\^{32}\text{P}\)]ATP.\(^{19}\) Therefore, the more a protein is phosphorylated by PKA in vivo, the less radioactivity will be incorporated by back-phosphorylation, resulting in less intense radioactive labeling of the protein.

In all cells, activation of the \(\beta\)-adrenergic pathway resulted in significantly decreased back-phosphorylation of the PKA substrates MBP-C, TnI, and PLB compared with baseline (Figures 4A through 4D), indicating increased in vivo phosphorylation of these sites, in accordance with past findings.\(^{1-5}\) Baseline and PKA-dependent phosphorylation of MBP-C, TnI, and PLB were not significantly different between uninfected and Ht31P-expressing myocytes; therefore, only Ht31- and Ht31P-expressing cells were compared. However, in Ht31-expressing myocytes, in the presence of 10 nmol/L Iso, back-phosphorylation of MBP-C (Figure 4B) and TnI (Figure 4C) was significantly greater than in Ht31P-expressing cells (15% and 33%, respectively), indicating a significantly reduced PKA-dependent phosphorylation of TnI and MBP-C in response to \(\beta\)-adrenergic stimulation in Ht31-expressing cells. There was no significant difference in baseline or PKA-dependent PLB phosphorylation between Ht31- and Ht31P-expressing myocytes (Figure 4D). To determine whether the reduction of in vivo phosphorylation of MBP-C and TnI in Ht31-expressing cells was due to a reduction of PKA C subunit, Western blot analysis was carried out. We found no difference in the level of C subunit between the 2 groups, assuming control cells expressed C subunit at 100% (n=6) and Ht31-expressing cells expressed C subunit at 102±2% (n=4).

### Discussion

We demonstrate, for the first time, that disruption of AKAP-mediated anchoring of PKA alters contractile function in cardiac myocytes during \(\beta\)-adrenergic stimulation. Expression of Ht31 in myocytes resulted in a redistribution of RII, which showed decreased compartmentalization of PKA in these cells, indicating that Ht31 competes with endogenous AKAPs for PKA. The functional studies suggest that Ht31-expressing myocytes are more responsive than control myocytes (uninfected and Ht31P-expressing cells) to the increased intracellular Ca\(^{2+}\) resulting from activation of the \(\beta\)-adrenergic signaling pathway. The back-phosphorylation experiments indicate that AKAP-mediated PKA anchoring regulates phosphorylation of a subset of PKA substrates, in particular the major PKA substrates localized at the myofi-
We can therefore conclude that AKAP-mediated anchoring of PKA is required for regulation of the contractile response to β-adrenergic stimulation.

This is the first study to examine the effect of blocking endogenous AKAP-PKA interactions on the phosphorylation of endogenous PKA substrates. We wished to determine the overall role of PKA anchoring in the regulation of cardiac contractility in isolated myocytes. This is a complex process because of the large number of PKA substrates in cardiac myocytes and the fact that the effects on contractility and relaxation in response to β-adrenergic stimulation are due to the interplay of several phosphorylation events.

The increased amplitude of cell shortening observed in myocytes with disrupted PKA targeting could be attributed, at least in part, to the reduction of PKA phosphorylation of TnI. Phosphorylation of TnI decreases the sensitivity of the actomyosin ATPase to cytosolic Ca²⁺ by decreasing the affinity of troponin C (TnC) for Ca²⁺. These findings are in agreement with a previous observation in transgenic mice expressing slow skeletal TnI that is not phosphorylated by PKA. In that study, a significant increase in the amplitude of cell shortening under stimulated conditions was observed. In addition, unphosphorylated MBP-C increased Ca²⁺ sensitivity in skinned fibers. Thus, in our experiments, the decreased PKA-dependent MBP-C phosphorylation may also have contributed to increased cell shortening in Ht31-expressing cells.

One might predict that increased myofibrillar Ca²⁺ sensitivity (due to decreased TnI and MBP-C phosphorylation) should lead to a longer relaxation time. However, we found that reduction of PKA-dependent TnI phosphorylation in Ht31-expressing myocytes was accompanied by a shorter relaxation time than in control cells. This apparent paradox may be explained in part by the results of Li et al., who recently showed that TnI phosphorylation did not participate in relaxation during unloaded shortening and that its contribution to accelerated relaxation during loaded shortening was minimal. Thus, the decrease of PKA-dependent TnI phosphorylation may have had little effect on the rate of relaxation in the unloaded myocytes used here. The reason for the shorter time of decline of the Ca²⁺ transient, accelerated rate of shortening, and accelerated rate of relaxation with loss of PKA anchoring during β-adrenergic stimulation is unclear. These effects may be due to changes in phosphorylation of other-as-yet unidentified PKA substrates. There may also be functions for MBP-C that have not yet been described. Nevertheless, our findings indicate that inhibition of PKA anchoring in cardiac myocytes affects cell shortening with minimal alterations in Ca²⁺ cycling.

To date, the only studies investigating PKA compartmentalization in cardiac myocytes determined the effect of PKA targeting on L-type Ca²⁺ currents. These studies suggest that regulation of the L-type Ca²⁺ channel by PKA is dependent on AKAP/PKA interaction, specifically PKA targeting by AKAP18. In addition, localization of mAKAP at the jSR and association of mAKAP and RyR, as demonstrated by communoprecipitation studies, suggests that mAKAP may regulate PKA phosphorylation of RyR and, thus, Ca²⁺-induced Ca²⁺ release. Because Ca²⁺ entering through the L-type Ca²⁺ channel and Ca²⁺-induced Ca²⁺ release from the RyR of the SR both contribute to the Ca²⁺ transient, we predicted that Ht31-expressing myocytes would display an altered Ca²⁺ transient under Iso-stimulated conditions as compared with control cells, most likely a reduced increase in amplitude.

The lack of significant difference in amplitude of the Ca²⁺ transient between Ht31-expressing and control cells under stimulated conditions suggests that the amount of Ca²⁺ entering the cytosol was not affected by loss of AKAP-mediated PKA anchoring. The lack of change in the Ca²⁺ transient in response to β-adrenergic stimulation in Ht31 cells is unlikely due to a failure to detect changes in Ca²⁺, because we did observe an increase in the amplitude of the Ca²⁺ transient in both Ht31-expressing and control cells in response to Iso treatment. One possible explanation is an
inability of expressed Ht31 peptide to compete with full-length endogenous AKAPs for RII. However, this is unlikely, because work from our laboratory shows that the affinity of Ht31 peptide for RII is 100-fold higher than AKAP18 and 10-fold higher than mAKAP. Overexpressed Ht31 is also likely to be present in the cardiac myocytes at much higher concentrations than endogenous AKAPs. Additionally, we have shown that periodicity of RII was lost in Ht31-expressing cells, thus verifying that Ht31 effectively competes with mAKAP for RII binding. Currently, we cannot rule out the possibility that in cultured adult cardiac myocytes, modulation of the L-type Ca\(^{2+}\) channel or RyR by targeted PKA is reduced.

PKA-dependent phosphorylation of at least 2 myofibrillar PKA substrates, Tnl and MBP-C, was altered in Ht31-expressing myocytes. Consequently, our data are best explained by 1 of 2 models. The first involves an unidentified myofibril-associated AKAP that functions to sequester PKA near its major targets, Tnl and MBP-C. Because a typical cell contains 5 to 10 distinct AKAPs (based on RII overlay assays), other AKAPs, in addition to the 2 identified to date, are expressed in cardiac myocytes. Furthermore, microdomains or gradients of cAMP exist in response to adenylyl cyclase activation. In cells such as cardiac myocytes, with multiple PKA pools and PKA substrates, sequestering PKA to different subcellular locations would allow the pool closest to activated adenylyl cyclases to be stimulated most rapidly by the local high concentrations of cAMP after receptor activation. This pool of PKA could then phosphorylate nearby protein substrates. Intriguing results from the laboratory of Johnson and colleagues suggest that under certain conditions the C subunits may not even dissociate from the R subunits of PKA, thus strengthening the argument for the need to anchor PKA holoenzyme near its targets.

In the second model, PKA is not targeted to the myofilaments by AKAPs, but primarily near the jSR. Feliciello et al found enhanced phosphorylation of a distant PKA substrate, nuclear cAMP response element binding protein, by PKA targeted to the cytoskeleton near the plasma membrane via expressed AKAP75. By analogy, targeting of PKA by mAKAP at the jSR may facilitate a local increase in the concentration of activated C subunit. This increased local pool of C subunit could then diffuse to distant locations such as the myofilaments. It is possible that the most abundant PKA substrates, ie, Tnl and MBP-C, would be most dependent on high concentrations of activated C subunit. Phosphorylation of these substrates would then be most affected by a decrease in the pool of activated PKA (near adenylyl cyclases), resulting from disruption of PKA targeting. Alternatively, it might be more difficult for the pool of activated C subunit to diffuse to myofilbrils than to other subcellular locations. The microenvironment of the myofilbrillar lattice, packed with thin and thick filaments and associated structural and regulatory proteins, may limit diffusion. If this is the case, then myofilbrillar substrates may be more susceptible to a decrease in activated C subunit than PKA substrates located in more accessible subcellular locations.

Intriguingly, under the conditions of our experiments, myocytes that expressed Ht31 displayed enhanced contractility with no major change in the intracellular Ca\(^{2+}\) transient. Thus, the effect of Ht31 expression is similar to the action of Ca\(^{2+}\) sensitizing agents. These drugs, under study as promising treatments for heart failure, increase the sensitivity of myofilaments to intracellular Ca\(^{2+}\) without increasing the level of Ca\(^{2+}\). Ca\(^{2+}\) sensitizing agents either increase Ca\(^{2+}\) binding to TnC or act directly on the actin-myosin complex. Unfortunately, there are some drawbacks to several of these drugs, such as a high level of phosphodiesterase inhibition and impairment of relaxation.

In conclusion, AKAP-anchored PKA regulates contractility under conditions of \(\beta\)-adrenergic stimulation in ventricular myocytes. This regulation occurs likely through phosphorylation of a subset of PKA substrates including MBP-C and Tnl but not PLB. Much work is still needed to completely unveil the mechanisms of this regulation.

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