Brief Communications

Neurohormonal Control of Calcium Sensitivity of Myofilaments in Rat Single Heart Cells

M. Puceat, O. Clement, P. Lechene, J. M. Pelosin, R. Ventura-Clapier, and G. Vassort

To investigate the changes in the properties of cardiac contractile proteins due to neurohormonal stimulation, different agonists were applied to single cells isolated from rat ventricle. Cells were then rapidly skinned by Triton X-100, and force was recorded after gluing the cells to a strain gauge. The skinned cells had mechanical properties very similar to those described for thin trabeculas. Tension-pCa relations were highly reproducible from one cell to another, with sarcomere length fixed at 2.1 μm. The application of α₁-adrenergic and muscarinic agonists, which increase the turnover of phosphatidylinositol, for 5 minutes before skinning the cells increased the sensitivity of the myofilaments to calcium, as indicated by a leftward shift of the tension-pCa relation, whereas β-adrenergic stimulation induced a rightward shift. The increase in calcium sensitivity was also evoked by protein kinase C activators such as 1,2-dioctanoylglycerol and phorbol 12-myristate 13-acetate but not by protein kinase C itself or by purinergic agonists, although the latter also increased the turnover of phosphatidylinositol. Incubation of the skinned cells with phosphatase reversed the alterations in calcium sensitivity induced by previous agonist stimulation of the intact cells. In conclusion, this study demonstrates a potentially influential mechanism for the physiological regulation of cardiac muscle contractility. (Circulation Research 1990;67:517-524)

It is evident that the contractility of cardiac muscle can be modulated by mechanisms that cause changes in the rise of cytosolic calcium during an action potential, but the physiological regulation of cardiac contraction could also involve changes in the properties of the contractile proteins themselves. It was found that the inhibitory subunit (troponin I) of cardiac troponin was phosphorylated by the cyclic AMP (cAMP)—dependent protein kinase. This was associated with a decrease in the calcium sensitivity of cardiac myofibrillar ATPase and tension. Muscarinic agonists, besides increasing a specific potassium conductance and thereby shortening the action potential, have been reported to antagonize the increase in calcium current after β-adrenergic stimulation by activating G, the guanine nucleotide binding protein that inhibits adenylate cyclase activity. In frog ventricular cells, acetylcholine also inhibits the opening of calcium channels by activating cyclic GMP (cGMP)—dependent phosphodiesterase that diminishes cAMP concentration. The reduced influx of calcium and the increased efflux of potassium could both contribute to the negative inotropic action of acetylcholine. However, in some cases it has been reported that muscarinic agonists induce a positive inotropy possibly related to an increase in intracellular sodium activity or in phosphoinositide turnover. In previous studies, it was reported that muscarinic cholinergic stimulation increases calcium sensitivity only in fibers that require high calcium concentration for 50% activation and that muscarinic stimulation induces only a weak leftward shift of the control tension-pCa relation compared with the large rightward shift elicited by β-adrenergic agonists. Furthermore, that part of the positive inotropic effect of sympathomimetic amines that is mediated via α₁-adrenoceptors has been suggested to increase myofibrillar sensitivity to calcium.

Our aim in this work was to investigate whether some of the agonists that increase phosphatidylinositol turnover and that have a positive inotropic effect may do so by increasing the affinity of cardiac myofilaments to calcium ions. Cells isolated from young rat hearts were bathed in the presence of α₁-adrenergic, muscarinic, and purinergic agonists. The force developed by these single myocytes after chemical skinning was measured at different calcium concentrations. All of these agents, with the excep-

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tion of the purinergic agonists, induced a large increase in myofilament sensitivity to calcium ions; β-adrenergic stimulation produced opposite results. This increased calcium sensitivity was reversed by phosphatase. Finally, when applied directly to skinned cells the cAMP-dependent protein kinase A (PKA) was able to reproduce the rightward shift induced by β-agonist stimulation; the addition of protein kinase C (PKC) was ineffective.

**Materials and Methods**

Cardiac ventricular cells were isolated from 200 g male Wistar rats according to the method of Wittenberg et al.,\textsuperscript{15} with slight modifications. Briefly, the heart was first perfused at 37°C with a Hanks-HEPES buffer solution containing (mM) NaCl 117, KCl 5.7, NaHCO\textsubscript{3} 4.4, KH\textsubscript{2}PO\textsubscript{4} 1.5, MgCl\textsubscript{2} 1.7, HEPES 21, glucose 11, creatine 10, taurine 20, and insulin (21 milliunits/ml) bubbled with 100% O\textsubscript{2}. After 5 minutes of washing to clear the blood from the heart, fresh buffer supplemented with 1.2–1.5 mg/ml collagenase and 20 μM calcium was recirculated for 50–60 minutes. The heart was gently disassociated with forceps in the same medium without collagenase. The cells were then filtered and incubated for 15 minutes. After decanting the supernatant, the myocytes were resuspended in a medium with 1 mM calcium and 0.5% bovine serum albumin. Seventy percent of the cells isolated with this procedure were calcium tolerant. The myocytes were then centrifuged in a Percoll gradient to increase the proportion of viable cells to 90–95%.

Before skimming, 10\textsuperscript{6} cells/ml were incubated for 5 minutes at 37°C in a buffer with 1 mM calcium and 0.5% bovine serum albumin in the presence of one of the following: 10 μM phenylephrine and 1 μM propanolol, 0.3 μM isoproterenol, 10 and 100 μM ATP, 100 μM carbachol, 50 μM 1,2-dioctanoylglycerol, (DOG), or 0.1 μM phorbol 12-myristate 13-acetate (PMA). Myocytes were then decanted and immediately incubated by shaking for 6 minutes at 20°C in a relaxing solution that contained 0.3% vol/vol Triton X-100 to allow for fast skimming. Skinned cells were then rinsed twice with the same solution without Triton X-100 and maintained at 4°C for up to 8 hours. To check for the adequacy of the skinning protocol, myosin ATPase was measured on a batch of skinned cells using a fluorometric coupled enzymatic assay; the lack of inhibition of the ATPase activity measured in the presence of 10 μM ouabain and 5 mM NaN\textsubscript{3} provided evidence for the absence of sarcolemmal and mitochondial membranes. The relaxing and activating solutions were calculated according to Fabiato,\textsuperscript{16} with the following exceptions: 30 mM imidazole and acetate acid were used as a buffer to adjust solutions to pH 7.1, and acetate was used instead of chloride anions.\textsuperscript{17} Some of the control skinned cells were exposed to a submaximal calcium solution that also contained cAMP-dependent PKA from bovine heart (Sigma Chemical Co., St. Louis, Mo.) or calcium- and phospholipid-dependent PKC (from bovine brain). In some other experiments, after the cells were stimulated by isoprenaline, phenylephrine, or DOG and skinned, they were incubated for 30 minutes at 20°C in relaxing solution with 50 units/ml alkaline phosphatase type VII NL (Sigma). They were then stored at 4°C before use. The same alkaline phosphatase treatment was applied to control skinned cells.

The system for recording tension consisted of a transducer (model AE 801, SensoNor a.s., Horten, Norway) with a thin 3-cm-long glass rod. It was connected to an amplifier to yield a sensitivity of 0.6 mV/nN with a noise level below 7 nN. A suspension of skinned cells in the relaxing solution was placed in a Petri dish on the stage of an inverted microscope. The tip of the glass rod, first soaked in a small drop of optical adhesive (Norland Products, Inc., North Brunswick, NJ) was gently put on one end of a myocyte. This end of the cell was then exposed to long wavelength ultraviolet light for 3 minutes to cure the adhesive. This adhesive had the advantage of remaining in the form of a drop at the tip of the glass rod when going through the air-water interface. The other end of the cell was sucked into the tip of a glass micropipette for fixation. The enzymatic activities of myosin ATPase and bound creatine kinase measured with a fluorometric coupled enzymatic assay were not changed by the exposure of a batch of skinned cells to ultraviolet light. Sarcomere length was adjusted under the microscope and was estimated by counting the number of sarcomeres in two different 25-μm-long areas of the cell (average length, 2.08±0.04 μm; n=33 cells). The attached myocyte was then superfused with solutions at 20–22°C. The cell was positioned at the tip of a conical microcapillary that received the outlets of seven microcapillaries connected to 10-ml syringes. Outflow (20 μl/min) was obtained by elevating a syringe 60 cm above the level of the dish. The dish was continuously superfused with the relaxing solution, and the drain for the bath perfusion was glued 2 mm to the rear of the opening of the conical microcapillary. The delay in response after elevating a syringe was 20 seconds.

**Results**

**Characterization of the Experimental Model**

Continuous records of the force developed by single chemically skinned cells when submitted to EGTA-buffered solutions of different calcium concentrations are shown in Figure 1. Both types of protocol, that is, decreases in pCa alternated with relaxing pCa (Figure 1A) and stepwise decreases in pCa (Figure 1B), were used with essentially the same results on these cells whose sarcomere length was initially fixed at 2.1 μm. The threshold pCa was 6, and the maximal force obtained at pCa 4.5 was between 1 and 1.5 mg. On average, maximal force was 180 mN/mm\textsuperscript{2} (See Table 1), assuming the thickness of the cell to be 60% of its width.\textsuperscript{16} This is within the range of the maximal force exerted by single
isolated rat cells and twice more than the average calcium-activated tension of a thin strip of isolated cardiac muscle. These effects were reproducible: a second application of pCa 4.5 solution gave rise to similar force within 10%. It should be noted, however, that in about 25% of the experiments cells detached upon the application of the lower pCa solution; these cells were discarded. Due to the unavoidable drift in baseline that resulted from minor temperature changes and instability of the strain gauge at the high gain in use, most of the experiments were performed using the protocol in Figure 1A.

Figure 2A shows an averaged tension-pCa relation obtained for control skinned cells at a sarcomere length of 2.1 μm. In these preparations this relation is well described by the Hill equation: %T = SⁿH/(K + SⁿH), where %T is the relative tension, S is the calcium concentration, K is the apparent affinity constant, and nH is the Hill coefficient. K and nH permit the calculation of pCa50, the concentration of Ca²⁺ needed to elicit half maximal tension since a pCa of 50 equals (−log K)/nH. Use of this equation and a linear regression fit provides a convenient and objective description of the tension-pCa relation.

**Figure 2.** Graph showing relative tension-pCa relation from single skinned cells in control conditions. The tension at each pCa is the average of results from 5 to 25 cells. The line was drawn using the Hill equation: %T=SⁿH/(K+SⁿH), where %T is the relative tension, S is the calcium concentration, K is the apparent affinity constant, and nH is the Hill coefficient. For this equation, pCa50 = −logCa50=5.80, and nH=2.78.

pCa50 and nH were 5.80±0.01 and 2.78±0.11, respectively (n=25 cells). There was only a very small scatter of the pCa50 when particular care was taken to estimate sarcomere length and when the same sequence of solutions was applied.

**Modulation of Myofibrillar Calcium Sensitivity**

The sensitivity of single skinned cells to calcium ions was markedly modified when the cells were stimulated for 5 minutes with different agonists before skinning. Figure 3 shows the traces obtained from three different cells exposed to isoprenaline, phenylephrine in the presence of propranolol, or the stable muscarinic agonist carbachol. The β-adrenergic agonist clearly reduced the calcium sensitivity so that the threshold pCa was around 5.87 (Figure 3A). On the other hand, both the α₁-adrenergic (Figure 3B) and the muscarinic (Figure 3C) agonists increased the myofibrillar calcium sensitivity; noticeable force was recorded at pCa 6.25. The use of skinned cells at variable times (up to 8 hours) after the skinning procedure confirms that the contractile proteins in relaxing solution maintained their previously acquired stimulated state.

The results from several experiments are reported in Figure 4 and Table 1. On average, phenylephrine and carbachol induced significant shifts toward the left

**Table 1.** Effects of Inotropic Agents on Calcium Sensitivity of Myofilaments

<table>
<thead>
<tr>
<th>Agent</th>
<th>Control</th>
<th>ISO (10 μM)</th>
<th>PHE (10 μM)</th>
<th>Cch (100 μM)</th>
<th>ATP (10 μM)</th>
<th>DOG (50 μM)</th>
<th>PKA</th>
<th>PKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tₘ₅₀ (mN/mm²)</td>
<td>178±8</td>
<td>186±11</td>
<td>188±9</td>
<td>174±7</td>
<td>164±28</td>
<td>214±21</td>
<td>162±15</td>
<td>165±18</td>
</tr>
<tr>
<td>pCa₅₀</td>
<td>5.80±0.01</td>
<td>5.63±0.02*</td>
<td>5.93±0.01*</td>
<td>6.00±0.02*</td>
<td>5.82±0.02</td>
<td>5.98±0.02*</td>
<td>5.67±0.01*</td>
<td>5.82±0.01</td>
</tr>
<tr>
<td>nH</td>
<td>2.78±0.11</td>
<td>2.52±0.10</td>
<td>2.71±0.14</td>
<td>2.48±0.28</td>
<td>2.78±0.37</td>
<td>2.89±0.17</td>
<td>2.89±0.14</td>
<td>3.23±0.34</td>
</tr>
<tr>
<td>n</td>
<td>25</td>
<td>8</td>
<td>10</td>
<td>6</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ISO, isoprenaline; PHE, phenylephrine; Cch, carbachol; ATP, adenosine triphosphate; DOG, 1,2-dioctanoylglycerol; PKA, protein kinase A; PKC, protein kinase C; Tₘ₅₀, maximal tension; pCa₅₀, concentration of Ca²⁺ needed to elicit half maximal tension; nH, the Hill coefficient; n, number of cells. pCa₅₀ and nH were determined according to the Hill equation using a linear regression fit of the values obtained from each skinned cell under the different conditions defined in the figures (in each case, correlation coefficients were greater than 0.995). Values for a given condition were obtained from at least two hearts. Statistical analysis was performed using Student’s t test for unpaired observations.

* pCa₅₀ significantly different from control at p≤0.01.
tension developed at pCa 5.75. This indicates that these compounds do not directly alter the sensitivity of the myofilaments to calcium ions.

In another series of experiments, none of the cells exhibited a change in the calcium sensitivity of tension when they were stimulated before skinning by ATP (at concentrations varying from $10^{-7}$ to $10^{-4}$ M) even though P$_2$-purinoceptor stimulation has been reported to increase mechanical activity and phosphatidinositol turnover in rat ventricle. This is in line with the lack of effect of extracellular ATP on the relation between changes in cell length and indo-1 fluorescence. However, in intact cells from the same cell-isolation procedure, ATP induced a large increase in the calcium current, indicating that our isolated cells had functional purinergic receptors.

The possibility that the inotropic effect of some of the nonpurinergic neurotransmitters that increase phosphoinositide turnover resulted from either an increase in inositol 1,4,5-trisphosphate (InsP$_3$) or from activation of PKC was checked. It has been reported that InsP$_3$ could affect the contractile mechanism of chemically skinned muscle. InsP$_3$ reversibly enhanced isometric steady-state force production at submaximal pCa in rabbit fast skeletal muscle and decreased it in freeze-dried frog skeletal muscle; InsP$_3$ had no effect on the calcium sensitivity or maximal force generated by the contractile apparatus of skinned cardiac muscle. Figure 5 shows that the addition of 10 µM InsP$_3$ to a submaximal activation solution did not alter the force developed by the skinned cell. The maximal tension was also not altered (not shown). On the other hand, the use of PKC activators such as a diacylglycerol analogue or a phorbol ester alters myofilament calcium sensitivity. Figure 6 shows the tension-pCa relation obtained from cells pretreated with DOG and compares it with the relation established in control cells. A large shift toward the left was observed. This was a consistent result in the eight cells investigated in the presence of DOG (Table 1). The results were more variable when the cells were subjected to PMA (pCa$_{50}$=5.90±0.04; n=4).

The decreased calcium sensitivity of tension development and calcium ATPase activity after β-adrenergic stimulation has been attributed to phosphorylation of troponin I by the cAMP-dependent PKA. This idea has recently received support from the observations of a decrease in the sensitivity to calcium of the Mg-ATPase of reconstituted regulated

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**Figure 3.** Continuous recordings of tension developed by single skinned cells that were exposed to different agonists for 5 minutes before skinning and were superfused with solutions of decreasing pCa alternated with relaxing solution. Panel A: Cells exposed to 0.3 µM isoprenaline. Panel B: Cells exposed to 10 µM phenylephrine in the presence of 1 µM propranolol. Panel C: Cells exposed to 100 µM carbachol.

by 0.13 and 0.20 pCa units, respectively; the rightward shift induced by isoprenaline was 0.17 pCa unit. No matter which agonist was used to stimulate the cell, there was no significant change in maximal developed tension when compared with control conditions.

In a skinned control cell, the application of 5 µM cAMP and 5 µM cGMP was without effect on the

**Figure 4.** Graph showing tension-pCa relations from single skinned cells previously exposed to different agonists. The relations, after averaging results obtained in cells stimulated by 10 µM phenylephrine (•), 0.3 µM isoprenaline (▲), or 100 µM carbachol (○) are compared with the control relation (■). Curves are drawn according to the Hill equation using the values reported in Table 1.
actomyosin in the presence of PKA. We also found that treatment of single skinned cells by PKA in the presence of cAMP decreased their calcium sensitivity (Figure 7A); the tension-pCa relation was shifted toward the right by 0.15 pCa unit (Figure 7C and Table 1). Phosphorylations of troponin I, troponin T, and myosin light chain by phospholipid-sensitive, Ca\(^{2+}\)-dependent PKC have been reported in intact studies. Therefore, after the skinning procedure, some cells were exposed to PKC in the presence of phosphatidylyserine and DOG. The calcium sensitivity of the myofilaments appeared unchanged even after different durations of incubation that varied from 5 to 60 minutes at pCa of 6 and 5.87 (Figures 7B and 7C). This also suggests that DOG, on its own, had no effects on the skinned cells.

The changes in myofilament calcium sensitivity that followed treatment of the cells by different agonists remained stable after the skinning procedure. However, all of these changes could be reversed with the addition of alkaline phosphatase (50 units/ml) to the relaxing solution for 30 minutes. The results of such a series of experiments are summarized in Table 2. In all cases, after the incubation with phosphatase, the pCa\(_{50}\) was very similar to the one obtained in control cells also treated with phosphatase.

**Discussion**

The tension developed by single skinned cardiac cells was measured, and the tension measured in untreated control cells was comparable with previously reported results from thin trabeculae. The sensitivity of the skinned cells to Ca\(^{2+}\) was very consistent in control conditions at a given sarcomere length. Ca\(^{2+}\) sensitivity was reduced after cells had been stimulated by isoprenaline but was significantly increased by pretreatment of the cells with phenylephrine, carbachol, and PKC activators. The effects of all these treatments were reversed by applying alkaline phosphatase to the skinned cells. There was no significant change in the maximal tension developed by the skinned cells under the different experimental conditions.

Major advantages of our technique are the reproducibility of the tension-pCa relation and the maintenance of the skinned cells in their stimulated state. The latter is probably the consequence of the fast skinning procedure in a buffered low-calcium solution, which prevents the action of phosphatases, phosphodiesterases, and other enzymes. Moreover, due to the small diameter of the preparations, accessibility and diffusion time should be favored. Since tension was sensitive to sarcomere length at submaximal calcium concentrations and less at maximal concentrations.
activation.25 we had been very careful when adjusting length at the beginning of each experiment.

Autonomic agonists produce changes in the regulation of calcium movements and in the sensitivity of myofilaments to calcium. These changes and their relative importance in sympathetic and parasympathetic regulation are unclear as yet. To avoid changes in the regulatory system specific for one or the other type of myosin,26 we chose to work with cardiac cells isolated from young rats in which the V1 isoform of myosin is virtually all that is expressed. A rightward shift in the relation between tension and pCa induced by PKA activation is generally in agreement with other studies5,27-29 (Figures 3 and 4). However, this shift was reported to be associated with29 or without3 (as in our results) changes in maximally activated force.

Tension developed in various calcium-containing solutions by skinned cells previously exposed to carbachol indicated that the calcium sensitivity was increased (Figures 3 and 4). In their recent work, McIvor et al5 demonstrated that acetylcholine at 1 µM could antagonize the β-adrenergic stimulation and that when applied alone could induce a slight leftward shift. Such an effect has already been observed in some hyperpermeable myocardial preparations from the rat.11 However, in these fibers, the effect of cholinergic agents on calcium sensitivity was particularly evident when the pCa90 of the preparation was lower than average in control conditions. It was attributed to cholinergic control of phosphatase through guanylate cyclase and cGMP.11 Moreover, there is a recent suggestion that muscarinic stimulation depresses the activity of inhibitor-1; this protein is an active inhibitor of type I phosphatase only when it has been phosphorylated by cAMP-dependent protein kinase.30 Our results clearly establish that cholinergic stimulation (by carbachol at 100 µM) induces a leftward shift; this could be related to the phosphorylation of one or several contractile proteins since the addition of alkaline phosphatase reverses the effect (Table 2).

Positive cardiac inotropism is also well described after α-adrenergic stimulation. Endoh and Blinks12 clearly demonstrated that this occurs without changes in the transient variations in intracellular calcium after loading their preparations with aequorin. They proposed that the affinity of the myofilaments to calcium ions was increased. However, this could have been the result of an intracellular alkalization, as reported recently,31 after the stimulation of the Na-H exchanger by PKC, since it is known that high pH enhances calcium sensitivity of myofilaments.32 Our results unambiguously show that myofilament calcium sensitivity is increased after phenylephrine stimulation. A similar 0.2 pCa unit shift was also observed after application of a muscarinic agonist at 100 µM, a concentration that is required to fully stimulate phosphatidylinositol turnover6,33 or application of PKC activators. However, our results and those of Danziger et al18 indicate that ATP, a P2-purinergic agonist that also increases phosphatidylinositol turnover, does not affect the calcium sensitivity of the myofilaments.

PKC activators and agonists, which increase the phosphoinositide turnover (except ATP) and thus supposedly activate PKC; increased the calcium sensitivity of myofilaments (Figures 4 and 6). When applied directly to the skinned cells, PKC was without effect on the Ca2+ sensitivity of tension, whereas the application of PKA significantly reduced the tension at submaximal pCa. This lack of effect of PKC was unlikely to be related to tissue or species specificity; the brain bovine PKC we used was a crude extract and contained the three isoforms of PKC including PKC type III, which is common to brain and heart.34 The extract phosphorylates at least one protein (of approximately 30 kD) when added to cardiac myofilaments; we will pursue this finding in later studies. Activation of PKC by a phorbol ester greatly increased phosphorylation of troponin T and, to a lesser extent, troponin I in isolated myocytes.35 However, in vitro phosphorylation of troponin T and troponin I by PKC is prevented by the presence of troponin C and tropomyosin.24,36 These observations and our results suggest that there are unknown steps between activation of PKC and the increase in myofilament sensitivity to calcium.

Changes in the contractile properties of the cardiac myofilaments have been often attributed to protein phosphorylations since the initial demonstration that troponin I can be phosphorylated by the cAMP-dependent protein kinase and that cardiac myofibrillar ATPase activity was reduced.23 Later, many in vitro studies reported phosphorylations of troponin I and troponin T, among other proteins, by the cAMP-, cGMP-, and calcium-phosphatidylinositol-dependent protein kinases. However, withdrawal of the β-agonist reverses the inotropic state of intact cells, but it does not reverse troponin I phosphorylation.23,37 In this work we demonstrated that the effects of activation of one of these protein kinases in the intact cell can be reversed by applying alkaline phosphatase to the skinned cell. Furthermore, after phosphatase treatment, the pCa90 is slightly reduced in all cells, as well as in control skinned cells. This would suggest that our control skinned cells had a significant and reproducible level
of phosphorylation after dissociation and skinning. Notice that in cardiac myofibrils prepared from dog heart a similar treatment induced a slight increase in cardiac myofibrillar calcium sensitivity.38

In conclusion, several of the agonists that increased phosphoinositol turnover may each have activated PKC-induced positive inotropy by different pathways. Purinergic stimulation presumably increased the calcium current but did not alter the sensitivity of skinned cells to calcium. α1-Adrenergic and muscarinic stimulations, which do not significantly increase the calcium current, enhanced the myofilament sensitivity to calcium, as did PKC activators. This implies a secondary internal control mechanism to account for the specificity of each of these positive inotropic agents. Furthermore, the increase in myofilament sensitivity to calcium involved intermediate steps after PKC activation; these steps seem to be lost with the skinning procedure.

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