Modulation of Voltage-dependent Ca\textsuperscript{2+} Channels in Rabbit Colonic Smooth Muscle Cells by c-Src and Focal Adhesion Kinase

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There is emerging evidence indicating that smooth muscle contraction and Ca\textsuperscript{2+} influx through voltage-dependent L-type Ca\textsuperscript{2+} channels are regulated by tyrosine kinases; however, the specific kinases involved are largely unknown. In rabbit colonic muscularis mucosae cells, tyrosine-phosphorylated proteins of ~60 and 125 kDa were observed in immunoblots using an anti-phosphotyrosine antibody and were identified as c-Src and focal adhesion kinase (FAK) by immunoblotting with specific antibodies. FAK co-immunoprecipitated with c-Src, and the phosphorylation of the c-Src-FAK complex was markedly enhanced by platelet-derived growth factor (PDGF) BB. The presence of activated c-Src in unstimulated cells was identified in cell lysates by immunoblotting with an antibody recognizing the autophosphorylated site (P416Y). In whole-cell patch-clamp studies, intracellular dialysis of a Src substrate peptide and anti-c-Src and anti-FAK antibodies suppressed Ca\textsuperscript{2+} currents by 60, 62, and 43%, respectively. In contrast, intracellular dialysis of an anti-mouse IgG or anti-Kv1.5 antibody did not inhibit Ca\textsuperscript{2+} currents. Co-dialysis of anti-c-Src and anti-FAK antibodies inhibited Ca\textsuperscript{2+} currents (63%) equivalent to dialysis with the anti-c-Src antibody alone. PDGF-BB enhanced Ca\textsuperscript{2+} currents by 43%, which was abolished by the anti-c-Src and anti-FAK antibodies. Neither the MEK inhibitor PD 098059 nor an anti-Ras antibody inhibited basal Ca\textsuperscript{2+} currents or PDGF-stimulated Ca\textsuperscript{2+} currents. The \(\alpha_{\text{1C}}\) subunit of the L-type Ca\textsuperscript{2+} channel co-immunoprecipitated with anti-c-Src and anti-phosphotyrosine antibodies, indicating direct association of c-Src kinase with the \(\alpha_{\text{1C}}\) channel. These data suggest that c-Src and FAK, but not the Ras/mitogen-activated protein kinase cascade, modulate basal Ca\textsuperscript{2+} channel activity and mediate the PDGF-induced enhancement of L-type Ca\textsuperscript{2+} currents in differentiated smooth muscle cells.

The influx of extracellular Ca\textsuperscript{2+} is a prerequisite for many cellular functions including cell proliferation and motility. In gastrointestinal smooth muscle, the upstroke of action potential is principally mediated by Ca\textsuperscript{2+} influx through voltage-dependent L-type Ca\textsuperscript{2+} channels and is responsible for initiation of contraction. A variety of neurotransmitters and hormones modulate Ca\textsuperscript{2+} channel activity through protein phosphorylation (1). Modulation of Ca\textsuperscript{2+} channel activity by serine/threonine kinases such as cAMP-dependent protein kinase and protein kinase C has been well established (2, 3), and phosphorylation sites have been identified on the \(\alpha\) subunit of L-type Ca\textsuperscript{2+} channels in vascular smooth muscle (4). In addition to their roles in growth and differentiation, accumulating evidence suggests that tyrosine kinases are involved in the regulation of smooth muscle contraction. For instance, activation of both G protein-coupled receptors and growth factor receptors leads to smooth muscle contraction that is accompanied by tyrosine phosphorylation of a number of proteins (5, 6). Moreover, smooth muscle contraction can also be inhibited by structurally unrelated tyrosine kinase inhibitors (7). The fact that Ca\textsuperscript{2+} currents are attenuated by tyrosine kinase inhibitors and enhanced by growth factors in smooth muscle cells (8) points toward a novel mechanism for tyrosine kinases in the regulation of smooth muscle function.

One of the earlier signaling events associated with activation of G protein-coupled receptors, particularly G protein-coupled receptors, and receptor tyrosine kinases involves activation of c-Src (9–11). Downstream signaling events of c-Src include the formation of complexes among Src, Grb2, and Sos and activation of the Ras and mitogen-activated protein (MAP)1 kinase cascade (12). Focal adhesion kinase (FAK) is a potential substrate for c-Src, and once phosphorylated, it may provide a docking site for SH2 domains of the adaptor proteins such as Grb2. In addition, FAK may facilitate activation of c-Src by displacement of the inhibitory C-terminal tyrosine phosphorylation (13). Interestingly, activation of G protein-coupled receptors and receptor tyrosine kinases increases the activities of c-Src and FAK in smooth muscle (10, 14, 15). The inhibition of basal Ca\textsuperscript{2+} currents by tyrosine kinase inhibitors suggests that there may be constitutively activated tyrosine kinase(s) that up-regulates Ca\textsuperscript{2+} channel activity. A possible candidate is c-Src because of its high levels in smooth muscle (16). Recent studies in vascular smooth muscle cells indicate that c-Src may be involved in the regulation of Ca\textsuperscript{2+} channels based on the finding that intracellular dialysis of c-Src enhances Ca\textsuperscript{2+} currents (17). However, it is not known whether other downstream signaling molecules may be involved in the regulation of smooth muscle Ca\textsuperscript{2+} channels.

In this study, we have examined the kinase activity of c-Src and its association with FAK in rabbit colonic smooth muscle cells. We have also evaluated the roles of c-Src and FAK as well as their downstream components Ras and MAP kinase in the regulation of basal Ca\textsuperscript{2+} channel activity and their involvement in platelet-derived growth factor (PDGF)-induced enhancement of Ca\textsuperscript{2+} currents. Our results demonstrate that basal Ca\textsuperscript{2+} currents are modulated by c-Src and FAK, but not Ras/MAP kinase, in differentiated smooth muscle cells. Furthermore, c-Src and FAK are involved in the functional cou-
pling of PDGF receptors and Ca\(^{2+}\) current enhancement, which is consistent with an increased phosphorylation of these two kinases leading to the tyrosine phosphorylation of the Ca\(^{2+}\) channel.

**EXPERIMENTAL PROCEDURES**

**Electrophysiological Recordings**—Single smooth muscle cells were freshly dispersed from rabbit colonic muscularis mucosae as described previously (8). Ca\(^{2+}\) currents were recorded using the whole-cell configuration of the patch-clamp technique (18). All experiments were performed at room temperature (\(-25 ^\circ C\)) using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Inc., Foster City, CA). Patch pipettes were pulled from thin-walled borosilicate glass, and the resistance was 3–5 megohms when filled with internal solution. The internal solution consisted of 100 mM cesium aspartate, 2 mM MgCl\(_2\), 5 mM HEPES, 5 mM EGTA, 5 mM ATP, and 0.1 mM GTP (pH 7.2 with NaOH). The cells were continuously perfused with HEPES-buffered physiological salt solution (135 mM NaCl, 5.4 mM KCl, 0.33 mM NaH\(_2\)PO\(_4\), 5 mM HEPES, 1 mM MgCl\(_2\), 2 mM BaCl\(_2\), and 5.5 mM glucose (pH 7.4 with NaOH)). Antibodies were applied directly to the cells by means of diffusional exchange during standard whole-cell patch-clamp recording, and current recordings were initiated 4 min after rupture of the membrane to allow adequate intracellular dialysis. Pulse generation and data acquisition were performed with a PC computer (Deskpro 486/33M, Compaq, Houston, TX) with pclamp6.0 software (Axon Instruments, Inc., CA.). Currents were filtered at 1 kHz and normalized with respect to cell capacitance. Series resistance did not exceed 5 megohms and was not compensated. The average cell capacitance was 64.6 ± 0.9 pF (n = 128). Currents in the absence and presence of antibody dialysis were normalized on the same day within the same population of cells.

**Immunoprecipitations and Western Blotting**—Following a 5-min treatment with or without 50 ng/ml PDGF-BB, cell suspensions were centrifuged, and cell pellets were lysed in a Triton X-100 lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin A and B, and 10% Triton X-100). Lysates were centrifuged at 12,000 rpm at 4 °C for 10 min, and protein contents of the supernatants were determined. For immunoprecipitation study, equal amounts of lysate protein (500 µg) were incubated overnight at 4 °C with a monoclonal anti-c-Src antibody. Immune complexes were recovered with protein A-Sepharose beads. The beads were washed, resuspended in sample buffer (50 mM Tris-HCl (pH 6.0), 5% β-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol), and boiled at 100 °C for 5 min. Protein samples were separated by 8% SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were blocked using 2% nonfat dried milk in phosphate-buffered saline (pH 7.2) and incubated overnight with an anti-phosphotyrosine antibody (PY20) from Upstate Biotechnology, Inc. (Lake Placid, NY), monoclonal anti-c-Src, anti-pg60\(^{5338}\), or anti-c-Src (P416Y) antibodies from Transduction Laboratories (Lexington, KY). Anti-Kv1.5 antibodies were from Alomone Labs (Jerusalem, Israel). The anti-c-Src antibody (P416Y) was kindly provided by Dr. A. P. Laudano. PD 098059 was from Alexis Co. (San Diego, CA).

**Chemicals**—Collagenase was from Yakult (Tokyo, Japan). Trypsin was from Sigma. Anti-c-Src and anti-FAK antibodies as well as the Src kinase substrate peptide were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-mouse IgG, anti-Ras, and anti-Tyr(P) (PY20) antibodies were from Transduction Laboratories (Lexington, KY). Anti-Kv1.5 and anti-αtubulin antibodies were from Alomone Labs (Jerusalem, Israel). The anti-c-Src antibody (P416Y) was kindly provided by Dr. A. P. Laudano. PD 098059 was from Alexis Co. (San Diego, CA).

**Statistical Analysis**—Data are expressed as means ± S.E. Data analysis were performed using Student’s t test, and differences with p < 0.05 were considered significant.

**RESULTS**

**Presence of c-Src and FAK in Rabbit Colonic Smooth Muscle Cells**—Tyrosine-phosphorylated proteins were identified in freshly isolated smooth muscle cells of rabbit colonic muscularis mucosae by immunoblotting with the anti-phosphotyrosine antibody (PY20). Fig. 1A shows several tyrosine-phosphorylated proteins in unstimulated cells, including bands at 60 and 125 kDa. These two bands were identified as c-Src and FAK, respectively, by stripping the anti-phosphotyrosine blot and reprobing with specific antibodies (data not shown). The phosphorylation of both c-Src and FAK was enhanced following treatment of cells with PDGF-BB (50 ng/ml) for 5 min (Fig. 1A). Immunoprecipitation of the cell lysates with an anti-c-Src antibody followed by immunoblotting with PY20 showed that a tyrosine-phosphorylated 125-kDa protein co-precipitated with c-Src in unstimulated cells, and the association of the phosphorylated protein with c-Src was enhanced following PDGF-BB treatment (Fig. 1B).

To further demonstrate that c-Src and FAK co-precipitated, cell lysates were immunoprecipitated with the anti-c-Src antibody and blotted with an anti-FAK antibody. Fig. 2A shows that there was a significant association of c-Src with FAK in unstimulated cells, which was enhanced following PDGF-BB treatment. The presence of a c-Src/FAK complex in unstimulated cells suggests that a constitutively activated c-Src may be present under resting conditions. This was confirmed by immunoblotting with an anti-c-Src antibody (P416Y) (Fig. 2B) that recognizes c-Src that is phosphorylated at Tyr\(^{416}\) and correlates with activated c-Src (19). This observation is consistent with the previous findings of a high degree of activated c-Src in resting smooth muscle (16) and the ability of tyrosine kinase inhibitors to attenuate basal Ca\(^{2+}\) channel activity (8). In addition, activated c-Src was also enhanced by PDGF-BB (Fig. 2B).

**Inhibition of Ca\(^{2+}\) Currents by Src Substrate Peptide and Anti-c-Src and Anti-FAK Antibodies**—To demonstrate whether c-Src and FAK may be involved in the regulation of Ca\(^{2+}\) currents, we examined the effects of intracellular dialysis of a c-Src substrate peptide and anti-c-Src and anti-FAK antibodies on Ca\(^{2+}\) currents in single smooth muscle cells using the patch-clamp technique. Ca\(^{2+}\) currents were recorded from a holding potential of −50 mV using Ba\(^{2+}\) (2 mM) as the charge carrier and normalized with respect to cell capacitance (8). The synthetic Src substrate peptide at high concentrations can result in a significant inhibition of Src kinase activity (20) and has
previously been shown to inhibit Ca\(^{2+}\) currents in vascular smooth muscle cells (17). Intracellular dialysis of the Src substrate peptide Cdc2-(6–20)-NH\(_2\) (60 μM), derived from p34\(^{cdk2}\) (21), suppressed the Ca\(^{2+}\) currents from \(-6.19 \pm 0.65\) (n = 6) to \(-2.52 \pm 0.45\) (n = 8) pA/pF (p < 0.001), corresponding to a decrease of 59.3% at the test potential of +10 mV (Fig. 3, A and B). Neither the threshold nor the reversal potential was altered by the Src substrate peptide. Because the Src substrate peptide does not discriminate between members of the Src family, we studied the effects of a monoclonal c-Src antibody (anti-c-Src antibody) on Ca\(^{2+}\) currents to determine the involvement of c-Src in the modulation of Ca\(^{2+}\) channel activity. In these sets of experiments, the control Ca\(^{2+}\) current at the test potential of +10 mV was \(-6.88 \pm 0.70\) pA/pF (n = 6), whereas it was reduced to \(-2.64 \pm 0.54\) pA/pF (n = 10; p < 0.0005) by intracellular application of the anti-c-Src antibody (10 μg/ml), representing an inhibition of 61.6% (Fig. 4, A and C). As illustrated in Fig. 4B, inhibition began soon after the onset of the dialysis process and reached its maximum within 4 min. The steady-state inactivation kinetics of the Ca\(^{2+}\) currents were not altered by the anti-c-Src antibody (data not shown). To determine the specificity of the anti-c-Src antibody on Ca\(^{2+}\) currents, we examined the effects of intracellular dialysis of an anti-mouse IgG antibody (10 μg/ml) and an anti-Kv1.5 antibody (20 μg/ml). As shown in Fig. 5, the amplitudes of Ca\(^{2+}\) currents were not altered by either antibody.

Since FAK co-immunoprecipitates with c-Src in colonic smooth muscle cells, we examined whether FAK could modulate Ca\(^{2+}\) channel activity. Cells were dialyzed with an anti-FAK antibody raised against Tyr 416 corresponding to activated c-Src. Activated c-Src was present in unstimulated cells and was markedly enhanced by PDGF-BB. Moreover, the enhanced association of c-Src and FAK following PDGF-BB treatment was greater in FAK knockdown cells (Fig. 6A). This result suggests that FAK co-precipitates with c-Src. To confirm this observation, we performed Western blots of cell lysates from unstimulated and PDGF-BB-treated cells with the anti-c-Src antibody and immunoblotting with the anti-FAK antibody. Note the presence of FAK in the unstimulated cells and the enhanced association of c-Src and FAK following PDGF-BB treatment (Fig. 6B). This supports the idea that FAK and c-Src interact in specific cellular contexts.

The regulation of Ca\(^{2+}\) channel activity by c-Src and FAK was further investigated by examining the effects of intracellular dialysis of the Src substrate peptide. Because the Src substrate peptide does not discriminate between members of the Src family, we studied the roles of c-Src and FAK in PDGF-BB-enhanced Ca\(^{2+}\) currents. Immunoprecipitation of FAK with c-Src and presence of activated c-Src in unstimulated cells. The Western blots demonstrate that FAK co-precipitates with c-Src. A shows immunoprecipitation (I.p.) of c-Src from cell lysates of unstimulated (Con) and PDGF-BB-treated cells with the anti-c-Src antibody and immunoblotting with the anti-FAK antibody. Note the presence of FAK in the unstimulated cells and the enhanced association of c-Src and FAK following PDGF-BB treatment. B is an immunoblot of cell lysates with the anti-c-Src antibody raised against Tyr 416 corresponding to activated c-Src. Activated c-Src was present in unstimulated cells and was markedly enhanced by PDGF-BB.

FIG. 2. Immunoprecipitation of FAK with c-Src and presence of activated c-Src in unstimulated cells. The Western blots demonstrate that FAK co-precipitates with c-Src. A shows immunoprecipitation (I.p.) of c-Src from cell lysates of unstimulated (Con) and PDGF-BB-treated cells with the anti-c-Src antibody and immunoblotting with the anti-FAK antibody. Note the presence of FAK in the unstimulated cells and the enhanced association of c-Src and FAK following PDGF-BB treatment. B is an immunoblot of cell lysates with the anti-c-Src antibody raised against Tyr 416 corresponding to activated c-Src. Activated c-Src was present in unstimulated cells and was markedly enhanced by PDGF-BB.

Since activation of c-Src and FAK stimulates the Ras/MAP kinase cascade (12, 13), and the anti-FAK antibody used in this study might block the binding of Grb2 to FAK, we investigated the roles of the Ras/MAP kinase pathway in the regulation of Ca\(^{2+}\) currents using an anti-Ras antibody and the MEK inhibitor PD 098059 (24).

Effects of Anti-Ras Antibody and PD 098059 on Ca\(^{2+}\) Currents—Since activation of c-Src and FAK stimulates the Ras/MAP kinase cascade (12, 13), and the anti-FAK antibody used in this study might block the binding of Grb2 to FAK, we investigated the roles of the Ras/MAP kinase pathway in the regulation of Ca\(^{2+}\) currents using an anti-Ras antibody and the MEK inhibitor PD 098059 (24). As shown in Fig. 7, intracellular dialysis of the anti-Ras antibody (5 μg/ml) did not depress the basal Ca\(^{2+}\) currents or PDGF-BB-stimulated Ca\(^{2+}\) currents. In the presence of the anti-Ras antibody, PDGF-BB enhanced Ca\(^{2+}\) currents by 39.5 ± 6.7% (n = 5). Furthermore, perfusing the cells with 30 μM PD 098059 also did not inhibit basal Ca\(^{2+}\) currents or PDGF-mediated enhancement (37 ± 8.1%, n = 5).
Interaction of c-Src with $\alpha_{1C}$ Ca$^{2+}$ Channel—To determine whether c-Src or FAK directly associates with the Ca$^{2+}$ channel, rabbit colonic tissue was treated with PDGF-BB and homogenized in 1% digitonin. The samples were immunoprecipitated with cardiac anti-$\alpha_{1C}$, anti-Tyr(P) (PY20), anti-Src, and anti-FAK antibodies and immunoblotted with the anti-$\alpha_{1C}$ antibody. Fig. 8 shows the presence of the $\alpha_{1C}$ L-type Ca$^{2+}$ channel (first lane), corresponding to a band of $\sim$210 kDa. Bands of similar size were also observed following immunoprecipitation with anti-phosphotyrosine (PY20) (second lane) and anti-c-Src (third lane) antibodies and immunoblotting with the anti-$\alpha_{1C}$ antibody, suggesting that the Ca$^{2+}$ channel is phosphorylated by tyrosine kinases and associates with c-Src kinase. However, the anti-FAK antibody failed to immunoprecipitate the $\alpha_{1C}$ subunit (data not shown).

DISCUSSION

In this study, we have examined the roles of the non-receptor tyrosine kinases c-Src and FAK in the regulation of Ca$^{2+}$ channel activity in differentiated smooth muscle cells of rabbit colon. The results demonstrate the presence of activated c-Src, which interacts with FAK to form a c-Src-FAK complex, in unstimulated smooth muscle cells. The phosphorylation of the c-Src-FAK complex is enhanced by PDGF-BB. Furthermore, we provide evidence that Ca$^{2+}$ currents are constitutively modulated by c-Src, and enhanced phosphorylation of the c-Src-FAK complex by PDGF-BB correlates with enhanced Ca$^{2+}$ currents.

This study also shows that c-Src directly associates with the $\alpha_{1C}$ subunit of the Ca$^{2+}$ channels in smooth muscle. The modulation of Ca$^{2+}$ channel activity by c-Src and FAK implicates an important role for these two kinases in regulating excitability of smooth muscle cells.

A significant finding of this study is that PDGF-BB-induced enhancement of Ca$^{2+}$ channel activity was blocked by anti-c-Src and anti-FAK antibodies. These data provide the first evidence for a direct involvement of c-Src and FAK in the regulation of Ca$^{2+}$ currents in differentiated cells by PDGF. The activities of c-Src and FAK are elevated by PDGF (9, 14), and c-Src is required for PDGF mitogenic signaling (25, 26). We confirmed these findings in colonic smooth muscle cells by...
showing that phosphorylation of these two kinases is enhanced by PDGF-BB, which is consistent with the observations in the electrophysiological study. Two binding sites for c-Src have been identified in the c-Src and FAK antibodies on Ca²⁺ currents. Cells were dialyzed with the anti-FAK antibody (FAK-Ab; 7.3 µg/ml; A) or co-dialyzed with the anti-c-Src antibody (c-Src-Ab; 10 µg/ml) and the anti-FAK antibody (FAK-Ab; 7.3 µg/ml; B). A: panel a, trace showing the effects of the anti-FAK antibody on Ca²⁺ currents obtained at +10 mV. Data are expressed as means ± S.E. of six control cells and six cells dialyzed with the anti-FAK antibody. *, p < 0.05 versus control. B: panel a, trace showing the effects of the anti-c-Src and anti-FAK antibodies on Ca²⁺ currents. The cell capacitance is 75 pF in control cell and 69 pF in cells dialyzed with the anti-FAK antibody. *, p < 0.05 versus control. Panel b, bar graph summarizing the effects of the anti-c-Src and anti-FAK antibodies on Ca²⁺ currents obtained at +10 mV. Data are expressed as means ± S.E. of four control cells and four cells dialyzed with the anti-c-Src and anti-FAK antibodies.

The formation of a c-Src-FAK complex is crucial for cell adhesion and integrin signaling (12) and may also be required for modulating Ca²⁺ channels. While FAK does not directly associate with the Ca²⁺ channel, its involvement is of particular relevance to smooth muscle contraction since one of the potential targets for FAK are the cytoskeletal proteins talin and paxillin, which are phosphorylated following activation of G protein-coupled receptors and receptor tyrosine kinases (6, 14, 15, 29). The membrane-associated dense plaques of smooth muscle are structurally similar to the focal adhesion sites of cultured cells in that both contain the cytoskeletal proteins.
Tyr\(^{576}\) and Tyr\(^{577}\) enhances the activity of FAK (32). By forming such a complex, c-Src activity is also up-regulated (13) and leads to modulation of the smooth muscle Ca\(^{2+}\) channel. A recent study in HEK 293 cells also suggests that formation of the c-Src-FAK complex is essential for coupling FAK to the Ras signaling pathway (33). In rat glomerular mesangial cells, PDGF was shown to enhance voltage-independent Ca\(^{2+}\) channels through Ras (34). The failure of the anti-Ras antibody and the MEK inhibitor PD 098059 to suppress basal Ca\(^{2+}\) currents or to alter PDGF-induced enhancement of Ca\(^{2+}\) currents suggests that the Ras/MAP kinase cascade, the downstream signaling components of c-Src and FAK, is not involved in the regulation of voltage-dependent Ca\(^{2+}\) channels.

In transfected HEK 293 cells, v-Src associates with the human delayed rectifier-type K\(^{+}\) channel Kv1.5, and tyrosine phosphorylation of the channels is accompanied by an inhibition of K\(^{+}\) currents (35). Furthermore, the association of c-Src with N-methyl-D-aspartate channels has been observed in rat central neurons (36). Our studies show similar modulation of smooth muscle L-type Ca\(^{2+}\) currents by c-Src. The L-type Ca\(^{2+}\) channel is tyrosine-phosphorylated and associates with c-Src following PDGF treatment (Fig. 8). The full cDNA sequence of the L-type Ca\(^{2+}\) channel (a\(_{1C}\) subunit) from rat vascular smooth muscle reveals a potential phosphorylation site of tyrosine kinases, which is located at residues 1869–1876 (RLP\(_{575}\)XP) in the target protein (37), XP), a sequence similar to the SH3 domain-binding motif, is present in the rat aorta Ca\(^{2+}\) channel (4).

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