

Activation of a Potassium Conductance by Extracellular Alkaline pH in Oocytes of *Xenopus laevis*

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ABSTRACT—Electrophysiological properties of *Xenopus* oocytes exposed to alkaline extracellular pH (pH_o) were investigated by measuring whole-cell currents using the two-electrode voltage-clamp method. Alkaline pH_o (8.5–10.5) elicited an outward current in a pH_o-dependent manner with a concomitant increase in the membrane conductance. This outward-current response was dependent on K⁺ because it was suppressed by a K⁺ channel blocker tetraethylammonium⁺ (20 mM), and the reversal potential of the response was in good agreement with the Nernst equation for K⁺. The response was not affected by pretreatment of oocytes with the acetoxymethyl ester of bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (10 μM), a membrane-permeant intracellular Ca²⁺ chelator, but it was augmented by forskolin (0.4 μM), a stimulant of adenylate cyclase. The outward-current response originates in the oocyte but not in the surrounding follicle cells because the current could still be evoked when follicle cells were removed by collagenase or when gap junctions connecting the oocyte membrane and follicle cells were blocked by 1-octanol (1 mM). It is concluded that the outward current elicited by alkaline pH_o in *Xenopus* oocytes is dependent on the activation of K⁺ channels via the cAMP pathway and that the outward current originates in the oocyte rather than the surrounding follicle cells.

Keywords: *Xenopus* oocyte, pH, Potassium channel, Tetraethylammonium, Forskolin

Oocytes of the South African clawed frog *Xenopus laevis* have been used as one of the indispensable tools for conducting molecular biological studies of ion channels and receptors, since they can synthesize exogenous proteins when injected with foreign RNA or DNA (1, 2). Although electrical recording techniques are useful for assessing the expression of such exogenous ion channels or receptors, the experiments should be carried out with great caution concerning the pH of the solutions used. Oocytes often produce currents in response to pH changes of bathing solutions and perfusates. For example, *Xenopus* oocytes produce inward current responses via activation of Ca²⁺-dependent Cl[−] channels in response to acid solutions (3, 4). Therefore the present work was carried out 1) to examine whether alkaline solution itself can evoke any current responses, and 2) if so, to elucidate the basic properties of *Xenopus* oocytes under alkaline pH conditions.

MATERIALS AND METHODS

Preparation of Xenopus oocytes

Mature oocytes, at stages V and VI, were collected from the ovary of adult frogs of *Xenopus laevis* that were anesthetized by cooling with ice (5, 6). Oocytes were freed from the connective tissue and follicle cell layer with 2 mg/ml collagenase (Type IA; Sigma, St. Louis, MO, USA) in Ca²⁺-free saline for 1.5–2.5 h at room temperature (20–24°C). These defolliculated oocytes were stored at 19°C in modified Barth's solution supplemented with penicillin (100 i.u./ml) and streptomycin (100 μg/ml) before electrophysiological study (5).

Electrophysiology

The conventional two-electrode voltage-clamp method was used to measure whole-cell currents in *Xenopus* oocytes as described previously (5–7). DC resistances of glass microelectrodes, filled with 3 M KCl, ranged between 0.6 and 1.5 MΩ. Voltage and current signals from oocytes were displayed on a chart recorder (SR6335-2L; Graphtec, Yokohama) and simultaneously stored on magnetic tapes using a pulse code modulation data recorder (RD-101T,

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sampling frequency of 48 kHz; TEAC, Tokyo). Experiments were carried out at room temperature (20–24°C).

Solutions

The composition of the extracellular solutions used for experiments was: 80 mM Na⁺, 2.5 mM K⁺, 1 mM Ca²⁺, 1 mM Mg²⁺, 64 mM Cl⁻, 5 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES, pK_a = 6.1), 5 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES, pK_a = 7.5), 5 mM *N*-tris(hydroxymethyl)methyl-3-aminopropane-sulfonic acid (TAPS, pK_a = 8.4), 5 mM 2-(*N*-cyclohexylamino)ethane-sulfonic acid (CHES, pK_a = 9.3) and 5 mM 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS, pK_a = 10.4); all pK_a values are at 25°C. The pH of these solutions was adjusted either to 5.5, 6.5, 7.5, 8.5, 9.5 or 10.5 with membrane-impermeant anion methanesulfonic acid⁻; thus the concentration of Cl⁻ was kept constant in all solutions. The pH 7.5 solution was used as a standard or control solution. Approximate amount of methanesulfonic acid used for titrating the solutions of pH 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5 was 21, 18, 14, 10, 5 and 1 mM, respectively. The pH 5.5 solution required the largest amount of methanesulfonic acid⁻ to adjust the pH and hence the osmolarity of the pH 5.5 solution was approximately 1.15 times that of the pH 10.5 solution due to the addition of methanesulfonic acid⁻. However, methanesulfonic acid⁻ itself did not evoke any appreciable response. Also, an increase in the osmolarity itself did not produce any response; this was confirmed by adding up to 15% sucrose to solutions.

The acetoxymethyl ester of bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA/AM; Calbiochem, La Jolla, CA) and 1-octanol (Sigma) were water-insoluble and they were firstly dissolved in dimethyl sulphoxide (DMSO) and were made up just before application. The final concentration of DMSO was 0.1% (v/v) which had no effect at this concentration. The Ca²⁺ chelator ethylene glycol bis-(β -aminoethylether)-*N,N'*-tetraacetic acid (EGTA) was also obtained from Sigma. All solutions were applied to oocytes by perfusion.

RESULTS

Whole-cell currents evoked by changing pH_o

The two-electrode voltage-clamp method was applied to *Xenopus* oocytes to measure whole-cell currents (5–7). Since the average value of the resting potential measured in the standard extracellular solution (i.e. pH 7.5 solution) was -55.4 ± 8.4 mV (mean \pm S.D., $n = 20$), oocytes were routinely clamped at -60 mV. The input resistance was 2.34 ± 0.69 M Ω ($n = 20$). As shown in Figs. 1A and 2B, an outward current was evoked in a *Xenopus* oocyte when it was exposed to alkaline pH. The amplitude of the outward

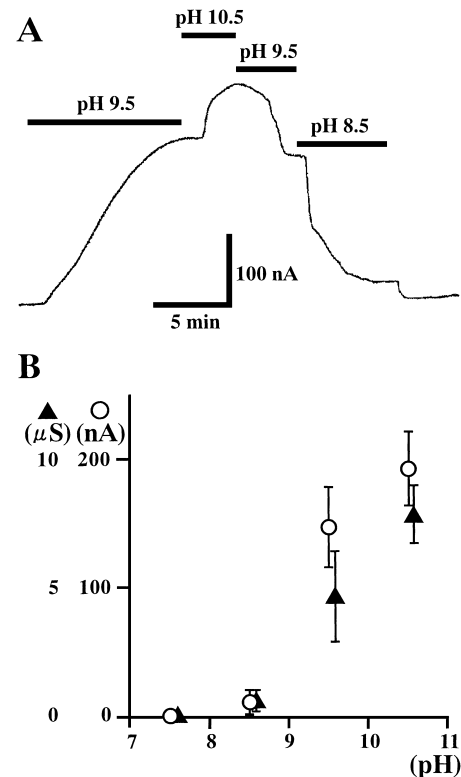


Fig. 1. Responses produced by alkaline extracellular pH (pH_o) solutions in *Xenopus* oocytes. A: Outward-current responses evoked in an oocyte by successively applying different alkaline-pH solutions by perfusion. The two-electrode voltage-clamp method was used to record whole-cell membrane currents, and the holding potential was -60 mV. Outward currents are shown as upward deflections. B: Plots of the peak amplitude of the outward-current (open circles: nA scale) and the increase in the membrane conductance (closed triangles: μ S scale) when pH_o was elevated from control 7.5 to the indicated value by 1 unit pH steps. Attached bars show the S.D. ($n = 5$).

current became larger when the pH of the solution was made more alkaline (Fig. 1A and open circles in the nA scale in Fig. 1B, $n = 5$). The membrane conductance was measured by applying -10 mV pulses of 2–5 s duration at 10–30-s intervals under voltage-clamp conditions, and the difference between the steady-state conductance at a given pH and that at pH 7.5 was plotted against the value of pH (closed triangles in μ S scale in Fig. 1B, $n = 5$). These plots show that the membrane conductance concomitantly increased with the amplitude of the response when pH_o became more alkaline and indicate that the current response occurred in a pH_o-dependent manner. In addition, it was found that the extracellular pH did not have to be alkaline to generate an outward current, i.e., a shift of pH in an alkaline direction could induce a response even though its amplitude is small. For instance, the size of the outward current evoked when pH_o was changed from 5.5 to 7.5 was 13.7 ± 5.5 nA ($n = 12$).

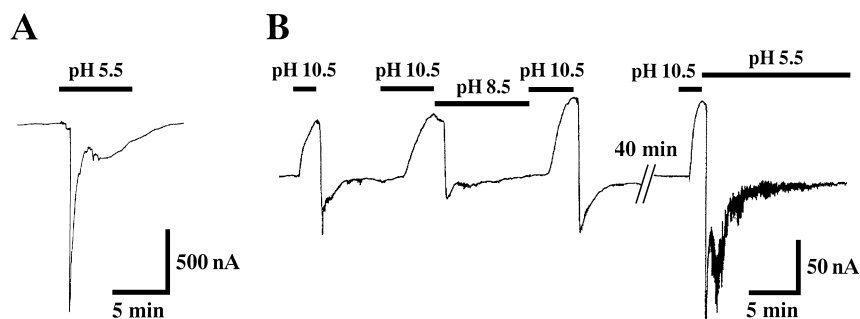


Fig. 2. Whole-cell membrane currents elicited in *Xenopus* oocytes by altering pH_o from control 7.5 to the indicated values shown by horizontal bars. A: An example of a large inward current which was induced when pH_o was changed from control (7.5) to acidic (5.5). B: The amplitude of outward current responses was approximately constant upon repetitive alkaline stimulation. Inward-current responses could be generated when the pH_o shift was in an acidic direction. The last response was obtained after 40-min wash with the control pH 7.5 solution. Parallel bars indicate a break in the record. Outward and inward currents are shown as upward and inward deflections, respectively.

In contrast to the outward current elicited by alkaline pH_o , an inward current was evoked in *Xenopus* oocytes when pH_o was changed from control (7.5) to acidic (5.5–6.5) conditions (Fig. 2A). The inward current consisted of a spike-like fast component followed by a slow component with superimposed inward-current fluctuations. It is to be noted again that the pH_o did not need to be acidic to evoke such an inward current. For example, the oocyte displayed in Fig. 2B generated inward currents in response to alkaline pH (7.5 and 8.5) when the oocyte had been pre-exposed to a more alkaline-pH solution (10.5 in this case). Figure 2B also shows that the inward-current response was larger in amplitude when the oocyte was challenged with a more acidic solution (pH 5.5).

Ionic basis of the alkaline pH_o -evoked current responses

Experiments were carried out to examine the ionic basis of the alkaline pH_o -induced outward current. The oocyte illustrated in Fig. 3 produced an outward current in response to pH 10.5 solution, and it generated a small inward current when the pH of the perfusate was switched to control pH 7.5. After a period of wash with this pH 7.5 solution, the oocyte was exposed to the same alkaline solution supplemented with 20 mM tetraethylammonium⁺

(TEA⁺), a K⁺ channel blocker in various types of cells including *Xenopus* oocytes (5). The amplitude of the outward-current response was considerably smaller than that which was elicited in the absence of TEA⁺. The normalized value of the outward-current response (with TEA⁺ / without TEA⁺) was 0.36 ± 0.14 ($n = 4$). The response recovered when TEA⁺ was washed away (Fig. 3). The reduction in the outward current amplitude in the presence of TEA⁺ cannot be ascribed to desensitization of the response, because no appreciable reduction was observed upon repetitive application of alkaline solution as displayed in Fig. 2B. The results suggest that the outward current response is dependent on the activity of K⁺ channels.

Further supporting data concerning the dependence of the outward current on K⁺ were obtained by measuring the reversal potential of the outward current using the ramp method in different external concentrations of K⁺, i.e., repetitive voltage ramps, 0.8–1.0 s in duration with slopes of 80–120 mV/s, were applied to an oocyte during a response under voltage-clamp conditions (5, 6). The average value of the reversal potential obtained from oocytes bathed in standard extracellular solution was -103.3 ± 9.4 mV ($n = 4$); the membrane potential was changed approximately between -140 and -40 mV. This reversal

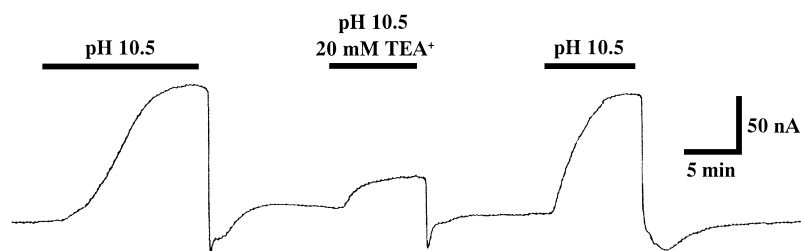


Fig. 3. Inhibition of an alkaline pH_o -evoked outward current response by the K⁺ channel blocker tetraethylammonium⁺ (TEA⁺). The applications of pH 10.5 solution with or without TEA⁺ are indicated by the horizontal bars.

potential is close to the reported equilibrium potential of -100 mV for *Xenopus* oocytes (1). In addition, a depolarizing shift in the membrane potential was observed when the extracellular K^+ concentration was increased from the control 2.5 to 25.0 mM, the average shift was 59.1 ± 8.2 mV ($n = 3$); the membrane potential was altered between -100 and 0 mV. This value of the depolarizing shift agrees well with the expected shift of 58.6 mV based on the Nernst equation at 23°C . These data for reversal potentials of the outward current indicate that the response is dependent on K^+ and that the contribution of other ions is negligible. The contribution of Na^+ to the alkaline pH_o -evoked outward current, e.g., by the Na^+/H^+ exchanger or by the Na^+/Ca^{2+} exchange system, was also negligible because even when Na^+ was totally replaced by Li^+ there was no significant change in the reversal potential for the outward current (-102.7 ± 11.4 mV; $n = 3$) (Mann-Whitney U test, $P < 0.01$).

In contrast, the ionic basis of acid pH_o -evoked inward current was different from that of alkaline pH_o -elicited outward current. The reversal potential of the acid-induced current response was -22.2 ± 6.1 mV ($n = 4$) and this was compatible with the reported value for a Cl^- current in *Xenopus* oocytes (1). The data show that the inward current response was predominantly dependent on Cl^- (7).

Is pH_o -evoked current response dependent on Ca^{2+} ?

In order to examine whether pH_o -elicited ionic currents are dependent on Ca^{2+} , intracellular free Ca^{2+} was chelated by a membrane-permeable Ca^{2+} chelator, BAPTA/AM (8). Extracellularly-applied BAPTA/AM penetrates the cell membrane, is cleaved by cytoplasmic esterases, and becomes free BAPTA which remains trapped inside the cell; this form of BAPTA can efficiently chelate intracellular free Ca^{2+} in *Xenopus* oocytes (6). The acid pH_o -evoked inward current response was greatly suppressed in *Xenopus* oocytes by reducing intracellular free Ca^{2+} ; oocytes were pretreated with BAPTA/AM ($10 \mu\text{M}$) for $2 - 5.5$ h and then exposed to acid-pH and alkaline-pH solutions. A representative example is shown in Fig. 4. This oocyte was pretreated with $10 \mu\text{M}$ BAPTA/AM for 3 h. The initial application of pH 5.5 saline failed to elicit an inward current (see Fig. 2A for comparison). Furthermore, a small inward current was produced when the pH_o was shifted from 10.5 to 5.5 , but it was much smaller than the control inward current response observed in BAPTA/AM-untreated oocytes as shown in Fig. 2B ($n = 4$). In contrast, pretreatment with BAPTA/AM did not significantly affect the alkaline pH_o -evoked outward current response; the amplitude of the outward-current response was 151.3 ± 49.9 nA (Fig. 4, $n = 4$). The data indicate that the acid pH_o -evoked inward-current is dependent on a rise in $[Ca^{2+}]_i$ while the alkaline pH_o -induced outward-

current is not.

Effect of forskolin on the alkaline pH_o -evoked outward current

It has been shown that the outward K^+ current usually occurs via the adenosine 3'5'-cyclic monophosphate pathway (cAMP pathway) in *Xenopus* oocytes (5, 9). This possibility was tested using forskolin which stimulates adenylate cyclase and increases the intracellular concentration of cAMP.

A *Xenopus* oocyte was firstly challenged by alkaline-pH solution (pH 9.5 solution), washed with standard pH 7.5 solution and treated with $1 \mu\text{M}$ forskolin for approximately 15 min. The oocyte was then exposed to alkaline pH solution again (Fig. 5). The current trace obtained from the same oocyte indicates the facilitatory effect of forskolin on the alkaline pH_o -induced outward K^+ current (Fig. 5). The average amplitude of the alkaline pH_o -elicited response in forskolin-pretreated oocytes was 1.48 ± 0.30 times that of control oocytes ($n = 3$), indicating the contribution of the cAMP pathway to the K^+ current.

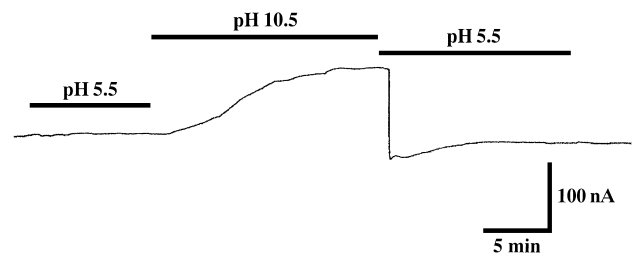


Fig. 4. Effect of the membrane-permeant intracellular Ca^{2+} chelator BAPTA/AM, on the current responses produced by alkaline and acid pH solutions. The oocyte was pretreated with $10 \mu\text{M}$ BAPTA/AM for 3 h.

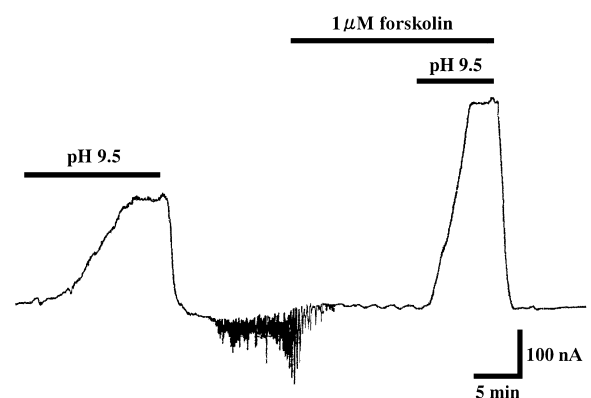


Fig. 5. Augmentation of the alkaline pH_o -induced outward current response by pretreatment with forskolin. A control response evoked by pH 9.5 solution was followed by a larger response recorded from the same oocyte after treatment with $1 \mu\text{M}$ forskolin.

Origin of the alkaline pH_o -induced outward current

The origin of the alkaline pH_o -induced outward current was examined because current occurring in the surrounding follicle cells can be recorded from a *Xenopus* oocyte through the electrical connection made between follicle cells and the oocyte via gap junctions (10). Therefore, surrounding follicle cells were enzymatically removed from oocytes with collagenase prior to experiments (see Materials and Methods). However, it is reported that such "defolliculated" oocytes still possess a small number of follicle cells (10). Thus, collagenase-treated oocytes were further exposed to 1-octanol (1 mM), a blocker of gap junctions between the oocyte and the surrounding follicle cells (5, 11). An outward current response could still be recorded from 1-octanol-treated oocytes (treated for 10–20 min) by changing pH_o from 7.5 to 9.5 (Fig. 6). The mean values of the peak amplitude of the outward-current response were 195.3 ± 29.8 and 187.5 ± 37.5 nA in control and 1-octanol-pretreated oocytes, respectively ($n = 4$). No significant difference was observed between these values ($P < 0.01$). It is concluded that the alkaline pH_o -evoked outward K^+ currents originate in the oocyte and not in the follicle cells.

DISCUSSION

Outward K^+ current elicited by alkaline pH_o

The present work was carried out to examine the physiological properties of oocytes under alkaline pH_o conditions. The results have shown that extracellular alkaline pH induces an outward current that is dependent on K^+ . It was also demonstrated that an outward current could be generated when the pH was shifted in the alkaline direction even at an acid pH, i.e. from pH 5.5 to 7.5. However, the amplitude of the outward current evoked by the 2 unit-pH shift from 5.5 to 7.5 was only 13.7 ± 5.5 nA ($n = 12$), and the value was less than 1/10 of the response induced by the pH change from 7.5 to 9.5 (195.3 ± 29.8 nA, $n = 4$). It is suggested that the absolute alkaline pH value is

more efficient at activating K^+ channels than a shift in the alkaline direction.

Outward currents dependent on K^+ have also been reported in follicular oocytes of *Xenopus* (i.e., oocytes surrounded by follicle cells), activated by various types of ligands such as adenosine (12) and growth hormone-releasing hormone (5). In these cases, K^+ currents were considered to originate not in the oocyte but in the follicle cells, because the K^+ currents were abolished when gap junctions connecting the oocyte and follicle cells were blocked by 1-octanol or when follicle cells were mechanically or enzymatically removed. However, the present study suggests that the alkaline pH_o -induced K^+ currents occur in the oocyte since the currents were not affected by 1-octanol (Fig. 6) or by removing the follicle cell layer. Also the present data suggest that the alkaline pH_o -induced K^+ currents occur via the cAMP pathway because these currents were not dependent on intracellular Ca^{2+} (Fig. 4) but were augmented by pretreatment with forskolin (Fig. 5), which stimulates adenylate cyclase and increases the intracellular concentration of cAMP (5).

The pH of solutions should be checked before electrophysiological experiments

The present work has described results that should be borne in mind when we conduct electrophysiological experiments on *Xenopus* oocytes. When we prepare expensive drugs in tiny volumes of solution, we often omit checking their pH simply because conventional pH meters are not useful for such measurements. It is possible therefore that we may be led to faulty conclusions due to artifacts induced by the altered pH of solutions applied to oocytes. Acidic solution produces inward currents, and alkaline solution generates outward currents. If oocytes showed signs of morphological change when exposed to altered pH, we would notice our mistakes in preparing solutions. Unfortunately, however, the present work has shown that *Xenopus* oocytes can survive great pH ranges (pH 5.5–10.5) without much noticeable change in morphology. An example of misinterpretation of experimental results is the case of GABA_B receptors which were thought to be expressed in *Xenopus* oocytes after the injection of guinea pig cerebral mRNA (3). Inward current responses were obtained when baclofen solution was applied to oocytes. Woodward and Miledi noticed that hydrochloride salts of baclofen caused acidification of the solution and repeated the same experiments after re-adjusting the pH of the baclofen-containing solution to neutral (4). Then, no inward-current responses to baclofen could be observed. It was therefore concluded that the apparent expression of GABA_B receptors by cerebral mRNA might be an artifact caused by the acidification of the solutions and not by baclofen itself. Similarly, the present study has shown

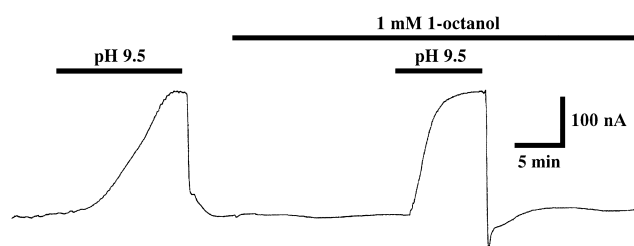


Fig. 6. Effect of 1-octanol, a blocker of gap junctions, on the alkaline pH_o -induced outward current response. No apparent suppression of the response was observed even when an oocyte was treated with 1 mM 1-octanol.

that alkaline pH solution can generate outward K^+ current responses in *Xenopus* oocytes. Thus, the pH of solutions should neither be acidic nor alkaline to analyze genuine effects of drug applications. For this purpose, the pH of solutions must be carefully adjusted using an appropriate compact pH meter which needs only a drop of solution for measurement.

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