Slow calcium oscillations in human spermatozoa

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We have used single-cell imaging to investigate intracellular Ca^{2+} signalling in human spermatozoa stimulated with progesterone ($3 \mu\text{M}$). In approx. 9 % of cells progesterone caused the activation of slow repetitive $[\text{Ca}^{2+}]_i$ (intracellular Ca^{2+} concentration) oscillations, with a period of 1–4 min, which persisted for the duration of recording (20–30 min). Pretreatment with nifedipine, which blocks T- and L-type voltage-operated Ca^{2+} channels in spermatogenic cells, did not modify the characteristics of the oscillations, but reduced the proportion of cells in which they were observed. Stimulation with Bay K 8644 or FPL64176 induced

$[\text{Ca}^{2+}]_i$ oscillations in 5–10 % of cells, but their frequency was low (period, 4–5 min). Application of valinomycin ($1 \mu\text{M}$) to clamp membrane potential at E_K (equilibrium potential for potassium) did not modify activity in oscillating cells, showing that plasma membrane potential and activation of voltage-operated conductances are not involved in the mechanism by which sperm $[\text{Ca}^{2+}]_i$ oscillations are generated.

Key words: channel, fertilization, intracellular calcium concentration ($[\text{Ca}^{2+}]_i$), progesterone, store.

INTRODUCTION

Intracellular Ca^{2+} signalling is of great significance in spermatozoa. There is an extensive literature on $[\text{Ca}^{2+}]_i$ (intracellular Ca^{2+} concentration) signals activated by agonists of the acrosome reaction and, more recently, by identified or putative chemotactic factors [1–3]. In mammalian spermatozoa elevation of $[\text{Ca}^{2+}]_i$ regulates hyperactivation, possibly contributing to ‘sperm-trapping’ in regions of high progesterone concentration, a factor derived from the cumulus cells surrounding the oocyte [4,5]. Though monitoring of $[\text{Ca}^{2+}]_i$ in individual spermatozoa is inherently difficult, there is a growing literature on the nature of single-cell $[\text{Ca}^{2+}]_i$ signals in spermatozoa. In contrast with $[\text{Ca}^{2+}]_i$ signalling in somatic cells, which often involves generation of complex waves and oscillations, recorded signals from spermatozoa have been, in almost every case, ‘simple’ signals generated by Ca^{2+} influx in which the characteristics of the signal are determined by the kinetics of the activation and inactivation of the influx pathway and the Ca^{2+} -clearance mechanisms of the sperm [6]. This may reflect the ‘simple’ nature of spermatozoa, compared with large complex somatic cells. The only detailed descriptions, to date, of complex $[\text{Ca}^{2+}]_i$ signals in spermatozoa have been rapid (3.5 Hz) oscillations of Ca^{2+} in the tail of mouse spermatozoa (associated with flagellar motility [7]) and the recently described rapid saw-tooth-like Ca^{2+} waves induced by speract in the tail of sea urchin spermatozoa, again associated with regulation of tail activity [8]. It appears likely that both of these observations reflect rapid regulation of local plasma-membrane Ca^{2+} -influx pathways and ‘readjustments of local gradients’ [8,9].

Recently Fukami et al. [10] commented that, “on occasions” slow $[\text{Ca}^{2+}]_i$ oscillations were induced in mouse spermatozoa stimulated with high concentrations of progesterone. Here we report that slow $[\text{Ca}^{2+}]_i$ oscillations are generated in human spermatozoa stimulated with progesterone at physiological (micromolar) concentrations and also by other, non-physiological, inducers of Ca^{2+} influx, and show that these oscillations are not generated by regulation of voltage-sensitive Ca^{2+} channels.

Our observations show that human spermatozoa have a cellular machinery capable of generating complex Ca^{2+} signals and suggest the possible involvement of sperm $[\text{Ca}^{2+}]_i$ oscillations in mammalian inter-gamete signalling.

EXPERIMENTAL

Preparation and capacitation of spermatozoa

All donors were recruited at the Birmingham Women's Hospital (U.K. Human Fertilisation and Embryology Authority centre number 0119), in accordance with the Human Fertilisation and Embryology Authority Code of Practice. Human ejaculated spermatozoa were obtained from healthy donors of proven fertility by masturbation. After semen liquefaction (approx. 30 min), motile spermatozoa were harvested by direct swim-up. Briefly, 2 ml of sEBSS (supplemented Earle's balanced salt solution) + 0.3 % BSA was under-layered with 1 ml of liquefied semen in a 15 ml Falcon 2095 tube (Becton Dickinson, Mountain View, CA, U.S.A.). The tube was then incubated at an angle of 45° for 1 h at 37°C under 5 % CO_2 . After 1 h the upper 1.75 ml of medium (containing the motile fraction of spermatozoa) was carefully removed using a sterile pipette. The concentration of the collected spermatozoa was assessed using an improved Neubauer counting chamber and adjusted to 6×10^6 cells/ml with sEBSS + 0.3 % BSA. Aliquots of spermatozoa were capacitated in 200 μl aliquots for confocal microscopy studies of calcium influx for 6 h at 37°C under 5 % CO_2 .

Confocal imaging

Aliquots of capacitated spermatozoa (200 μl) were labelled in the incubator (5 % CO_2 , 37°C) with 15 μM Calcium Green-1 acetoxymethyl ester. After 45 min, the entire aliquot was gently introduced into a purpose-built, perfusable, imaging chamber (volume, 200 μl), the lower surface of which consisted of a ‘1.5’ coverslip (previously coated with 10 % poly-L-lysine solution and

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; E_K , equilibrium potential for potassium; sEBSS, supplemented Earle's balanced salt solution.

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air-dried) for viewing on an inverted microscope. The chamber was then placed in the incubator for a further 30 min. This period of time allowed further labelling and for the spermatozoa to adhere to the coverslip.

After the second labelling period the chamber was placed on the stage of the confocal microscope (Bio-Rad MRC 600 system on Nikon Diaphot inverted) and perfused with at least 10 ml of sEBSS medium at the standard perfusion rate (0.4 ml/min) to remove all traces of extracellular dye. Cells were imaged using a $\times 40$ objective. Fluorescence excitation was from an argon-ion laser filtered at 488 nm narrow bandpass (488DF10). Emitted fluorescence was filtered at 540 nm with a bandpass filter (540DF30). In order to minimize bleaching, intensity of exciting illumination was reduced to 1 % of maximum by a neutral density filter. Perfusion and subsequent experiments were carried out at room temperature ($24 \pm 1^\circ\text{C}$).

In experiments in which an agonist was used, an initial 5 min control period of perfusion with sEBSS was followed by perfusion of the recording chamber with progesterone ($3.2 \mu\text{M}$) or Bay K 8644 ($1 \mu\text{M}$) in sEBSS. Other compounds were also applied in the perfusate after diluting in EBSS to the required final concentration. Progesterone (Sigma, Poole, Dorset, U.K.), Bay K 8644 (a gift from Bayer, Wuppertal, Germany), FPL 64176 (RBI, Natick, MA, U.S.A.) and valinomycin (Sigma) were made up as concentrated stock solutions in DMSO and stored at 4°C . Trial experiments were carried out to observe the time of arrival of coloured dyes at the area scanned by the microscope. Allowance was made for this delay, and the time shown on the Figures as the point of application indicates the estimated time of arrival of progesterone at the cell membrane. Images were captured at intervals of 15 s.

Confocal data processing and analysis

Data was processed offline using Lucida software (Kinetic Imaging, Nottingham, U.K.). Using the computer mouse, an ovoid was drawn around the head of each spermatozoon in the field of view. For each cell the average intensity within the head was obtained for every image in the series (typically 100 images over a 25 min recording period) and plotted against time. To exclude any cells that moved significantly during the experiment, images throughout each series were compared. If the cell had moved such that the defined area no longer contained the entire sperm head, the data from that cell were not included in analysis.

Raw intensity values were imported into Microsoft Excel and normalized using the equation

$$R = [(F - F_{\text{rest}})/F_{\text{rest}}] \times 100 \%$$

where R is normalized fluorescence intensity, F is fluorescence intensity at time t and F_{rest} is the mean of at least 10 determinations of F taken during the control period.

Traces were examined in Excel and sorted by visual inspection to identify those in which oscillations were occurring. Cells were only considered to be oscillating when two or more cycles (or part cycles, depending upon termination of the experiment) could be identified, with a regular amplitude, frequency and shape (allowing for the inherent noise generated by movement of the sperm tail). Frequency of occurrence was defined as the number of cells identified by the criteria stated above, divided by the total number of cells examined.

To assess population effects of agonists and channel blockers, the normalized fluorescence intensity values (R) for each cell were compiled to generate an overall average normalized head fluorescence (R_{tot}) at each time point. The total series of R_{tot}

was then plotted to give the mean normalized response of head fluorescent intensity for that experiment. To assess the effect on individual cells, Excel was used to calculate the mean and 95 % confidence interval of fluorescent intensity of each sperm head for at least 10 images during the control period ($C \pm c$). This was then compared with fluorescence intensity for an appropriate period after application of the drug.

To generate data for mean period of the oscillation for a given treatment, a random sample of the oscillating cells was taken across three or more experiments. The mean period within each selected oscillation train was used to calculate treatment means.

Criteria for acceptance/rejection of data

In some experiments there was a drift in fluorescence intensity, possibly reflecting bleaching of the fluorochrome or loss of dye retention. These experiments/cells were not used. The criteria applied were as described previously [11].

RESULTS

Progesterone-induced $[\text{Ca}^{2+}]_i$ oscillations

In preparations of human spermatozoa that had received no drug or agonist treatments, the majority of cells showed little, if any, variation in $[\text{Ca}^{2+}]_i$, beyond the tendency in a minority of cells for $[\text{Ca}^{2+}]_i$ to increase or decrease slowly. However, after treatment with $3 \mu\text{M}$ progesterone (present from the time of application to the end of the experiment), a product of the cumulus cells [4] which induces biphasic elevation of $[\text{Ca}^{2+}]_i$ in human spermatozoa [11,12], we observed the occurrence of $[\text{Ca}^{2+}]_i$ oscillations. Following the initial transient response (which occurs in $> 90\%$ of cells [11,13]), $8.1 \pm 1.1\%$ of cells ($n = 25$; 5809 cells total) generated a continuing series of transients with a period of 1–9 min (3.61 ± 0.16 ; $n = 100$ cells), the amplitude and duration being similar to, but not identical with, that of the initial response to progesterone (Figure 1). These repeated $[\text{Ca}^{2+}]_i$ rises appeared to be generated in the rear of the head, similarly to the initial progesterone transient. Though the shape of the oscillations varied between cells, they typically included a rising phase of 40 s–1 min duration, followed in some instances by a plateau and then a decay phase lasting 1–2 min (Figure 1). The interval between oscillations varied slightly between cycles and was usually 'flat', though a 'pacemaker' ramp was discernible in some cells (Figure 1). The amplitude of the oscillations in an individual cell was consistent (Figure 1), implying that the calcium rise is quantal or is terminated by a feedback mechanism.

During analysis it became apparent that, in a number of cells, spontaneous oscillations occurred before application of progesterone. In four experiments (969 cells) in which this was analysed in detail, less than 0.5 % of cells were generating slow oscillations before application of any treatment.

Effect of duration of capacitation on generation of $[\text{Ca}^{2+}]_i$ oscillations

The proportion of cells in which oscillations were observed varied greatly between experiments (0–27 %), possibly reflecting differing levels of capacitation. To investigate the possible effect of capacitation on progesterone-induced $[\text{Ca}^{2+}]_i$ oscillations, we investigated their occurrence in cells incubated under capacitating conditions for a prolonged period (20–26 h). After prolonged capacitation, the proportion of cells in which oscillations were generated upon treatment with $3 \mu\text{M}$ progesterone was higher

Figure 1 Progesterone induces slow $[Ca^{2+}]_i$ oscillations in human spermatozoa

Traces in (a) and (b) show time-normalized fluorescence plots for four individual spermatozoa (from two different experiments) loaded with Calcium Green-1. Before stimulation $[Ca^{2+}]_i$ (fluorescence) is stable, but after stimulation with progesterone (arrow in each panel) the $[Ca^{2+}]_i$ transient is followed by a series of oscillations of consistent size and kinetics, which continue for the duration of recording. Period of the cycle can vary between 60–100 s (a) and 300 s or more (b). Occasionally the cycle 'freezes' and then restarts (a, ■). When the interval between oscillations is extended, a 'pacemaker' $[Ca^{2+}]_i$ ramp can sometimes be seen. Insets show cells from the same experiments in which stimulation with progesterone induced a 'stable' sustained response rather than repetitive oscillations. (c) shows a series of pseudo-coloured images of two adjacent Calcium Green-1-labelled cells (see also <http://www.BiochemJ.org/bj/378/bj3780827add.htm>). The cell marked by the blue arrow (blue trace in fluorescence intensity plot) gives a simple biphasic response but the cell marked by the red arrowhead generates a series of $[Ca^{2+}]_i$ oscillations in the rear of the head (red trace in fluorescence intensity plot). Numbers below the images show the time in seconds at which each frame was captured and relate to the time axis on the fluorescence intensity plot. Progesterone was added at 250 s (arrow).

than in cells capacitated for only 6 h before experimentation ($18 \pm 4\%$; $n = 3$; 639 cells).

Effect of modulators of voltage-operated Ca^{2+} channels

Various voltage-operated Ca^{2+} channels are expressed in mammalian spermatozoa [14], including T- and possibly L-type channels [15,16]. To investigate the possible involvement of these channels in the oscillatory response we carried out experiments in the presence of $10 \mu\text{M}$ nifedipine, a blocker of L-type channels which is also extremely potent in blocking the T-type channel of spermatogenic cells [17]. Exposure of human spermatozoa to progesterone in the presence of nifedipine causes a biphasic elevation of $[\text{Ca}^{2+}]_i$, similar to that in non-pretreated cells, though the kinetics of the transient phase are markedly altered [18]. In the presence of $10 \mu\text{M}$ nifedipine, progesterone still caused the generation of $[\text{Ca}^{2+}]_i$ oscillations, and their characteristics (amplitude, kinetics and period) were indistinguishable from those in non-pretreated preparations, suggesting that nifedipine-sensitive channels are not intimately involved in the cyclical mechanism by which the oscillations are generated. However, the frequency of occurrence of oscillating cells was reduced to $4.7 \pm 0.5\%$ ($n = 6$; 1078 cells total; $P < 0.05$), an effect consistent with a contribution of nifedipine-sensitive channels to processes by which $[\text{Ca}^{2+}]_i$ oscillations are triggered, possibly by mediating low levels of passive Ca^{2+} influx. We have described previously a fall in $[\text{Ca}^{2+}]_i$ in human spermatozoa upon application of $10 \mu\text{M}$ nifedipine [18], consistent with such nifedipine-sensitive tonic Ca^{2+} influx. We therefore investigated the effect of enhancing Ca^{2+} influx through such channels using the L-type voltage-operated Ca^{2+} -channel agonists Bay K 8644 and FPL 64176 [19].

Upon application of Bay K 8644 (500 nM) the majority of spermatozoa ($63 \pm 4.5\%$; $n = 6$; 1585 cells total) showed a sustained rise in $[\text{Ca}^{2+}]_i$, the latency of the response varying between cells (Figure 2a). R_{tot} (mean normalized fluorescence of all sperm heads) increased by approx. 10% (Figure 2b). Examination of single-cell records showed that, upon application of Bay K 8644, although most responsive cells showed a sustained rise in $[\text{Ca}^{2+}]_i$ (Figure 2a), within any experiment 5–10% of cells generated slow $[\text{Ca}^{2+}]_i$ oscillations (Figure 3). The period of the oscillations induced by Bay K 8644 was typically 3–5 min (3.57 ± 0.19 ; $n = 50$ cells), similar to the oscillations induced by progesterone, but 'fast' oscillations (period ≤ 2 min) were very rare in Bay K 8644-treated cells. In some cells the $[\text{Ca}^{2+}]_i$ oscillations became disorganized after the first three or four cycles, but in most cases the series of oscillations continued, with no marked change in frequency or amplitude, to the end of the recording period (15–20 min after application of Bay K 8644). Similar results were observed with the non-dihydropyridine L-channel agonist FPL64176 ($1 \mu\text{M}$; results not shown).

Contribution of membrane potential to $[\text{Ca}^{2+}]_i$ oscillations in human spermatozoa

To examine whether cyclical changes in membrane potential might drive the observed oscillations of $[\text{Ca}^{2+}]_i$, possibly by regulating nifedipine-insensitive voltage-operated Ca^{2+} channels, we investigated the effects of clamping sperm membrane potential at E_K (equilibrium potential for potassium) with the K^+ ionophore valinomycin. Valinomycin ($1 \mu\text{M}$) has been shown to shift membrane potential of sperm populations rapidly (within seconds [20]) to E_K and is commonly used for calibration of membrane-potential probes. After stimulating cells with progesterone to establish $[\text{Ca}^{2+}]_i$ oscillation in a population of cells, valinomycin (1 – $10 \mu\text{M}$) was applied in the continuing presence of the agonist.

Figure 2 Bay K 8644 increases $[\text{Ca}^{2+}]_i$ in human spermatozoa

(a) Increase in $[\text{Ca}^{2+}]_i$ in five cells from a population stimulated with 500 nM Bay K 8644 (added at arrow). In these five cells the latency of the rise in $[\text{Ca}^{2+}]_i$ varies from 50 to >150 s after introduction of Bay K 8644 to the imaging chamber. The kinetics of the rise in $[\text{Ca}^{2+}]_i$ also vary markedly between cells. (b) Calculation of the mean normalized fluorescence from all sperm heads in the field of view for this experiment (R_{tot}) gave a smooth rise in $[\text{Ca}^{2+}]_i$, which began within 45 s of exposure to Bay K 8644 (shown by arrow) and saturated after approx. 300 s.

Figure 3 Bay K 8644 induces $[\text{Ca}^{2+}]_i$ oscillations in a subset of spermatozoa

Time-normalized fluorescence ($[\text{Ca}^{2+}]_i$) plots from three individual cells which responded to application of Bay K 8644 (500 nM) with a series of $[\text{Ca}^{2+}]_i$ oscillations similar to those induced by progesterone.

In a small number of cells there was a 'reset' of the cycle (Figure 4b, $\square$) and occasionally oscillation ceased. We also occasionally observed an increase in amplitude of the $[\text{Ca}^{2+}]_i$ peaks after application of valinomycin (Figure 4a, $\blacksquare$). However, in the vast majority of cells no significant effects upon the oscillatory activity

Figure 4 'Clamping' membrane potential with valinomycin does not prevent $[Ca^{2+}]_i$ oscillations

Traces show time-normalized fluorescence ($[Ca^{2+}]_i$) plots from four individual spermatozoa. Application of progesterone (first arrow) causes repeated $[Ca^{2+}]_i$ oscillation. Subsequent application of 1 μ M valinomycin (second arrow), to 'clamp' membrane potential at E_K , fails to arrest ongoing $[Ca^{2+}]_i$ oscillations. Some effect on characteristics of the oscillations can be seen, such as an increase in amplitude (a, $\blacksquare$) and a resetting of the oscillatory rhythm (b, $\square$).

of the cells were observed, the period and amplitude of oscillation being maintained.

DISCUSSION

In the present paper, we describe the generation of slow oscillations (period typically several minutes) of $[Ca^{2+}]_i$ in human spermatozoa. These oscillations are of physiologically significant amplitude (comparable with the transient phase of the progesterone-induced $[Ca^{2+}]_i$ signal, up to 1 μ M $[Ca^{2+}]_i$) and, in most cells, occur cyclically for the duration of recording. Their kinetics are slow, taking up to 1 min to rise to a peak and decaying over 1–2 min, consistent with the global Ca^{2+} -clearance mechanisms recently characterized in mouse spermatozoa by Wennemuth et al. [6].

This is the first description of such activity in human spermatozoa, though there is evidence that such oscillations, activated by high (arguably non-physiological) concentrations of progesterone (100 μ M) occur in mouse spermatozoa [10]. Rapid $[Ca^{2+}]_i$ oscillations associated with motility occur in the tail of mouse [7] and sea urchin [8] spermatozoa. In the sea urchin $[Ca^{2+}]_i$ oscillations are activated by speract and spread to the sperm head [8]. However, these were clearly of a different nature from the oscillations described in the present paper, being briefer, of higher frequency (1–2 orders of magnitude) and originating in the sperm tail. Interestingly, unlike the slow oscillations in human spermatozoa, speract-induced oscillations were blocked by Ca^{2+} -channel antagonists (including dihydropyridines; see below). Progesterone-induced oscillations in human spermatozoa were seen in response to stimulation with 3 μ M progesterone, a concentration present within the follicular fluid, and therefore biologically relevant. Induction of $[Ca^{2+}]_i$ oscillations in human spermatozoa during

gamete interaction is therefore a possibility. However, the frequency of occurrence of $[Ca^{2+}]_i$ oscillations in populations of human spermatozoa was low. In our experiments this averaged less than 10% of cells. Extended periods of incubation under capacitating conditions increased this to 18%, but this was often associated with developing bacterial contamination. This increased occurrence could therefore represent a response to bacterial products rather than to extended incubation under capacitating conditions. Although a role for $[Ca^{2+}]_i$ oscillation *in vivo* is therefore a real possibility, their physiological significance is still an open question and a matter for further study.

With the exception of one description of thapsigargin-mediated mobilization of stored Ca^{2+} in a suspension of cells bathed in 'Ca²⁺-free' medium [21], all reports of $[Ca^{2+}]_i$ signalling in human spermatozoa appear to reflect influx of extracellular Ca^{2+} . Though the characteristics of the oscillations described here are consistent with those of store-mediated oscillations in somatic cells, other mechanisms could generate such signals. In particular, cyclical changes in membrane potential or action potential bursting, leading to cyclical activation of voltage-activated Ca^{2+} -influx pathways, have been described (e.g. [22]), and generation of sperm $[Ca^{2+}]_i$ oscillations in this way is possible. We therefore investigated the possible involvement of membrane potential in the generation of $[Ca^{2+}]_i$ oscillations. Not only did blockade of voltage-operated Ca^{2+} channels with nifedipine fail to prevent $[Ca^{2+}]_i$ oscillations, but application of valinomycin (to 'clamp' membrane potential at E_K) had no significant effect. We conclude that oscillation of membrane potential does not underlie the observed oscillations of $[Ca^{2+}]_i$. Of other potential mechanisms, we consider participation of the putative $[Ca^{2+}]_i$ stores of spermatozoa [21,23,24] to be the strongest candidate.

During our experiments with nifedipine it became apparent that, although the drug does not prevent $[Ca^{2+}]_i$ oscillations or change their characteristics, the frequency of occurrence (as a proportion of cells in the population) was reduced in nifedipine-pretreated populations. Conversely, treatment of human spermatozoa with Bay K 8644 or FPL 64176, agonists of voltage-operated Ca^{2+} channels, induced $[Ca^{2+}]_i$ oscillations. These opposing effects are correlated with a sustained decrease or increase in $[Ca^{2+}]_i$, respectively, almost certainly reflecting tonic effects on plasma-membrane Ca^{2+} channels. It thus appears that modulating Ca^{2+} influx is sufficient to regulate the generation of oscillations. We tentatively suggest that background Ca^{2+} influx is required for continuous 'priming' of the processes by which slow $[Ca^{2+}]_i$ oscillations are generated in spermatozoa. Whether the sustained phase of the progesterone-induced Ca^{2+} influx is solely responsible for progesterone-induced $[Ca^{2+}]_i$ oscillations, or whether there are other factors which also contribute to this effect, cannot be determined from these data. However, it is worthy of note that the maximum frequency of $[Ca^{2+}]_i$ oscillations induced by direct activation of Ca^{2+} influx was generally slower than that induced by the more complex action of progesterone, which may stimulate several signalling pathways [25].

In summary, we have shown that human spermatozoa can generate complex $[Ca^{2+}]_i$ signals in response to activation by an egg-induced factor, that occurrence of these oscillations is regulated, at least in part, by Ca^{2+} influx, but that the cyclical 'injection' of Ca^{2+} into the cytoplasm is not dependent upon regulation of membrane voltage-operated Ca^{2+} channels. In the future, examination of the acrosomal status and motility of the oscillating cells would be interesting. Intriguingly, these observations raise the possibility that, upon gamete fusion, the sperm may not only provide a sperm factor which activates $[Ca^{2+}]_i$ oscillations in the egg [26,27], but may be already oscillating, contributing to triggering of the oocyte $[Ca^{2+}]_i$ signal.

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