Novel activation stimulus of chloride channels by potassium in human osteoblasts and human leukaemic T lymphocytes

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1. The whole-cell recording mode of the patch-clamp technique was used to study the effect of extracellular K$^+$ and Rb$^+$ on membrane currents in human osteoblasts, in a human osteoblast-like cell line, and in the Jurkat human leukaemic T cell line.

2. Increasing the extracellular concentration of K$^+$ increased the membrane conductance of the cells in a concentration-dependent manner. This increase in membrane conductance was due to the activation of a Cl$^-$ conductance. Rb$^+$ also induced this conductance, but conductance was less than half that seen in K$^+$.

3. The Cl$^-$ channel blockers 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and 4-acetamido-4'-isothiocyanatostilbene 2,2'-disulphonic acid (SITS) blocked the K$^+$-induced Cl$^-$ current in a voltage-dependent manner. The degree of blockade increased with membrane depolarization to a maximum level at 40 mV. At potentials above this value the block appeared to decrease.

4. Both tonicity and K$^+$ were required for maximal activation of the Cl$^-$ conductance since the K$^+$-induced Cl$^-$ conductance could be inhibited by hypertonic solutions and the activation of a volume-sensitive Cl$^-$ conductance by hypotonic solutions could be enhanced by extracellular K$^+$.

5. We conclude that an outwardly rectifying Cl$^-$ conductance can be activated either upon osmotic swelling or by an increase in extracellular K$^+$. Both activation pathways may be involved in cell volume regulation and seem to apply to volume-sensitive Cl$^-$ channels in general since we observe this phenomenon in two different cell types, in human osteoblasts as well as in human leukaemic T lymphocytes.

Mechanical stimulation by uniform and cyclic elongation has been shown to influence proliferation and differentiation of human osteoblasts (Neidlinger-Wilke, Wilke & Claes, 1994). One possible mechanism by which the mechanical stimulation could be transduced is via mechanosensitive channels. As an alternative approach to stretching the membrane of the cell directly, the osmolarity of the bath solution can be lowered to produce cell swelling and therefore produce membrane stretch. This procedure activates a volume-sensitive Cl$^-$ conductance in a variety of cell types (Grinstein, Clarke, Dupre & Rothstein, 1982; Worrell, Butt, Cliff & Frizzell, 1989; Lang, Völk & Häussinger, 1990; Lewis & Cahalan, 1990; Sole & Wine, 1991; Tseng, 1992; Lewis, Ross & Cahalan, 1993; Botchkin & Matthews, 1993; Diaz, Valverde, Higgins, Rucareanu & Sepulveda, 1993; Ross, Garber & Cahalan, 1994; Anderson, Jirsch & Fedida, 1995; Gosling, Smith & Poyner, 1995). We therefore started out to characterize electrophysiologically human osteoblasts and a human osteoblast-like cell line in order to assay the ion channels normally expressed in those cells with the intent to detect mechanosensitive channels that might be involved in the control of proliferation. We present evidence that human osteoblasts have a Cl$^-$ conductance activated by hypotonic solutions, and that this Cl$^-$ conductance is also activated by extracellular K$^+$. A similar activation stimulus of a Cl$^-$ conductance could be observed in cells from a human leukaemic T cell line.

Some of the results have been reported in preliminary communications (Steinert, Hanselmann & Grissmer, 1996a,b; Steinert & Grissmer, 1997).

METHODS

Cells

Human osteoblasts were obtained from anonymous patients treated for bone fractures. Since the cells were removed during surgery and would have been disposed of as organic waste no informed consent and human ethics committee procedures needed to be followed. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum (FCS), were identified as osteoblasts as mononuclear cells with high content of alkaline phosphatase and, from the second passage on, were used for the

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electrophysiological experiments by plating onto coverslips. C1 cells, immortalized human osteogenic precursor cells, were obtained from Dr Brian A. Ashton (The Robert Jones and Agnes Hunt Orthopaedic and District Hospital, Oswestry, Shropshire, UK) and cultured in DMEM−10% FCS with 2 mM glutamine and maintained continuously in a humidified, 5% CO₂ incubator at 37 °C. The human T lymphoma cell line Jurkat E6-1 was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained continuously in a culture medium of RPMI 1640 supplemented with 1 mM glutamine and 10% heat-inactivated fetal bovine serum in a humidified, 5% CO₂ incubator at 37 °C.

Solutions
All experiments were done at room temperature (21–25 °C). The cells under investigation were normally bathed in mammalian Na⁺ solutions containing (mM): 160 NaCl, 4-5 KCl, 2 CaCl₂, 1 MgCl₂, 5 Heps; pH adjusted to 7.4 with NaOH; osmolarity adjusted to 330 mosmol l⁻¹ with either sucrose or mannose. In mammalian K⁺ solutions all the Na⁺ was replaced by an equal amount of K⁺. In Rb⁺ solutions all the Na⁺ and K⁺ was replaced by an equal amount of Rb⁺. In anion-substituted mammalian K⁺ solution all the KCl was replaced by KI, KBr, KNO₃, or potassium aspartate. The composition of the internal pipette solution was (mM): 160 potassium aspartate, 1 CaCl₂, 2 MgCl₂, 10 Hepes, 10 EGTA, 3 ATP, 0.5 GTP; pH 7.2; 320 mosmol l⁻¹; adjusted with either sucrose or mannose. 4-Acetamido-4'-isothiocyanatostilbene 2,2'-disulphonic acid (SITS) and 4,4' disothiocyanatostilbene-2,2'-disulphonic acid (DIDS) were purchased from Sigma, dissolved in DMSO as stock solutions (10 mM) and applied at concentrations of 10 or 100 μM.

Electrophysiology
Experiments were carried out using the whole-cell recording mode of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Grissmer & Cahalan, 1989; Grissmer, Nguyen & Cahalan, 1993; Hanselmann & Grissmer, 1996). Electrodes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) in two stages, coated with Sylgard (Dow Corning), and fire-polished to resistances measured in the bath of 2.5–6 MΩ. Membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) interfaced to a

![Figure 1. Cl⁻ current induction in C1 cells by a K⁺-rich external solution](image)

A, current was elicited by voltage ramps from −120 to 40 mV within 400 ms at 10 s intervals. Current traces shown were measured before and after changing the bath solution from a normal Na⁺ solution (160 mM Na⁺, 4-5 mM K⁺) to a K⁺ solution (164-5 mM K⁺). Traces were collected at the times shown in B. B, time course of Cl⁻ current induction by K⁺-rich external solutions. Conductance obtained from the slope of the Cl⁻ current was determined by fitting a line through the ramp current between −60 and −30 mV from those records shown in A and additional records, switching the bath solution several times from normal Na⁺ solution (Na⁺) to a K⁺-rich external solution (K⁺). The slope of the ramp current was plotted against the absolute time after establishing the whole-cell recording mode. a, b, c and d correspond to the traces shown in A.
Macintosh computer running acquisition and analysis software (Pulse/Pulsefit). All potentials due to the liquid junction potential that develops at the tip of the pipette if the pipette solution is different from that of the bath were less than 5 mV. Each illustrated response was observed at least four times.

**Presentation of data**

Unless otherwise stated, data are presented as means ± s.e.m.

**RESULTS**

**K⁺ activation**

The basic observation is illustrated in Fig. 1A, which shows a series of currents elicited by voltage ramps from −120 to 40 mV in a Cl cell. In this cell no outwardly rectifying current was observed unless Na⁺ in the extracellular solution was replaced by K⁺. The induced current reversed at approximately −50 mV indicating Cl⁻ selectivity rather than an increase in non-selective leak. The time course of this induction can best be seen in Fig. 1B, where the slope of the ramp current, measured at potentials between −60 and −30 mV, was plotted against the absolute time after establishing the whole-cell recording mode. The Cl⁻ conductance could be induced repeatedly by application of the K⁺ solution, although the magnitude of the response was diminished with repeated K⁺ application. A Rb⁺ solution was also able to induce this current, but current amplitudes were less than half with Rb⁺ than K⁺ (data not shown). In addition, a similar induction of the Cl⁻ conductance by application of a K⁺ solution could be observed in human leukaemic T lymphocytes as well as in human osteoblasts (data not shown).

In similar experiments we used lower bath [K⁺] than 164·5 mM to find out whether these bath solutions were also able to induce the Cl⁻ conductance. An example of such an experiment can be seen in Fig. 2A, which shows currents elicited similar to the records in Fig. 1A. Little outward rectifying current was observed with 4·5 mM K⁺ in the extracellular solution. Increasing the bath [K⁺] from 4·5 mM to 40 and to 164·5 mM increased the slope conductance determined (as described in the legend of Fig. 1) from ~1 nS to ~4 and ~10 nS, respectively. The time course of this Cl⁻ current increase by the applied solutions is shown in Fig. 2B.

The K⁺-induced current seemed reminiscent of earlier described volume-sensitive Cl⁻ conductances induced by hypo-osmotic extracellular solutions in other cell types (Grinstein et al. 1982; Worrell et al. 1989; Lang et al. 1990; Lewis & Cahalan, 1990; Sole & Wine, 1991; Tseng, 1992; Lewis et al. 1993; Botchkina & Matthews, 1993; Diaz et al. 1993; Ross et al. 1994; Anderson et al. 1995; Gosling et al.

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**Figure 2. Cl⁻ current induction in Cl cells depends on [K⁺].**

A, current was elicited as described in legend to Fig. 1. Current traces shown were measured before and after changing the bath solution from a normal Na⁺ solution containing 160 mM Na⁺ with 4·5 mM K⁺ (a, 4·5 K⁺) to a solution containing 40 mM K⁺ with 120 mM Na⁺ (b, 40 K⁺) and to a solution containing 164·5 mM K⁺ without Na⁺ (c, 164·5 K⁺). Traces were collected at the times shown in B.

B, time course of Cl⁻ current induction by 40 and 164·5 mM K⁺-containing external solutions. Conductance was obtained similarly to the records in Fig. 1B. The slope of the ramp current was plotted against the absolute time after establishing the whole-cell recording mode. a, b and c correspond to the traces shown in A.
1995) although in our case the extracellular solutions were specifically made slightly hypertonic with either sucrose or mannose in order to prevent the activation of this conductance.

**Pharmacology**

It had been shown that the volume-sensitive Cl\(^-\) conductance was blocked by the stilbene disulphonates DIDS and SITS in a time- and voltage-dependent manner (Lewis et al. 1993; Gosling et al. 1993). We therefore determined the sensitivity of block by DIDS and SITS of the K\(^+\)-induced Cl\(^-\) conductance in cells from the human osteoblast-like cell line. Figure 3A shows ramp currents in the absence and presence of 100 \(\mu\)M DIDS after the activation of the current by the external application of the K\(^+\) solution. It is obvious from these records that DIDS is able to block the K\(^+\)-induced Cl\(^-\) conductance in a voltage-dependent manner, i.e. DIDS block was stronger at more depolarized potentials. Drug block after washout was usually only partially reversible (data not shown; compare Lewis et al. 1993). To quantify the voltage dependence of the block as well as a possible time dependence of block that cannot be seen using voltage ramps, we performed a family of voltage steps in the absence and presence of 100 \(\mu\)M DIDS (Fig. 3B) and SITS (data not shown). At potentials more positive than \(-40\) mV the blockade also clearly showed a time dependence after the step similar to that seen in experiments with the volume-sensitive Cl\(^-\) conductance (Lewis et al. 1993; Gosling et al. 1995). To determine the voltage dependence of the DIDS (and SITS) block of the K\(^+\)-induced Cl\(^-\) conductance, we determined the amplitude of the steady-state current (as described in the legend) in the solutions with and without DIDS and plotted it against the applied membrane potential (Fig. 3C, left). The steady-state current in the presence of

![Figure 3. Inhibition of K\(^+\)-induced Cl\(^-\) current in C1 cells by DIDS](image-url)

A, currents were elicited using the voltage ramp protocol described in the legend to Fig. 1 in the absence and presence of 100 \(\mu\)M DIDS, after a 3 min exposure to the drug. B, currents were elicited by 100 ms voltage steps from \(-80\) to \(80\) mV in 20 mV increments from a holding potential of \(-80\) mV in the absence (left) and the presence (right) of 100 \(\mu\)M DIDS (3 min exposure). Time between pulses was 1 s. C, voltage dependence of steady-state block by DIDS for the experiment shown in B. Block at each test potential was determined by dividing the steady-state current during drug application by the control steady-state current. Steady-state current was determined as the plateau current measured between 80 and 95 ms after the beginning of the pulse. A Boltzmann curve of the form

\[
\frac{I_{\text{drug}}}{I_{\text{control}}} = \frac{1}{1 + \exp[(V - V_\theta)/k]}
\]

was fitted by eye to the data, indicating an e-fold change in blockade per 20 mV (k) and \(V_\theta = -35\) mV.
external DIDS was divided by the steady-state current obtained in the absence of DIDS and plotted against the membrane potential (Fig. 3C, right). The smooth curve through the ratio $I_{\text{DIDS}}/I_{\text{control}}$ represents a fit of a Boltzmann equation to the data between $-60$ and $40$ mV. The steepness factor was $20$ mV per e-fold change for both DIDS and SITS block. This block of the $K^+$-activated $Cl^-$ conductance is therefore as steep as expected from the movement of a single divalent anion about halfway into the electric field. From the maximal degree of block (measured at $40$ mV) with different drug concentrations, we calculated dissociation constants, $K_d$, for the drug–channel complex assuming a single binding site isotherm with a $K_d$ of $21 \pm 2 \mu M$ ($n = 7$) for DIDS and $72 \pm 3 \mu M$ ($n = 3$) for SITS.

DIDS and SITS blockade increased with depolarization to a maximum level at $40$ mV (Fig. 3C). At potentials above this value the block appears to decrease again. One possible explanation for this result is that block of the $K^+$-activated $Cl^-$ channels with DIDS and SITS is relieved at highly positive potentials. The voltage dependence of the DIDS and SITS block of the $K^+$-activated $Cl^-$ channel as well as the relief of block at positive potentials ($>40$ mV) was similar to that of the volume-sensitive $Cl^-$ conductance in human osteoblasts (this paper, data not shown), in a rat osteoblast-like (ROS 17/2.8) cell line (Gosling et al. 1995) and in lymphocytes (Lewis et al. 1993).

**Ion selectivity**

To obtain information about the selectivity of the $K^+$-induced $Cl^-$ conductance, we activated the channels with a $164-5$ mM $K^+$ solution, varied the external anion species, and measured at the same time changes in the reversal potentials, $E_{\text{rev}}$, during whole-cell recording. Reversal potentials were determined as zero-current potentials. $E_{\text{rev}}$ changed in $\Gamma^-$, $\text{Br}^-$, $\text{NO}_3^-$, and aspartate-containing solutions by $-4.8 \pm 1.6$ mV ($n = 4$), $-1.5 \pm 0.9$ mV ($n = 3$), $6.4 \pm 2.9$ mV ($n = 3$), and $36.7 \pm 2.1$ mV ($n = 3$), respectively (data not shown). From these changes in $E_{\text{rev}}$ the estimated permeability sequence of this anion conductance is $\Gamma^->\text{Br}^-\approx\text{Cl}^-\approx\text{NO}_3^->\text{aspartate}$. This permeability sequence is similar to the permeability sequence described for volume-sensitive $Cl^-$ conductances in other cells (Lewis et al. 1993).

**$K^+$ activation and tonicity**

The simplest explanation for the similarity of the $K^+$-induced $Cl^-$ conductance and the volume-sensitive $Cl^-$ conductance is that there are two different types of stimuli that can activate a single class of $Cl^-$ channels by either

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**Figure 4.** Modulation of the volume-sensitive $Cl^-$ current in C1 cells by a hypotonic $K^+$-rich external solution

A, current was elicited by voltage ramps from $-120$ to $40$ mV within $400$ ms at $10$ s intervals. Current traces shown were measured before and after changing the bath solution from a normal $Na^+$ solution ($Na^+$) to a $75\%$ $Na^+$ solution to a $75\%$ $K^+$-rich solution (normal $Na^+$ and $K^+$ solutions diluted with distilled water). Traces were collected at the times shown in B. $B$, time course of $Cl^-$ current induction by hypotonic and $K^+$-rich external solution. The slope of the ramp current, determined as described in the legend to Fig. 1 was plotted against the absolute time after establishing the whole-cell recording mode. $a$, $b$, $c$, $d$ and $e$ correspond to the traces shown in A.
hypotonic external solutions through cell swelling or by an increase in $[K^+]_o$. To test this hypothesis we first activated the volume-sensitive $Cl^-$ conductance by application of a hypotonic solution (75% normal mammalian Na+ solution) and then changed to a hypotonic K+-rich external solution (75% mammalian K+ solution). The result of such an experiment can be seen in Fig. 4. Hypotonic solutions activate the volume-sensitive $Cl^-$ conductance in cells from the human osteoblast-like cell line, while replacement of Na+ by K+ in the extracellular solution is able to increase this activation.

To find out whether high-K+ solutions can maximally activate the $Cl^-$ conductance or whether both high [K+] and hypotonicity are required for maximal current activation we performed an experiment in which we activated the current with a high-K+ solution (120 mM K+, 40 mM Na+) and then removed the Na+ to make the solution hypotonic. The result of this experiment, shown in Fig. 5, clearly demonstrates that both high [K+] and hypotonicity are required for maximal current activation. This was the case for all three different cell types investigated.

In further experiments we wanted to find out whether the two activation stimuli, hypotonic and K+-rich solutions, can act independently of each other. In that case one would expect that the activation by a K+-rich solution should be independent of the osmolarity of the solution. This, however, is not the case as can be seen in Fig. 6.

In the experiment shown in Fig. 6 we first activated the $Cl^-$ conductance with a 164-5 mM K+ solution (330 mosmol l-1) and then changed the bath solution to the same K+ solution with 50 mM added glucose (380 mosmol l-1). This solution change resulted in an almost complete loss of $Cl^-$ conductance that was induced by the K+ solution indicating that the $Cl^-$ conductance activated by the K+ solution was not independent of the osmolarity of the solution.

The idea of the identity of the K+-induced and the volume-activated $Cl^-$ conductance is further supported by the observation that in about one out of four investigated cells, from a total of eighty cells, we could not induce a $Cl^-$ conductance by the 164-5 mM K+ solution and in most of those non-responders (> 80%) we were also unable to induce the volume-sensitive $Cl^-$ conductance by hypotonic solutions.

**DISCUSSION**

We have characterized a $Cl^-$ conductance in human osteoblasts, in cells from a human osteoblast-like cell line, and in human leukaemic T lymphocytes. In all three cell

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**Figure 5.** Activation of a $Cl^-$ current in Cl cells by either a K+-rich isotonic or a K+-rich hypotonic solution

_A_, current was elicited by voltage ramps from -120 to 40 mV within 400 ms at 10 s intervals. Current traces shown were measured before and after changing the bath solution from a normal Na+ solution (160 mM Na+, 4-5 mM K+) to a K+-rich isotonic solution (120 mM K+, 40 mM Na+) to a K+-rich hypotonic solution (120 mM K+, 0 mM Na+). Traces were collected at the times shown in _B_, time course of $Cl^-$ current induction by the K+-rich isotonic and hypotonic external solution described in _A_. The slope of the ramp current, determined as described in legend to Fig. 1 was plotted against the absolute time after establishing the whole-cell recording mode. _a_, _b_ and _c_ correspond to the traces shown in _A_.

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types the Cl\(^-\) conductance was activated by an increase in [K\(^+\)]\(_o\). Similar Cl\(^-\) channels have been characterized in a number of cell types, including epithelial cells (Botchkin & Matthews, 1993; Anderson et al. 1995), lymphocytes (Lewis et al. 1993), colon cells (Worrell et al. 1989), HeLa cells (Diaz et al. 1993), cardiac cells (Tseng, 1992), and a rat osteoblast-like cell line (Gosling et al. 1995). In all those cells the activation mechanism was thought to occur via cell swelling through hypotonic extracellular or hypertonic intracellular solutions.

Volume-sensitive Cl\(^-\) channels have been implicated as one limb in the regulatory volume changes that follow cell swelling and form part of the mechanism by which osmotically active solutes leave the cell. Here we report a novel activation stimulus of these volume-activated Cl\(^-\) channels: the activation by extracellular K\(^+\). Since the other limb of the regulatory volume decrease (RVD) often involves a K\(^+\) efflux, this activation pathway might provide an additional feedback mechanism after the initial volume activation to keep this channel open and could fine-tune the regulatory volume decrease. This activation mechanism seems to apply to volume-sensitive Cl\(^-\) channels in general since we observe this phenomenon in two different cell types, in human osteoblasts and in human leukaemic T lymphocytes.

Molecular cloning has revealed a variety of Cl\(^-\) channel types, but only two of the cloned Cl\(^-\) channel types, that is CIC-2 and I\(_{Clm}\), show a volume-sensitive activation pattern (Gründer, Thiemann, Pusch, Jentsch, 1992; Paulmichl et al. 1993). Since CIC-2 is inward rectifying, has a selectivity sequence of Cl\(^-\) > Br\(^-\) > I\(^-\), and is not sensitive to block by DIDS, we conclude that our K\(^+\)-induced or volume-sensitive Cl\(^-\) conductance is not CIC-2. The Cl\(^-\) conductance described in this paper more closely resembles I\(_{Clm}\) with regard to the block by DIDS, the selectivity sequence, and the outward rectification (Paulmichl, Wickmann, Ackermann, Peralta & Clapham, 1992). If the two Cl\(^-\) conductances were identical, we would suggest that I\(_{Clm}\) would also be activated by K\(^+\)-rich solutions.

The novel activation stimulus by extracellular potassium of this Cl\(^-\) conductance has not been shown experimentally up until now and sheds new light on the physiological role of this Cl\(^-\) conductance. An opposite activation stimulus, i.e. a Cl\(^-\)-dependent cation conductance activated during cellular shrinkage, has been described in human airway epithelial cells (Chan & Nelson, 1992). In addition, in human epithelial cancer cells (Anderson et al. 1995) a regulation of Cl\(^-\) current activated by cell swelling through divalent cations has been found, but the divalent cations block cell swelling-activated Cl\(^-\) channels in these cells rather than activate them.

The K\(^+\) dependence of the Cl\(^-\) conductance described in this paper provides a means for the regulation of Cl\(^-\) efflux when K\(^+\) accumulates in the extracellular medium through K\(^+\) loss from the same cell. This is the case after the initial activation of this conductance via cell swelling, an increase in K\(^+\) efflux and consequently the efflux of water leading to a reduction in cell volume. Thus, the K\(^+\)-induced Cl\(^-\) conductance seems to have complementary functions in cell volume regulation. In addition, K\(^+\) accumulation in the extracellular medium can occur when cells in the vicinity die or are destroyed and release their intracellular content, mainly K\(^+\), into the extracellular solution as is the case with bone fractures.

The unusual dependence of this Cl\(^-\) conductance on the extracellular monovalent cation species may be a mechanism whereby cells under either osmotic stress or an increase in [K\(^+\)]\(_o\) could control Cl\(^-\) flux thereby fine-tuning regulatory volume decrease. The result also suggests the presence of an external K\(^+\) binding site on the Cl\(^-\) channel that may act as a modulator/activator for this anion conductance.

![Figure 6. Reduction of the K\(^+\)-induced Cl\(^-\) current in C1 cells by hypertonic solutions](image)

After the induction of the Cl\(^-\) current by a 164.5 mM K\(^+\) solution, current was elicited by voltage ramps from -120 to 40 mV within 400 ms at 10 s intervals. Current traces shown were measured after full induction of the current and after changing the bath solution from the K\(^+\) solution (330 mosmol l\(^-\)) to the same solution with 50 mM added glucose (380 mosmol l\(^-\)).


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