Differentiation of Inotropic Action from Chronotropic Action of Sympathetic Agents in Cultured Cells of Chick Embryonic Ventricles

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Abstract The rate of the spontaneous activity of cultured cells from the chick embryonic ventricle was only slightly increased by norepinephrine (NE) or epinephrine (EP), even if it was applied close to the cell. On the other hand, the amplitude of contraction, measured from the shortening of the diameter of the cell aggregate, was clearly augmented by up to 50%. Intracellularly injected cAMP also potentiated contraction. Namely, NE, EP, or cAMP showed positive inotropic action but slight chronotropic action on cultured ventricle cells. NE produced an increase in the action potential amplitude, shortening of action potential, and slight hyperpolarization in 17- to 19-day-old but not in 7- to 9-day-old embryo. If NE was applied to the cells whose plateau potential was inhibited by the previous treatment with nifedipine, NE recovered the plateau level to the control. Ionophoretically injected cAMP also recovered the plateau level once it had been inhibited by nifedipine or verapamil. In some cells, the membrane was transiently depolarized after the end of injection and turned back to the control level. This transient depolarization took place spontaneously again. It was suggested that the Ca^{2+} -induced Ca^{2+} release mechanism from the sarcoplasmic reticulum was involved in the cyclic depolarization.

Key words: cultured heart cells, chick embryo, norepinephrine, epinephrine, iontophoresis of cAMP.

The effects of norepinephrine (NE) and epinephrine (EP) on the spontaneous beating rates of cultured cardiac cells have already been investigated by several authors. But, these previous results are not always consistent with each other. SPERELAKIS and LEHMKUHL (1965) first reported that the cultured embryonic ventricular cells were insensitive to NE, but ERTEL *et al.* (1971) demonstrated the positive chronotropic action of the adrenergic agents. GOSHIMA (1976) found that the cultured heart cells of the embryonic mouse were also insensitive to NE whereas

those of the newborn mouse responded to NE, similar to the adult heart cells. SHIGENOBU *et al.* (1980) described the transient increase in spontaneous beating rate by NE but sustained decrease by dibutyl cyclic AMP (DBcAMP). According to HERMSMEYER and ROBINSON (1977), the cells were sensitive to the autonomic agents if they were applied close to the single cell which was beating spontaneously. They explained that the desensitization of the cells was the principal reason for low effectiveness of NE applied to the bulk solution of culture medium. Furthermore, it was shown that newborn rat cells cultured from sinoatrial node were 100 or 1,000 times as sensitive as those from ventricle (MARVIN *et al.*, 1984). The effect of the sympathetic agents on contraction was only briefly reported by SPERELAKIS (1972, p. 32), and has not been further investigated.

The present work was carried out to investigate the effects of NE or EP on contraction, measured from the edge movement of a cell aggregate (CLUSIN, 1981). The effect of intracellularly injected cAMP was also examined. Spontaneous action potentials were recorded to find the changes in plateau under the influence of the sympathetic agents, because it has been known that NE and EP increase slow inward current in adult cardiac cells (REUTER, 1974; REUTER and SCHOLTZ, 1977; KASS and WIEGERS, 1982; REUTER *et al.*, 1983). It was demonstrated that the ventricular cells exhibited the lasting sensitivity to NE or EP in maintaining the augmented contraction.

METHODS

The procedures for tissue culture of chick embryonic cells and for recording the electrical and mechanical activities were described in the previous paper (TOYOTA and MATSUMURA, 1989). Usually, 7- to 9-day-old embryos were used, because the cells younger than 5 days old were easily damaged during microelectrode insertion. Seventeen- to 19-day-old cells were also used to determine the developmental changes in sensitivity to the sympathetic agents. Norepinephrine hydrochloride, *l*-epinephrine bitartrate, Na salt of DBcAMP, and Na salt of cAMP were supplied from Sigma Chem. Co. NE, EP, and DBcAMP were applied to the cells by two methods. In the steady chamber method, the drug was dissolved in distilled water at the concentration of 10^{-4} – 10^{-5} M and 0.02 ml of the solution was dropped into the medium of 2 ml, distant from the cells from which membrane potential was recorded. In the ejection method, 10^{-4} M solution was poured into a thin glass pipette and was ejected close to the cell aggregate at the rate of $0.5 \mu\text{l/s}$ by hydrostatic pressure from a syringe. During the period for the ejection to be off, the negative pressure was applied to prevent the leakage of the test solution. The second method prevented the cells from being desensitized to NE or EP and was convenient for observation of the reversibility of drug action.

Injection of cAMP was performed following the method of TSIEN *et al.* (1972). The intracellular glass microelectrode was filled with 1.5 M Na-cAMP and was inserted into one of the cells within an aggregate. The negative current of

$1-5 \times 10^{-8}$ A was passed intermittently with a 2-s injection period and an intervening 1-s interval with total duration for 3–8 min. This cAMP electrode was connected either to current source or to an amplifier through an electromagnetic switch and it was provided both to injection and to the membrane potential measurement. Thus, the membrane potential was recorded from the cell into which cAMP was injected. For control, Na salt of adenosine 5'-monophosphate (Sigma Chem. Co.) was injected with the same procedure as cAMP injection, as was described in the paper of TSIEN *et al.* (1972). In early experiments, the membrane potential was simultaneously recorded during cAMP injection with a double-barreled electrode. The results showed that the membrane potential was shifted by more than 50 mV or even 100 mV towards more negative potential, but the spontaneous activities were not completely inhibited meanwhile. At the time of the end of current pass the action potential was generated, namely, anode break excitation. The disadvantage in double-barreled electrode was that the leakage of injection current through barreled wall changed the tip potential of the 3 M KCl electrode and distorted measurement of the injection current.

VOGEL and SPERELAKIS (1981) obtained the value of 0.024 for the transport number of cAMP in free solution. When electrophoretic current of 1×10^{-8} A was passed for 60 s then the total amount of injected cAMP was 1.5×10^{-13} mol. The common size of the cell aggregate was assumed to be a disk of 0.3–0.5 mm in diameter and 0.1 mm in thickness, so the concentration of injected cAMP would be $20-8 \times 10^{-6}$ M. This calculation came from the assumptions that the injected cAMP was not dissociated and that it was distributed uniformly within the cell aggregate. It was probable that injected cAMP diffused to neighboring cells within a cell aggregate through close junctions, as was proved for adult Purkinje fibers (TSIEN and WEINGART, 1976). Therefore, several minutes of injection time would be enough to obtain the effective concentration of the order of 10^{-6} M (FABIATO and FABIATO, 1975, 1978; FLITNEY and SINGH, 1980).

RESULTS

1. *Rhythm and shape of spontaneous action potentials in NE and EP solutions*

The spontaneous activity of 7-day-old embryonic cells continued to beat at 72–116/min at 33°C. In about 80% of these young cells, the action potential had a low amplitude, a smooth plateau, and a distinct pacemaker potential. On the other hand, most of 17- to 19-day-old embryonic cells showed low beating rates of 60–92/min, with the action potentials having a deep diastolic potential, a large overshoot, a marked plateau but a small pacemaker potential. These differences in action potential parameters between 7- to 9-day-old cells and 17- to 19-day-old ones are presented in Table 1. The irregularly beating cells are excluded from the results.

NE changed the rate of the spontaneous activity only slightly. Figure 1 shows a train of action potentials when NE was applied following the ejection method. The rate of the spontaneous activity was 76/min in control and it was increased to

Table 1. Rhythm and shape of action potentials as influenced by NE and EP.

Age (day)	Conc. of NE or EP (10^{-6} M)	Rhythm (min^{-1}) control test ¹⁾	Peak action potential (mV) control test	Time for 1/4 repolarization (ms) control test	Time for 3/4 repolarization (ms) control test
7-9	2.0-5.0	83	96	88	160
	<i>n</i> =5	88	100	94	164
	20-30	85	90	83	149
	<i>n</i> =3	89	93	87	153
	Ejection ²⁾	102	92	79	145
	<i>n</i> =7	109	94	79	140
	Total of ³⁾ the above	92 ± 4.8 98 ± 5.8	93 ± 2.3 95 ± 1.9	83 ± 2.9 85 ± 3.5	151 ± 6.1 151 ± 7.1
*	2.6-5.2	82	90	87	152
	<i>n</i> =3	86	94	87	150
17-19	1.0-2.3	84	101	138	229
	<i>n</i> =5	85	108	141	215
	23	68	114	150	240
	<i>n</i> =1	70	114	150	205
	Ejection	66	104	147	241
	<i>n</i> =6	70	106	148	216
	Total of the above	74 ± 3.8 76 ± 3.3	104 ± 2.3 107 ± 1.5	143 ± 2.2 145 ± 2.9	236 ± 3.8 215 ± 5.7

* shows the results on EP, and others on NE. ¹⁾ All of the values in NE or EP medium are obtained from the same cells as the control cells. ²⁾ In ejection method, the concentration of NE was not determined but supposed to be less than 10^{-5} M. ³⁾ The values are expressed as mean $\pm$ S.E.

85/min 5 min after starting the ejection. The transient increase in the spontaneous activity, as was reported by SHIGENOBU *et al.* (1980), was not observed. Rhythm and shape of action potentials in NE and EP medium is shown in Table 1. The positive chronotropic action on NE or EP was not so clearly observed whether it was applied into the culture medium by the steady chamber method or close to the cell aggregate by the ejection method. Table 1 indicates that NE increased the peak action potential, shortened duration of its tail, and shifted the diastolic potential to hyperpolarized direction in old cells but not in young ones.

If the solution of high concentration of NE was ejected close to the cell so that the concentration around the cells was temporarily more than 10^{-4} M, the maximum diastolic potential was shifted toward less negative by 10-15 mV, as illustrated in Fig. 2. The rate was increased, but it was not certain whether the increase took place as the result of NE action or the change in diastolic potential. After the transient

Fig. 1. Continuous recording of action potentials (lower trace) before and after application of NE. The rate of activities per minute is represented by a tachograph (upper trace). Record A1 is continued by A2. At the time indicated by an arrow, 3×10^{-4} M NE solution was applied so that the final concentration was 3×10^{-6} M. 9-day-old embryo. Vertical calibration is 40 mV and horizontal one is 1 min. 33°C.

Fig. 2. A: continuous recording of action potentials (lower trace) and of diastolic potential level (upper trace). At the arrow, 3.5×10^{-4} M NE was ejected close to the cell aggregate. 9-day-old embryo. Vertical calibrations are 10 mV (upper trace) and 40 mV (lower trace), and horizontal calibration is 1 min. B: B1 and B2 are cathode ray oscilloscope (CRO) recordings of the action potentials marked by triangles in A. 33.5°C.

depolarization period, as ejected NE diffused away, the maximum diastolic potential became gradually more negative than the control. The overshoot potential increased and plateau increased and lengthened (Fig. 2B), but the rate of the spontaneous activity decreased.

2. *Rhythm and shape of spontaneous action potential following cAMP injection*

To investigate whether very little effect of NE on the spontaneous rate originated in catecholamine reception mechanism or in intracellular enzymatic

Fig. 3. A: continuous recordings of a train of action potentials (lower trace) and of diastolic action potentials (upper trace). During interrupted period of potential recording cAMP is intracellularly injected with the iontophoretic current of 2×10^{-8} A. The numbers indicate the duration of injection in seconds. Vertical calibrations are 10 mV and 40 mV, and horizontal calibration is 1 min. B: B1 through B4 are the CRO recordings of action potentials marked by triangles in A. Calibrations are 20 mV and 50 ms. 10-day-old embryo. 32°C .

mechanism, cAMP was directly injected into the cell through the intracellular microelectrode. Figure 3 shows the membrane potential change during spontaneous activities before and after cAMP injection. The rate was 53–54/min throughout the experiment. Careful examination revealed that the maximum diastolic potential was shifted toward hyperpolarized direction by 3–5 mV. This hyperpolarization was small but it was certainly observed after the injection with the iontophoretic currents of $1\text{--}5 \times 10^{-8}$ A lasting for more than 5 min, as is shown in also Fig. 4B. Addition of theophylline at 1 mM was ineffective to modulate the effect of cAMP. The effect of theophylline at higher concentration has not yet been tested.

In 3 out of more than 20 cell aggregates, the membrane was transiently depolarized immediately after the interruption of injection current (Fig. 4). The depolarization lasted for 30 s and was accompanied by the increase in the rate of the spontaneous activity. The transient changes were not observed when the negative current was passed through the microelectrode filled with 2 M $\text{K}_3\text{-citrate}$. Therefore, it is quite certain that the membrane potential change results from the effect of cAMP and not from an artifact due to strong injection current itself. The maximum diastolic potential after recovery was a little more negative than the control. The interesting finding was that the depolarization took place spontaneously again 60 or 90 s after the period of the regular spontaneous activities. The time course of the second depolarization was the same as that of the first one and terminated in 30 s or so. The third depolarization has not been observed as far as examined for 20 cell aggregates.

Fig. 4. A: continuous recordings of a train of action potentials (lower trace) and of diastolic potentials (upper trace). cAMP is intracellularly injected with the current of 4.5×10^{-8} A between interrupted period of potential recording, 170 s between A1 and A2; 45 s between A2 and A3, 70 s between A3 and A4. Vertical calibrations are 10 and 40 mV, and horizontal one is 1 min. B1 is the superimposed CRO recording of action potentials in A1 and A3, and B2 is that in A1 and A4. 9-day-old embryo.

Fig. 5. Contraction of a cell aggregate. 1.0×10^{-4} M NE was ejected close to the cell aggregate during the period between the two arrow marks. Two experiments from different 7-day-old embryos are shown. Horizontal calibration is 10 s. 33.5°C.

3. Effects of NE, EP, and of cAMP on contraction

The contractions were detected as the change in diameter of the cell aggregate (TOYOTA and MATSUMURA, 1989). When NE was applied briefly by ejection method, the contraction increased by 50%, and after ejection was turned off, as the NE solution diffused away, it decreased back to the control (Fig. 5A). The beating rates

Table 2. Potentiation of contraction by sympathetic agents.

Age (day)	Agents	Conc. ($\times 10^{-6}$ M)	Contraction (% of control)
7-8	NE <i>n</i> = 2	0.9-1.0	140
	NE <i>n</i> = 3	Ejection ¹⁾	137
7	EP <i>n</i> = 3	Ejection	130
7-8	DBcAMP <i>n</i> = 3	2-3	133
7,10	cAMP <i>n</i> = 4		145
17	cAMP <i>n</i> = 2		200

¹⁾ In ejection method, the concentration of agents was supposed to be less than 10^{-5} M.

remained at nearly constant value of 108-114/min throughout the action of and recovery from NE. Another result obtained from separate cell aggregate is shown in Fig. 5B. Here, also the amplitude of contraction increased by around 30%, although the beating rate remained nearly unchanged.

The contraction was increased also by EP, if applied by ejection method. The potentiation of contraction was observed by extracellular application of DBcAMP at the concentrations of the order of 10^{-6} M. The action of NE, EP, and DBcAMP on contraction is shown in Table 2.

Intracellular injection of cAMP also potentiated contraction. Figure 6A shows the simultaneous recordings of the contraction and the action potential during injection of cAMP. Edge movement was recorded at the positions 70-100 μ m apart from the injection electrode. Contraction started to increase gradually within 1 min after beginning of injection and reached the steady strength in another 2 min. Meanwhile, the action potential configuration was changed only a little, as was described in the previous section. Figure 6B shows another result of potentiation of contraction during cAMP injection. Here, action potentials are not recorded. The maximum potentiation attained 145% of the control. These results appear also in Table 2. Contrary to the slight effects on the rhythm and shape of the action potentials, the effects on contraction were clearly demonstrated. It is concluded that the adrenergic agents exhibited the inotropic action on ventricular cells more clearly than they did the chronotropic action.

4. Recovery of plateau from treatment with nifedipine

The experiments described in this section were carried out to obtain clear results of actions of sympathetic agents on the spontaneous action potential

Fig. 6. A: contraction and action potential of cell aggregate. cAMP was injected with trains of the iontophoretic current pulses with 3×10^{-8} A strength and 2-s duration interrupted by 1-s interval. The action potential recording was disturbed during injection period. 10-day-old embryo. B: contraction of another cell aggregate. The current was passed in the same way as in A. 17-day-old embryo. Horizontal calibration is 1 min (for A and B) and vertical one is 40 mV (for A only). 32°C .

Fig. 7. A: action potentials in control medium; B: after application of 3×10^{-8} M nifedipine; and C: 5 min after further addition of NE at the concentration of 1.5×10^{-6} M. 19-day-old embryo. Calibrations are 40 mV and 50 ms. 33.5°C .

parameters.

Figure 7 shows the inhibition of plateau in nifedipine solution and the recovery by NE. The plateau level was lowered after addition of 3×10^{-8} M nifedipine. The intervals of the spontaneous activities were unchanged or a little shortened at low concentrations of nifedipine. If NE was applied at the final concentration of 1.5×10^{-6} M, the time course of the action potential including plateau level turned back to the one before nifedipine treatment.

The recovery of the plateau once it had been reduced by nifedipine was also observed by intracellular injection of cAMP, as is shown in Fig. 8. After injection of cAMP with the iontophoretic current of 2×10^{-8} A for 3 min, the shape of the action potential was nearly the same as the control (Fig. 8A-C). Usually, the upper limit of the strength of injection current was 5×10^{-8} A and stronger current pass caused the irreversible membrane depolarization, but a few cells showed resistance

Fig. 8. A: action potentials in control medium; B: 80 s after application of 7×10^{-8} M nifedipine, and C: after iontophoretic injection of cAMP with the injecting current of 4.0×10^{-8} A for 3 min. 17-day-old embryo. Calibrations are 40 mV and 50 ms. 33.5°C. D, E, and F show another series of experiment. D: action potentials in 1×10^{-7} M nifedipine medium. E: after iontophoretic injection of cAMP with the injection current of 4.0×10^{-7} A for 17 s. F: further injection of cAMP for 20 s. 17-day-old embryo. Calibrations are 40 mV and 50 ms. 33.5°C.

to current as strong as 10^{-7} A. The plateau level recovered within 17 s, which, of course, was much shorter than the time for weak injection current (Fig. 8D–F). The recovery of plateau by cAMP injection was observed also for the cells previously treated by verapamil.

DISCUSSION

SPERELAKIS and SHIGENOBU (1972), McDONALD and SACHS (1975), and NATHAN and DEHAAN (1979) investigated the changes in electrical activities during development. Consistent with their results, the present study has shown that the 17- to 19-day-old cells possessed more negative diastolic potential, larger overshoot, longer plateau, and lower rates of activity than the 7- to 9-day-old cells. In addition, our result also shows that the NE sensitivity was developed in old cells.

The effects of NE on the electrical activities of cultured embryonic heart cells have been extensively investigated, but those of other catecholamines such as EP or isoproterenol have not. One of the reasons for the selective usage of NE is to study the developmental change in NE sensitivity of the cells, where sympathetic innervation is not completed (JAMES, 1970; KUPFER *et al.*, 1982). In the present work, the effects of EP or injected cAMP were studied in addition to those of NE.

The spontaneous rate was only slightly increased by NE or EP. This result is consistent with that of SPERELAKIS and LEHMKUHL (1965). The values listed in Table 1 were obtained at the concentrations of 10^{-6} or 10^{-5} M when the agent was applied

by the steady chamber method. These concentrations were 10 times as high as that used by ERTEL *et al.* (1971), who showed that 10^{-7} or 10^{-6} M of NE was most effective, lower or higher concentrations being less effective. In the present study, however, NE or EP at 10^{-7} M order was found to be much less effective than at 10^{-6} or 10^{-5} M in modulating action potential parameters. In the ejection method, the NE or EP pipette was placed 0.5–1.0 mm apart from the cell aggregate and the solution was ejected at the flow rate of 0.5 μ l/s. If the solution was ejected more rapidly and closer to the cells, the fluctuation of culture medium took place and it disturbed the inserted electrode within the cell and might stimulate the cell mechanically which lead to increased beating rate. Moreover, if the NE concentration was raised immediately after the injection, the cells were transiently depolarized (cf. Fig. 2), which also brought about increased rate. This depolarization at high concentration of NE can be explained as the increase in non-selective cation permeability (COLQUHOUN *et al.*, 1981; JOSEPHSON and SPERELAKIS, 1982). Therefore, the desensitization of the cell to NE or EP was not so completely excluded as it was in the work of HERMSMEYER and ROBINSON (1977). This may be one reason why clear chronotropic action is lacking.

The NE receptor process, however, seems not to be of importance in understanding the low effectiveness, because the intracellularly injected cAMP also was not effective to exhibit the chronotropic action. According to CLAY and SHRIER (1981a, b), the decay of K_2^+ outward current contributes to the generation of the pacemaker potential of the cultured embryonic cell and the progressive diminution of this current during development is the origin of the decreased spontaneous rates. This K_2^+ current is now termed i_f in Purkinje fibers and can probably be so termed in embryonic ventricles, and it is activated by EP (DiFRANCESCO, 1985). The low sensitivity to NE or EP can be ascribed at least in part to the immature property of i_f channel. It is also known that the adrenergic innervation starts at the later embryonic stage and is completed after birth (JAMES, 1970). The channel still has not yet fully developed in 17- to 19-day-old embryos, even though they are a little more sensitive to NE than 7- to 9-day-old ones. In sinoatrial node cells, the Ca^{2+} current is involved in pacemaker current (NOMA *et al.*, 1980; HAGIWARA *et al.*, 1988). It is not certain whether Ca^{2+} current, in addition to i_{K_2} of CLAY and SHRIER (1981a) or i_f of DiFRANCESCO (1985), contributes to the pacemaker current of cultured ventricular cells.

All of NE, EP, DBcAMP, and injected cAMP potentiated contraction measured as the shortening of the diameter of a cell aggregate. As was discussed in the previous paper, the contraction was evaluated less quantitatively and might be underestimated. Nevertheless, the contraction was clearly increased by the sympathetic agents. The strength of contraction was inversely proportional to the beating rate (TOYOTA and MATSUMURA, 1989) and therefore, its positive chronotropic action must be independent of the change in beating rate. The potentiated contraction lasted at least 15 min if NE or EP was applied by the steady chamber method. The result means that the ventricular cells do not show the desensitization

to these agents, so far as the inotropic action is considered. It is concluded that the principal action of the sympathetic agents on the ventricular cells is to potentiate contraction rather than to increase the spontaneous beating rate.

It was expected that, since NE, EP, or cAMP increased contraction, the inward Ca^{2+} current must be increased and the plateau level must be elevated by NE. This expectation will be valid although the changes in action potential parameters are only slight. The evidence is that the plateau level is well recovered from the previous treatment with nifedipine or verapamil, indicating activation of the Ca^{2+} current during plateau. The restoration of the plateau by EP after treatment with D600 was also reported by McDONALD and SACHS (1975). Intracellularly injected cAMP also recovered the plateau level from nifedipine or verapamil treatment. It is already known that in adult cells cAMP prolongs the duration of and raises the level of plateau in some kinds of adult cells (TSIEN, 1973; FLITNEY and SINGH, 1980; TRAUTWEIN *et al.*, 1982), and increases Ca^{2+} current of the isolated single cell (OSTERRIDER *et al.*, 1982; REUTER *et al.*, 1983; FISCHMEISTER and HARTZELL, 1987), and that cAMP inhibitors reduce Ca^{2+} current (BKAILEY and SPERELAKIS, 1984). Although Ca^{2+} current is not measured directly, it is supposed that cAMP increases Ca^{2+} current and consequently potentiates contraction.

The interesting result after cAMP injection is that the transient shift of the maximum diastolic potential to the depolarized direction takes place repetitively. The repetitive contractions and relaxations are reported in skinned cardiac and skeletal muscle fibers at relatively low concentrations of free Ca^{2+} and they are increased in frequency and peak tension by cAMP (FABIATO and FABIATO, 1975, 1978). These spontaneously repetitive contractions are considered to reflect intracellular Ca movement between myoplasm and sarcoplasmic reticulum (SR) through Ca^{2+} -induced Ca release mechanism. In the presence of cAMP, more Ca^{2+} is accumulated into SR and more Ca^{2+} is released from a more fully loaded SR. The present results on cyclic depolarization and repolarization show the similar frequency to the transient tension changes. Injection of cAMP into the cells augments Ca uptake by SR and increase Ca contents within SR. The heavily loaded SR by Ca^{2+} will release Ca^{2+} into myoplasm, and the Ca^{2+} -induced Ca release mechanism will be facilitated. The rise of myoplasmic Ca^{2+} concentration activates non-selective cation channel (COLQUHOUN *et al.*, 1981), which results in the depolarization. Increase in inward Na-outward Ca exchange due to a rise of $[\text{Ca}^{2+}]_i$ may be involved in this depolarization.

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