Capacitative Calcium Entry Contributes to Nuclear Factor of Activated T-cells Nuclear Translocation and Hypertrophy in Cardiomyocytes

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In nonexcitable cells, depletion of endoplasmic reticulum Ca\(^{2+}\) stores leads to activation of plasma membrane Ca\(^{2+}\) channels, a process termed capacitative Ca\(^{2+}\) entry. Here, we demonstrate that this pathway functions in cells that also contain voltage-gated Ca\(^{2+}\) channels, neonatal rat ventricular myocytes. The depletion of sarcoplasmic reticulum Ca\(^{2+}\) stores elicited a prolonged increase in cytoplasmic Ca\(^{2+}\) dependent extracellular Ca\(^{2+}\). Inhibitors of store-operated channels but not L-type channels diminished this response. The importance of this pathway to cardiac hypertrophy, which often is dependent on Ca\(^{2+}\)/calmodulin-dependent transcription factors, was also assessed in this model. Hypertrophy and atrial natriuretic factor expression induced by angiotensin II or phenylephrine was more effectively attenuated by inhibitors of capacitative entry than of L-type channels. Additionally, cardiomyocytes were transfected with a construct encoding a fluorescent nuclear factor of activated T-cells chimeric protein to follow nuclear localization in response to thapsigargin, angiotensin II, and phenylephrine. This translocation was completely prevented by inhibitors of capacitative Ca\(^{2+}\) entry and only partially abrogated by inhibitors of L-type channels. In contrast, a hypertrophic response induced by overexpression of the transcription factor MEK1 was unaffected by inhibitors of capacitative entry. Together, these data suggest a role for CCE in cardiomyocyte physiology and, in particular, in Ca\(^{2+}\)-mediated cardiac hypertrophy.

Regulators of cardiac function such as \(\alpha\)-adrenergic agonists and vasoactive peptide hormones activate phosphoinositide-specific phospholipase C (PLC)\(^1\) and thereby generate inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol. These agonists have been shown to elevate the concentration of cytoplasmic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in cardiomyocytes and to have positive inotropic effects on the heart (1, 2). In addition to their importance in the acute regulation of cardiac function, their significance has increased further as the transcriptional pathways that lead to cardiac hypertrophy have been elucidated. Although initially compensatory, prolonged hypertrophy is often associated with decomposition, dilated cardiomyopathy, arrhythmia, fibrotic disease, and heart failure (3).

The importance of agonists that activate PLC to cardiac hypertrophy is now well established (4). One well studied mouse model of cardiac hypertrophy involves the overexpression of a constitutively active form of a G protein subunit, Goq, which leads to chronic activation of PLC and the continuous production of IP\(_3\) and diacylglycerol (5). In addition, overexpression in the heart of the PLC-activating angiotensin II (Ang II) type I receptor also leads to hypertrophy (6, 7). More clinically relevant, hypertrophied hearts induced by volume overload are commonly characterized by high levels of IP\(_3\)-generating agonists such as Ang II (8).

There are multiple signaling pathways downstream of PLC leading to cardiac hypertrophy. One involves diacylglycerol, protein kinase C, small guanine nucleotide-binding proteins (9), the MEK1-ERK1/2 branch of the mitogen-activated protein kinase pathway (10), and the transcription factor GATA4 (11, 12). Two others involve IP\(_3\) and elevated levels of [Ca\(^{2+}\)]\(_i\). One of these is dependent on Ca\(^{2+}\)/calmodulin-dependent calmodulin kinase and the transcription factor MEF2 (13), and the other is mediated by the Ca\(^{2+}\)/calmodulin-activated protein phosphatase calcineurin and the transcription factor NFAT3 (14). The latter signaling pathway was first defined in lymphocytes (15) and is fundamental to an array of biological responses in a variety of cell types (16, 17). A rise in [Ca\(^{2+}\)]\(_i\) triggered by ligands generating IP\(_3\) leads to the activation of the phosphatase activity of calcineurin, the dephosphorylation of NFAT family members, and their translocation to the nucleus to initiate transcription. Rapid export of NFAT from the nucleus when [Ca\(^{2+}\)]\(_i\) levels drop prevents brief [Ca\(^{2+}\)]\(_i\) pulses from initiating transcription of NFAT-dependent genes (15, 16).

A critical, unresolved issue for cardiac hypertrophy is the mechanism leading from IP\(_3\)-mediated stimuli to elevated [Ca\(^{2+}\)]\(_i\). In most cell types, the initial increase in [Ca\(^{2+}\)]\(_i\), in response to IP\(_3\)-generating agonists is due to the release of Ca\(^{2+}\) from the endoplasmic reticulum (ER). The subsequent depletion of ER Ca\(^{2+}\) stores results in an influx of extracellular Ca\(^{2+}\) into the cytoplasm, causing sustained elevations in [Ca\(^{2+}\)]\(_i\), a process termed store-operated or capacitative Ca\(^{2+}\) entry (CCE) (18). Although highly characterized in nonexcit-
able cells, this pathway has not previously been identified in cardiomyocytes. Interestingly, it has recently been shown to coexist with L-type voltage-gated Ca\(^{2+}\) channels in both smooth (19) and skeletal (20) muscle cells. Here, we sought to determine whether a CCE pathway was present in NRVMs and to investigate whether this pathway contributes to the elevated [Ca\(^{2+}\)], associated with cardiac hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Primary Cardiomyocyte Cultures**—Animal procedures conformed to the Guide for the Care and Use of Laboratory Animals, issued by the National Academy Press. Primary cultures of NRVMs were obtained from 1-day-old Sprague-Dawley rats and isolated from the United States Institute for Laboratory Resources. Primary cultures of NRVMs (medium).

**Medium and Culture Conditions**—Cultured primary NRVMs were maintained for 48 h in medium alone or in the presence of agonists and/or blockers as described for the various experimental conditions. The media and drug treatments were replaced daily. For immunocytochemistry NRVMs were fixed in ice-cold 100% methanol for 15 min, washed three times in phosphate-buffered saline, and blocked in saline containing 5% bovine serum albumin for 30 min. Secondary antibodies (Alexa Fluor 488 goat anti-rabbit and 568 goat anti-mouse; Molecular Probes) were used at a dilution of 1:400 in saline containing 5% rat serum and incubated for 30 min. Cell size was determined using Universal Imaging Image 1 software. Mean cell areas were determined for >100 cells in each of the treatment groups for each experiment. Percentages of NRVMs positive for ANF were determined by counting the total number of NRVMs in a field based on the nuclear localization signal of the NFAT4 isoform instead of the NFAT3 isoform that is more highly expressed in cardiomyocytes (17). Both of these isoforms have calcium/calmodulin/calcineurin-dependent nuclear localization signals (17, 24).

**Replication-deficient Adenovirus Production**—A recombinant adenovirus expressing EGFP-NFAT was constructed (25). The cDNA encoding the chimeric construct (24) was cloned into the adenovirus transfer vector pCA15 (Microbix Biosystems), which allows expression of the target gene under the control of the cytomegalovirus promoter. The plasmid containing the cDNA was cotransfected into low passage 293 cells (26) along with pJM17 (containing the entire Ad5 genome except the E1 region) (27) to allow in vivo homologous recombination of the two plasmids. Following the recombinant, replication-deficient adenovirus expressing EGFP-NFAT forming plaques or more homogeneous infected cells. Individual plaques were picked and screened for recombinant adenovirus by PCR using cDNA-specific primers. Recombinant adenovirus was identified and purified through several rounds of plaque purification. High titer stocks of purified recombinant adenoviruses were generated by the virus production core at the University of Alabama at Birmingham. The MEK1 adenovirus was kindly provided by Dr. J. D. Molkenop (University of Minnesota, Minneapolis). The MEK1 and EGFP-NFAT recombinant adenoviruses were infected with MEK1 and EGFP-NFAT recombinant adenoviruses at a multiplicity of infection of 25 plaque-forming units/cell in 250 μl of Dulbecco’s modified Eagle’s medium for 18 h at 37°C and cultured for an additional 36 h.

**RESULTS**

**NRVMs Display CCE**—Cardiomyocyte hypertrophy is associated with elevated [Ca\(^{2+}\)] (28). Previous work has focused on enhanced Ca\(^{2+}\) influx through L-type channels (29) or via the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (30). Here, however, we asked whether sarcoplasmic reticulum (SR/ER Ca\(^{2+}\) store depletion activates an influx of extracellular Ca\(^{2+}\) that manifests as a sustained increase in [Ca\(^{2+}\)]. In initial experiments we utilized confocal microscopy and sparsely plated NRVMs that were not exhibiting spontaneous beating. We compared [Ca\(^{2+}\)], responses to SR/ER store depletion achieved using the irreversible SR/ER Ca\(^{2+}\) ATPase inhibitor thapsigargin to [Ca\(^{2+}\)], increases because of KCl-induced depolarization. Sections of quiescent NRVMs detecting the Ca\(^{2+}\)-sensitive indicator Oregon Green were examined prior to exposure to either thapsigargin or KCl and then at selected intervals. Both stimuli resulted in significant increases in [Ca\(^{2+}\)], that persisted for more than 10 min with extracellular Ca\(^{2+}\) present (Fig. 1A). Control experiments with thapsigargin in the absence of extracellular Ca\(^{2+}\) detected increases in [Ca\(^{2+}\)], that persisted only for up to 1 min (data not shown). When extracellular Ca\(^{2+}\) was chelated, both the signal caused by thapsigargin and that caused by KCl rapidly decreased (Fig. 1B), further illustrating the dependence of the sustained elevation of [Ca\(^{2+}\)] on extracellular Ca\(^{2+}\). The experiments with thapsigargin thus support the premise that SR/ER Ca\(^{2+}\) store depletion, even in the absence of agonist-induced second messengers, leads to Ca\(^{2+}\) influx, CCE, in cardiomyocytes.

We next examined the effects of various inhibitors on the sustained [Ca\(^{2+}\)], increases induced by thapsigargin or KCl. Increases caused by thapsigargin treatment were more sensitive to inhibition by the CCE inhibitors glucosamine (31) and SKF96365 (18) than to the L-type Ca\(^{2+}\) channel inhibitor verapamil (Fig. 1, C–E). In contrast, increases caused by KCl were relatively insensitive to the CCE inhibitors but were effectively blocked by verapamil (Fig. 1, C–E). These data (summarized in Fig. 1F) are consistent with a CCE pathway for extracellular Ca\(^{2+}\) that is dependent upon a class of Ca\(^{2+}\)-permeant channels distinct from voltage-gated channels.

A conventional protocol for defining CCE calls for the depleting ER/ER Ca\(^{2+}\) stores in the absence of extracellular Ca\(^{2+}\), resulting in an increase in [Ca\(^{2+}\)], that returns to baseline after the stores are thoroughly depleted (18). The subsequent addition of extracellular Ca\(^{2+}\) then results in a sustained increase in [Ca\(^{2+}\)], if CCE has been activated. This protocol was carried out with NRVMs, employing the Ca\(^{2+}\)-sensitive dye Fura-2 and whole cell digital imaging that allowed for more rapid detection of [Ca\(^{2+}\)], changes.

Treatment with thapsigargin resulted in a transient increase in [Ca\(^{2+}\)], that lasted for about 90 s as Ca\(^{2+}\) in the SR stores released to the cytoplasm. When 1.8 mM Ca\(^{2+}\) was restored to
Physiological Agonists Induce CCE in NRVMs—We next determined whether physiological agonists also induce an apparent CCE in cardiomyocytes. As noted above and by previous authors (33), substantial cell-to-cell variability in \([\text{Ca}^{2+}]_i\) response profiles depends in large part on the local density of the NRVMs being examined. Two \(\text{IP}_3\)-generating hypertrophic agonists, phenylephrine (PE) and Ang II, produced \([\text{Ca}^{2+}]_i\) increases consistent with CCE across this spectrum of phenotypes. In sparsely plated cells there was usually no spontaneous activity prior to treatment (Fig. 3A), whereas in more dense cultures spontaneous beating produced correlative fluctuations in \([\text{Ca}^{2+}]_i\) (Fig. 3B, C, E, and F). Despite this variability, \(\text{IP}_3\)-inducing agonists produced an increase in resting \([\text{Ca}^{2+}]_i\) that in all cases was insensitive to verapamil and inhibited by blockers of CCE, suggesting a common mechanism. In the example shown in Fig. 3A, PE led to an increase in \([\text{Ca}^{2+}]_i\), spikes, followed by an acceleration of the beat amplitude and frequency. Again, the
addition of verapamil abolished the spikes, but [Ca\(^{2+}\)], remained elevated. In even more dense cultures, spontaneous beating was usually more rapid and resulted in larger fluxes in [Ca\(^{2+}\)]. Agonists led to an increase in time-averaged [Ca\(^{2+}\)], as shown for Ang II in Fig. 3C. The addition of verapamil stopped the beating, although [Ca\(^{2+}\)], remained elevated.

To further address whether the increase in time-averaged [Ca\(^{2+}\)], was through L-type channels, we added PE in the presence of verapamil. PE still led to a rapid elevation in [Ca\(^{2+}\)], (Fig. 3D), indicating that the time-averaged increase was not dependent on L-type channels. However, the subsequent addition of the CCE inhibitor glucosamine dropped [Ca\(^{2+}\)], levels precipitously, although they returned to the elevated level when the inhibitor was washed out. When glucosamine treatment preceded the addition of PE (data not shown) or Ang II (Fig. 3E), there was little or no increase in base-line [Ca\(^{2+}\)], and the frequency of the [Ca\(^{2+}\)], spikes failed to accelerate. Lastly, resting [Ca\(^{2+}\)], increases caused by Ang II in rapidly beating cells were reversed by glucosamine (Fig. 3F), although beating was unaffected. Thus, in NRVMs exhibiting a wide variety of spontaneous phenotypes, both of these IP\(_3\)-mediated agonists led to increases in [Ca\(^{2+}\)], (43 of 52 cells) that were relatively insensitive to inhibitors of L-type channels (19 of 20 cells) and sensitive to inhibitors of CCE (13 of 17 cells). Taken together, these data define a Ca\(^{2+}\) entry pathway independent of L-type channels and implicate a role for CCE in cardiomyocytes following treatment with IP\(_3\)-mediated agonists.

CCE Inhibitors Reduce Hypertrophy in NRVMs—Cultured NRVMs undergo cellular responses that parallel many of the responses seen in vivo in hypertrophying hearts (10, 34). These include an increase in contractile protein content, an increase in cell size, elevated [Ca\(^{2+}\)], enhanced sarcomeric organization, and the induction of fetal isoforms of cardiac genes (35). The induction of ANF gene expression is a highly conserved and cardinal feature of ventricular hypertrophy (35) and is readily detectable with immunocytochemistry as perinuclear staining (22). NRVMs were plated overnight and then cultured for 48 h in the presence of agonists and/or inhibitors. In the experiment shown in Fig. 4, NRVMs were stained for \(\alpha\)-actinin (red) and ANF (green). The selected photomicrographs represent data from at least three different cell preparations, and the averaged data are presented in Fig. 4K. Both Ang II and PE markedly increased the percentage of cells expressing ANF. Inclusion of CCE inhibitors more potently blunted ANF expression than verapamil or nifedipine. In addition, both Ang II and PE also led to cell size increases that were attenuated more effectively by inhibitors of CCE than of L-type channels (Fig. 4L). In other experiments, the cells were cultured for 48 h in thapsigargin. This proved to be completely lethal in controls and in the presence of all inhibitors except glucosamine, in which an estimated one-third of the cells survived (data not shown).

CCE Inhibitors Prevent EGFP-NFAT Translocation—To further establish the involvement of CCE in NFAT-mediated hypertrophic responses, we transfected NRVMs with an EGFP-NFAT plasmid (24) and were able to fluorescently monitor the stimulus-induced translocation of the NFAT chimera into the nucleus. Transfected NRVMs were subjected to a 15-min treatment with Ang II, PE, or thapsigargin in the presence or absence of inhibitors and then fixed and assessed by fluorescence microscopy. Without treatment, fluorescence was restricted to the cytoplasm in >90% of the transfected cells (Fig. 5, A and F). Treatment with Ang II or PE for 15 min led to the translocation of EGFP-NFAT into the nucleus in the majority of transfected cells (Fig. 5, B and G). The presence of either CCE inhibitor, glucosamine or SKF96365, prevented the nuclear translocation of the NFAT chimera in response to either agonist (Fig. 5, C, D, H, and I). In contrast, verapamil had only a partial effect (Fig. 5, E and J), and nifedipine had almost no effect (Fig. 5K).

CCE Inhibitors Do Not Affect Hypertrophy Induced by MEK1—The commitment to hypertrophy caused by a physiological stimulus such as Ang II or PE is likely to require the simultaneous involvement of multiple signaling pathways, all perhaps only modestly activated and likely to be essential (4, 9, 11). In contrast, an experimentally induced, high level of stimulation of a particular pathway, for example caused by phorbol ester (34), calmodulin kinase (13), the MEK1 kinase (10), a small guanine nucleotide-binding protein (9), calcineurin (14), or a transcription factor (11, 14), is often sufficient alone to cause a hypertrophic response. Although cross-talk among pathways can be a factor (36, 37), these findings allowed us to ask whether the abrogation by CCE inhibitors of the hypertrophic response caused by PE and Ang II was likely due to a

![Figure 2](http://www.jbc.org/)
rather general toxicity or, in contrast, could be overcome in a relatively Ca\(^{2+}\)-independent model of hypertrophy.

Bueno et al. (10) have established that the expression of a constitutively active MEK1 protein kinase is sufficient to cause hypertrophy in NRVMs. Ichida and Finkel (37) found that introduction of an activated RAS, directly upstream of MEK1, gave rise to 24% nuclear localization of NFAT3, so we first sought to determine whether MEK1-induced hypertrophy was accompanied by the nuclear localization of our NFAT indicator. In coinfection experiments utilizing two recombinant adenoviruses, one encoding the constitutively active MEK1 (10) and the other encoding EGFP-NFAT, we found that the EGFP-NFAT chimeric protein was localized to the nucleus in about 20% of the NRVMs (37). However, cell size determinations on the coinfected cells displaying nuclear EGFP-NFAT localization and those displaying cytoplasmic EGFP-NFAT localization determined that the extents of their hypertrophy were comparable (Fig. 6A). In a further study, we determined that the addition of Ang II or PE to the doubly infected NRVMs led to the rapid nuclear localization of EGFP-NFAT in more than 90% of the cells. This was prevented by the CCE inhibitors SKF96365 and glucosamine (data not shown). This established that IP\(_3\)-generating agonists are able to initiate NFAT nuclear translocation in the presence of constitutively active MEK1 but that the pathway leading from MEK1 to hypertrophy does not appear to require NFAT as a costimulatory molecule. This is consistent with the finding that GATA4, the transcription factor phosphorylated by MEK1, is alone sufficient to evoke the hypertrophic response (11, 12).

With these results, we infected cells with the MEK1-encoding virus and cultured them in the continuing presence of glucosamine or SKF96365. After 48 h the NRVMs were assessed for hypertrophy by cell size (Fig. 6B) and ANF expression (Fig. 6C). In contrast to the results seen in Fig. 4, glucosamine and SKF96365 had no effect on MEK1-induced hypertrophy or ANF expression. These results support the premise that the inhibitory effect of these agents on PE- and Ang II-induced hypertrophy is due to a blockade of the CCE pathway.

DISCUSSION

Given the importance of \(\alpha\)-adrenergic and vasoactive peptide agonists both to the acute regulation of cardiac physiology and to hypertrophic responses, it is perhaps surprising that the molecular mechanisms responsible for their effects are still not clearly understood. Some investigators have attributed their inotropic effects to an increase in myofilament responsiveness to \([\text{Ca}^{2+}]_i\) (38). However, the preponderance of evidence suggests that an increase in \([\text{Ca}^{2+}]_i\), is primarily responsible (2). Using \(\text{Ca}^{2+}\)-sensitive dyes, Touyz et al. (39), and Shao et al. (40) detected responses similar to those reported here for Ang II, as did De Jonge et al. (41) for PE. Several investigators have reported that L-type \(\text{Ca}^{2+}\) current is increased in response to such agonists (2, 42). Others have implicated \(\text{Ca}^{2+}\) entry via the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (43).

The present experiments support an explanation dependent upon the CCE described in nonexcitable cells (18) and in smooth (19) and skeletal muscle (20) that might function instead of or alongside other mechanisms. We established that both thapsigargin and IP\(_3\)-generating agonists cause an increase in \([\text{Ca}^{2+}]_i\), dependent upon extracellular \(\text{Ca}^{2+}\). In addition, we found that a sustained increase in \([\text{Ca}^{2+}]_i\), was more sensitive to inhibitors of CCE than of L-type channels and that the CCE inhibitors prevented hypertrophic responses and NFAT nuclear translocation. Although the data presented here were collected using NRVMs, our unpublished data confirm the continued expression of the CCE pathway in adult cardiomyocytes using both dye-based and whole cell patch clamp approaches.2 This underscores the potential relevance of these findings to the in vivo development of cardiac hypertrophy in adults.

The first description of a \(\text{Ca}^{2+}\) influx independent of L-type voltage-gated channels in adult cardiomyocytes appeared nearly 40 years ago (44). More recently, \(\text{Ca}^{2+}\)-permeant, non-voltage-gated, nonselective cation channels have been reported to be activated by stretch (45, 46), ryanodine treatment (47), and a myocarditis-associated antigen associated with dilated cardiomyopathy (48). Coulombe and coworkers (49) and Wang et al. (50) have defined \(\text{Ca}^{2+}\)-permeant channels that are activable in intact cells by metabolic poisoning and free radicals.

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The work most relevant to that presented here, in that channel activation depends on the generation of IP3, has been reported by Merle et al. (51) and Felzen et al. (52). The former group showed that an IP3-generating ligand, basic fibroblast growth factor, enhanced the opening of a voltage-independent, low conductance, Ca2⁺/H1001-permeant channel in the plasma membrane of cardiomyocytes. Similarly, Felzen et al. (52) found that binding to Fas on the cardiomyocyte surface by either Fas ligand or a Fas-specific monoclonal antibody led to the generation of IP3 and prolonged elevation of myocyte [Ca2⁺/H1001]. Importantly, these effects could be mimicked by the intracellular delivery of IP3, lessening the probability that the other metabolite resulting from PLC activation, diacylglycerol, was important to the process. Although none of these has linked the respective Ca2⁺ influx pathways with ER/SR store depletion, many of these observations could be explained by activation of Ca2⁺-permeant channels caused by such depletion.

In addition, the prime candidate genes for the channels responsible for CCE are members of the Trp (transient receptor potential) family (53, 54). mRNA transcripts for Trp1 and Trp4 have been detected in cardiac tissue from various species at high expression levels, and Trp2, Trp3, Trp5, and Trp6 have been amplified from cardiac tissue (55).

The finding that inhibitors of CCE are able to inhibit an agonist-induced, PLC-dependent in vitro model of hypertrophy despite the assumed production of diacylglycerol might seem surprising in light of the finding that the presence of a diacylglycerol analogue (34), an activated small guanine nucleotide binding protein (9), MEK1 (10), or the downstream transcription factor GATA 4 (11, 12) is sufficient to lead to the hypertrophic response. As suggested previously (9, 11), it is likely that physiological stimuli like Ang II and PE activate at relatively modest levels the multiple signaling pathways that contribute to hypertrophy. The inhibition of any one of these may be sufficient to abrogate the stimulus-induced response. These pathways clearly interact with [Ca2⁺/H1001]-controlled pathways (36, 37), and how they come together in response to physiological stimuli remains an unresolved question. Nonetheless, if, as we suggest, the elevation of [Ca2⁺/H1001] in cardiac hypertrophy is due in part to CCE, a new family of molecular targets for intervening in the progression to cardiac hypertrophy and failure will have been identified.
Localization of EGFP-NFAT in transfected NRVMs. A 15-min treatment with either 1 µM Ang II or 50 µM PE resulted in nuclear localization (B and G) of EGFP-NFAT. Both glucosamine (C and H) and SKF96365 (D and I) effectively prevented stimulus-induced translocation, whereas verapamil (E and J) and nifedipine (K) were less effective. K, the percentages of transfected cells with nuclear localization of EGFP-NFAT from three separate experiments, with error bars representing standard errors.

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