Analysis of the hyperpolarizing effect of catecholamines on canine cardiac Purkinje fibres

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1 The hyperpolarization induced by catecholamines on barium-depolarized (0.2–0.8 mM BaCl) canine cardiac Purkinje fibres, *in vitro*, was studied by use of conventional microelectrode recordings of transmembrane electrical potentials.

2 Noradrenaline, adrenaline and isoprenaline hyperpolarized Purkinje fibres in a concentration-dependent manner from a threshold concentration around 5 nM. The three catecholamines were shown to be approximately equipotent. Tachyphylaxis was observed when the interval between catecholamine applications was less than 15 min.

3 Atenolol (10 μ M) blocked the hyperpolarization reversibly and theophylline (0.5 mM) potentiated it.

4 Tetrodotoxin (5 μ M) did not affect the hyperpolarization induced by isoprenaline. Acetylcholine and histamine, up to 10 μ M, were not effective in hyperpolarizing Purkinje fibres.

5 Low extracellular potassium concentrations (zero and 1 mM) did not affect the hyperpolarization, but high extracellular potassium concentrations (10–20 mM), markedly reduced the effect of isoprenaline (100 nM).

6 Reduction of the extracellular sodium concentration produced a roughly proportional reduction in the isoprenaline-induced hyperpolarization. The hyperpolarization was reversibly blocked in 34 mM sodium Tris-Tyrode solution.

7 The hyperpolarization was not reduced in Tyrode solution containing 0.6 mM calcium, but was drastically reduced in zero-calcium Tyrode solution. This effect was reversible.

8 Addition of verapamil (5–10 μ M) diminished the hyperpolarization, in a concentration-dependent manner. This effect was partially reversed after washing.

9 Ouabain (0.7–1 μ M) significantly reduced the isoprenaline-induced hyperpolarization, but 2,4-dinitrophenol (0.2 mM) did not affect it.

10 Caesium chloride (20 mM) abolished the hyperpolarization. The blockade was only partially reversed upon washing.

11 It is suggested that the hyperpolarization induced by a short exposure to catecholamines is mainly due to an increase in potassium permeability (P_K). A mechanism involving calcium-dependent potassium channels might underlie the increase in P_K .

Introduction

Catecholamines have been shown to affect the resting potential of a great variety of excitable cells *in vitro* in a variable manner. For example, noradrenaline was reported to depolarize sartorius muscle but to hyperpolarize soleus muscle (Horowitz

et al., 1985). Noradrenaline and isoprenaline depolarize sheep cardiac Purkinje fibres (Glitsch & Rasch, 1986; Terris *et al.*, 1986), but hyperpolarize canine Purkinje fibres (Gadsby, 1983) and amphibian sympathetic ganglia (Koketsu & Nakamura, 1976). In those preparations in which catecholamines induce hyperpolarization, there has been a tendency to link

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this effect with a possible stimulation of the sodium-potassium pump (see also Eisner, 1986). Other investigations suggest a catecholamine-induced increase in K^+ conductance as the main factor responsible for the hyperpolarization in cardiac cells (Gadsby 1984, for review) and sympathetic ganglia (Smith, 1984a).

In a previous study (Riccioppo Neto & Sperelakis, 1985) it was found that isoprenaline hyperpolarized embryonic chick heart cells that have been depolarized by high concentrations of local anaesthetics/antiarrhythmic agents. The hyperpolarization was not affected by ouabain ($1 \mu\text{M}$), but was completely blocked by verapamil. The present study analyses the hyperpolarization produced by catecholamines in depolarized (barium-treated) canine Purkinje fibres.

Methods

Adult mongrel dogs (10–15 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} , i.v.), and their hearts were quickly removed through a lateral thoracotomy. Both ventricles were opened, and various pieces of muscle with attached false tendons were dissected and stored in cool oxygenated Tyrode solution. One piece was mounted in a 10 ml tissue chamber with a Sylgard-lined base and perfused with Tyrode solution aerated with 95% O_2 and 5% CO_2 (pH 7.4). The solution was kept at a temperature of $36 \pm 0.5^\circ\text{C}$ and flowed over the preparation at a rate of $6\text{--}7 \text{ ml min}^{-1}$.

The Tyrode solution had the following composition (mM): NaCl 137, CaCl_2 1.8, KCl 4, MgCl_2 0.45, NaHCO_3 12, NaH_2PO_4 0.32 and glucose 5.5. Experiments investigating the influence of various extracellular K^+ concentrations were performed by reducing or increasing the KCl concentration in the Tyrode solution. Low Na^+ solutions were made by isomolar substitution of Na^+ by Tris-HCl, the solutions being aerated with O_2 . Low Ca^{2+} solutions always contained compensating concentrations of MgCl_2 in order to avoid permanent damage to the membrane due to the reduction of divalent charges in the extracellular medium (Siegelbaum & Tsien, 1980). The great majority of experiments were done in Ba^{2+} -depolarized preparations by the addition of a concentration of BaCl_2 (0.2–0.8 mM) sufficient to depolarize Purkinje fibres to a maximum diastolic potential of -62 to -66 mV . In these conditions the fibres fired at a regular rhythm and hyperpolarized more markedly than normally polarized preparations when perfused with catecholamines (see Results). The enhancement of the hyperpolarization after catecholamine addition was the only reason for using BaCl_2 as a depolarizing agent. Ascorbic acid

(0.1 mM) was added to Tyrode solutions containing catecholamines.

Transmembrane potentials were recorded with conventional glass microelectrodes filled with 3 M KCl having resistances of 10–20 M Ω and displayed, via an input capacity preamplifier (Grass P 16) on an oscilloscope (Tektronix 5113). Oscilloscope traces were photographed on 35 mm film with a camera (Nihon-Kohden PC-3A). Pen-recordings were simultaneously made on a servo-driven potentiometer (E.C.B.). Maximum diastolic potentials (MDP) were measured by hand after enlargement of the film ($7\times$). Data are expressed as mean values $\pm$ s.e. mean and comparisons between two means were made by Student's paired t test. P values of less than 0.05 were considered to indicate significant differences.

Drugs used were: acetylcholine chloride (Merck); (–)-adrenaline hydrochloride (Sigma); atenolol hydrochloride (Sigma); caesium chloride (Sigma); 2,4-dinitrophenol (Sigma); histamine hydrochloride (Sigma); (–)-isoprenaline hydrochloride (Sigma); (–)-noradrenaline hydrochloride (Sigma); ouabain octahydrate (Sigma); tetrodotoxin (TTX, Sigma); theophylline (Sigma); verapamil (Knoll). Unless otherwise stated, the results describe the effects of drugs applied during a single stable impalement.

Results

Effects of isoprenaline, adrenaline and noradrenaline on maximum diastolic potential of normally polarized and barium-depolarized Purkinje fibres

Superfusion of canine Purkinje fibres with catecholamines induced hyperpolarization in the great majority of the experiments. The hyperpolarization was more marked in depolarized (barium-treated) than in normally polarized preparations (Figures 1 and 2). Normally polarized and depolarized preparations had MDP of $89.0 \pm 0.9 \text{ mV}$ ($n = 5$) and $63.5 \pm 0.2 \text{ mV}$ ($n = 56$), respectively. The rate of spontaneous discharge was also increased more by catecholamines in depolarized than in normally polarized fibres.

The hyperpolarization was concentration-dependent, attained its peak around 3 min after the beginning of the perfusion and subsided slowly, even in the presence of catecholamines. The development of tachyphylaxis was clearly observed in the first experiments, and a period of 20–25 min of perfusion with drug-free Tyrode solution always separated the addition of catecholamines to avoid this problem.

Isoprenaline (Iso), adrenaline and noradrenaline showed similar potency (Figure 1). In four experiments, atenolol ($10 \mu\text{M}$) blocked reversibly the hyperpolarization induced by adrenaline (500 nM) and

Figure 1 Peak hyperpolarization induced by isoprenaline (Iso) (●), adrenaline (■), noradrenaline (□) on Ba^{2+} -depolarized canine cardiac Purkinje fibres (n between 4 and 8); (▲) effects of Iso on preparations ($n = 3$) previously treated with theophylline (0.5 mM). Open (○): normally polarized preparations ($n = 5$) perfused with Iso. * $P < 0.05$ compared with Ba^{2+} -depolarized, Iso-treated preparations.

during the blockade, adrenaline did not induce any observable effect that could suggest an eventual action on different α -adrenoceptor sub-types. Isoprenaline, perfused for 3 min, was the catecholamine chosen in the following experiments due to its more specific cardiac β_1 -adrenoceptor agonist activity.

In three preparations, prior addition of TTX ($5 \mu\text{M}$) to the Tyrode solution did not affect the isoprenaline-induced hyperpolarization. In two experiments, superfusion of the preparations with a low-chloride Tyrode solution containing Ba^{2+} , also did not affect the hyperpolarization produced by isoprenaline.

As shown in Figure 1, addition of theophylline (0.5 mM) potentiated the effects of Iso. Theophylline by itself had no effect on the maximum diastolic potentials of the fibres.

In five Ba^{2+} -treated preparations, histamine and acetylcholine were tested up to a concentration of $10 \mu\text{M}$ without any observable effect.

Influence of the extracellular K^+ concentration

The hyperpolarizing effect of Iso (100 nM) was studied in nine Ba^{2+} -treated preparations in the pre-

Figure 2 Effects induced by isoprenaline (Iso) perfusion on maximum diastolic potential (a-e) and action potential (1-4) of a spontaneously beating cardiac Purkinje fibre. The preparation was exposed to Iso for periods of only 3 min indicated by the horizontal bars below each trace in the upper panel. In (a), a normally polarized fibre was perfused with two different concentrations of Iso (100 nM and 500 nM) and a time interval of 20 min separated the two records. From (b)-(e) the preparation was depolarized by Ba^{2+} (0.2 mM) and treated with Iso. (b) 5 nM; (c) 10 nM; (d) 100 nM and (e) 500 nM. The pen recording of the upper panel does not reproduce completely the upstroke of the action potential. The lower panel (1-4) shows oscilloscope traces taken in (d) and (e). Time intervals, of 25 min were allowed between Iso additions.

sence of various extracellular K concentrations ($[\text{K}]_o$). Each preparation was tested in control condition ($[\text{K}]_o = 4 \text{ mM}$) and the $[\text{K}]_o$ was either increased ($n = 4$) to 15 mM and then to 20 mM, the effect of Iso being tested in these conditions, or decreased ($n = 5$) to 1 mM and then to 0 mM, followed by the addition of the catecholamine in each condition.

As shown in Figure 3, the hyperpolarizing effects of Iso are similar in control and in low K conditions, but are reduced in high $[\text{K}]_o$.

Effects of solutions of low Na^+ concentrations on the hyperpolarization induced by isoprenaline

The hyperpolarizing effect of Iso on Ba^{2+} -treated Purkinje fibres was examined in 13 preparations superfused with Tyrode solution containing reduced (Tris-substituted) Na^+ concentrations. After control dose-response curves to Iso had been established, the extracellular sodium concentration ($[\text{Na}]_o$) was decreased to 103 mM, 68 mM and 34 mM and new curves obtained in each condition. The MDP changed little after Na^+ substitution (hyperpolarizations of 2-3 mV were found), but the rate of spontaneous discharges decreased gradually and almost disappeared in 34 mM $[\text{Na}]_o$. Multiple impalements were made in this set of experiments.

Figure 3 Effects of isoprenaline (Iso) on maximum diastolic potential (MDP) of Purkinje fibres bathed in Tyrode solution containing different extracellular potassium concentrations ($n = 5$ for $[K]_o = 0$ mm and 1 mm; $n = 4$ for $[K]_o = 15$ mm and 20 mm): (○) controls in a given $[K]_o$; (▲) after addition of Ba^{2+} (0.1–0.2 mM) to the Tyrode solutions; (●) addition of Iso (100 nM) to the Ba^{2+} -treated preparations. * $P < 0.01$ compared with preparations treated with Iso in $[K]_o = 4$ mm.

The hyperpolarizing effect of isoprenaline was gradually reduced in 103 mM and in 68 mM $[Na]_o$ and completely abolished in 34 mM, as shown in Figure 4. These effects were totally reversed after readmission of the normal Tyrode solution (Figure 4).

Influence of solutions of low Ca^{2+} concentrations and of verapamil

The hyperpolarizing effects of Iso in seven barium-treated preparations were compared when obtained in control conditions, after reducing Ca^{2+} to 0.6 mM and after omitting calcium from the Tyrode solution (equimolar amounts of Mg^{2+} were added in each condition). It can be seen from Figure 5 that external Ca^{2+} is necessary for the full development of the hyperpolarization.

The addition of verapamil to the bathing solution also decreased, in a concentration-dependent manner, the hyperpolarization produced by Iso on Ba^{2+} -treated Purkinje fibres ($n = 6$; Figure 6). Verapamil was added at an initial concentration of 5 μM , perfused for 20 min and after testing with Iso, its concentration was increased to 10 μM . The blocking

Figure 4 Effects of low Na^+ solutions on the isoprenaline (Iso)-induced hyperpolarizations in Ba^{2+} -treated cardiac Purkinje fibres: (●) control ($n = 13$); (○) 103 mM $[Na]_o$ ($n = 5$); (□) 68 mM $[Na]_o$ ($n = 5$); (■) 34 mM $[Na]_o$ ($n = 3$); (▲) recovery after readmission of normal Na^+ ($n = 8$).

effect of the higher concentration of verapamil was only partially reversed after 1 h washing in drug-free Tyrode solution (Figure 6). Three preparations treated with verapamil remained depolarized (around -65 mV) even after 1 h washing with verapamil-free, barium-free Tyrode solution. In these preparations, addition of $CaCl_2$ (0.1 ml; 1 M) directly into the perfusion chamber, promptly restored the resting potential (around -85 mV) and induced spontaneous discharges.

Effects of ouabain and of dinitrophenol

Ouabain (0.7–1.0 μM) perfused for 10 min induced arrhythmic activity and sometimes stopped spontaneous discharges in Ba^{2+} -depolarized Purkinje fibres. In these conditions, Iso (100 nM and 500 nM), produced (six preparations) hyperpolarizations of 8.2 ± 1.3 mV and 12.5 ± 2.2 mV, effects different ($P < 0.05$) from the controls 13.8 ± 3.0 and 18.7 ± 2.6 mV. These effects of ouabain were not completely reversed after 1 h washing.

On the other hand, continuous perfusion of dinitrophenol (DNP, 0.2 mM) did not reduce the Iso-induced hyperpolarization. The application of DNP

Figure 5 Effects of low Ca^{2+} Tyrode solutions on the hyperpolarization induced by isoprenaline (Iso) in seven Ba^{2+} -depolarized preparations: (●) control in 1.8 mM Ca^{2+} ; (○) 0.6 mM Ca^{2+} ; (▲) Ca^{2+} omitted from the Tyrode solution; (△) recovery in normal Tyrode solution. * $P = 0.01$ compared to controls in 1.8 mM Ca^{2+} .

Figure 6 Blocking effects of verapamil on the peak hyperpolarization produced by isoprenaline (Iso) in Ba^{2+} -treated Purkinje fibres ($n = 6$): (●) control; (○) verapamil 5 μM ; (▲) 10 μM ; (△) partial recovery after 1 h washing in verapamil-free Tyrode solution. * $P = 0.01$ compared with controls before addition of verapamil.

for 15 min usually stopped the spontaneous discharges in Ba^{2+} -treated Purkinje fibres, but the addition of Iso (50 nM and 100 nM) induced hyperpolarizations (14.6 ± 1.8 mV and 17.3 ± 2.2 mV, respectively) similar to those found in control, barium-treated preparations, and induced electrical rhythmic activity in all four preparations tested.

Blocking effect of caesium

Addition of CsCl (20 mM) to the Tyrode solution depolarized four preparations to around -60 mV. The hyperpolarizations induced by 100 nM and 500 nM Iso were completely inhibited in Cs^+ -treated preparations, and partially recovered their usual amplitudes after 1 h washing in Cs^+ -free Tyrode solution.

Discussion

Adrenaline, noradrenaline and isoprenaline induced a dose-dependent hyperpolarization in canine cardiac Purkinje fibres which was similar, in many respects, to that observed in cells of the canine coronary sinus (Boyden *et al.*, 1983). The effects appeared

in the nanomolar range and their amplitudes reached a maximum of 20–25 mV. Although isoprenaline is generally known as a more potent activator of β -adrenoceptors, the three catecholamines exhibited a somewhat unexpected equipotency in inducing hyperpolarization. Equivalent potencies between them were also found by Boyden *et al.* (1983). Similarly, the half maximal concentrations of isoprenaline and noradrenaline that depolarize sheep cardiac Purkinje fibres are between 1 and 3×10^{-7} M (cf. Glitsch & Rasch, 1986 and Terris *et al.*, 1986). The hyperpolarization was blocked by atenolol and potentiated by theophylline, suggesting that stimulation of β_1 -adrenoceptors and enhanced levels of cyclic AMP are involved in its development. The phenomenon under study exhibits some specificity difficult to explain since histamine (which also enhances cyclic AMP levels in cardiac muscle) and acetylcholine were not able to induce it even at high concentrations. It is not known to what extent the barium concentration used to depolarize the Purkinje fibres might have affected the responses to these agents.

Two main mechanisms (which are not mutually exclusive) have been proposed to explain the hyperpolarization induced by catecholamines on cardiac

tissues: (a) stimulation of the Na^+ - K^+ pump to generate more outward current (Trautwein & Schmidt, 1960; Vassalle & Barnabei, 1971; Wasserstrom *et al.*, 1982; Lee & Vassalle, 1983; Désilets & Baumgarten, 1986); (b) increase in the K^+ permeability (Boyden *et al.*, 1983; Gadsby, 1983). The present experiments, although not decisive in relation to these hypotheses are in favour of a catecholamine-induced increase in K^+ permeability, perhaps involving Ca^{2+} activated K^+ channels.

The results obtained under conditions in which the $[\text{K}]_o$ was varied between zero and 20 mM were not in favour of a catecholamine-induced increase in the electrogenic pump activity. It is well known that removal of external K^+ inhibits the sodium pump activity in cardiac tissues (Deitmer & Ellis, 1978; Eisner & Lederer, 1979; Gadsby & Cranefield, 1979). Zero $[\text{K}]_o$, however, did not reduce Iso-induced hyperpolarization, which agrees with the results of Boyden *et al.* (1983). On the other hand, an increase in $[\text{K}]_o$ up to 20 mM drastically reduced the hyperpolarization produced by Iso but it would not be expected to block electrogenic Na^+ pumping. It should be pointed out that, under no circumstances, did the catecholamine hyperpolarization reach a value more negative than the calculated equilibrium potential for K^+ (E_K); this also argues against a participation of an electrogenic Na^+ pump (Rasmussen *et al.*, 1984; 1986). In isolated myocardial cells from the guinea-pig, it has been shown that depolarization by 20 mM $[\text{K}]_o$ does not reduce electrogenic pumping (Gadsby *et al.*, 1985). However, a reduction in the magnitude of the hyperpolarization would be expected to occur, if it were due to outward K current flowing in conditions of high $[\text{K}]_o$, in which E_K would be less negative.

A decrease in $[\text{Na}]_o$, theoretically, should diminish the thermodynamic load to the sodium pump (Weer, 1984) and, perhaps, potentiate its possible stimulation by catecholamines. The contrary was observed, namely, low $[\text{Na}]_o$ diminished and blocked the hyperpolarization induced by isoprenaline.

The results obtained in low Ca^{2+} solutions suggest that a Ca^{2+} -dependent phenomenon underlies the hyperpolarization induced by catecholamines. Since an inwardly directed Ca^{2+} current would depolarize the fibre, it is reasonable to assume that a K^+ current flowing outward is responsible for the hyperpolarization. It is well established that β -adrenoceptor agonists cause an increase in transmembrane Ca^{2+} currents (see Reuter, 1983 and Tsien, 1983, for reviews). Calcium-dependent K^+ currents have been described in many cell types (see Schwarz & Passow, 1983, for a review) including sheep cardiac Purkinje fibres (Isenberg, 1977a). As with previous observations in embryonic chick

hearts (Riccioppo Neto & Sperelakis, 1985), the slow channel blocker, verapamil, at a high concentration (10 μM), blocked the hyperpolarization evoked by isoprenaline. This finding reinforces the hypothesis of a Ca^{2+} -dependent phenomenon and might help in understanding the puzzling depolarization of different preparations which follows *in vitro* superfusion with verapamil (Rosen *et al.*, 1974; Shigenobu *et al.*, 1974; Wit & Cranefield, 1974).

It could be argued that the outward hyperpolarizing current is also Na^+ -dependent since it was blocked in low sodium solutions. Our results showed that the fast Na^+ channels are not necessarily implicated since the hyperpolarization was not affected by tetrodotoxin. It seems that a certain amount of external sodium is necessary in order to assure proper functioning of voltage-dependent Ca^{2+} channels, since the Ca^{2+} -dependent slow responses induced in depolarized heart muscle by catecholamines and methylxanthines disappeared in low (30 mM) $[\text{Na}]_o$ (Schneider & Sperelakis, 1975). It was speculated that in certain types of Ca^{2+} -conducting channels, Na^+ ions must be bound externally in the membrane for adequate gating. However, studies in dialysed ventricular cells have recently shown that the Ca^{2+} channel current was not significantly changed when Tris replaced external Na^+ (Matsuda & Noma, 1984). A sodium-dependent Iso-activated inward current has been recently described in single guinea-pig ventricular myocytes (Egan *et al.*, 1987). This current was resistant to strophanthidin, and unaffected by caesium, calcium-channel blockers or zero Ca^{2+} . This current also provokes depolarization and is probably different from the one described in the present study. The explanation for the effect of low sodium solutions on the isoprenaline-induced hyperpolarization is therefore not known.

Cardiac glycosides have been shown to abolish the catecholamine-induced increase in cation pumping through the cardiac membrane (Lee & Vassalle, 1983; Pecker *et al.*, 1986), but a short exposure (2 min) to acetylstrophanthidin did not affect the hyperpolarization induced by catecholamines in canine coronary sinus (Boyden *et al.*, 1983) and Purkinje fibres (Gadsby, 1983). Ouabain also did not reduce the Iso-induced hyperpolarization in cultured embryonic chick heart cells (Riccioppo Neto & Sperelakis, 1985). In the present experiments ouabain significantly reduced the hyperpolarization. On the other hand, DNP, a well-known sodium pump inhibitor in cardiac tissues (Kleinfeld *et al.*, 1961), which has been shown to block the hyperpolarization caused by high-frequency stimulation of sheep cardiac Purkinje fibres (and, therefore, assumed to be electrogenic Na^+ extrusion) (Vassalle, 1970), did not reduce the Iso-induced hyperpolar-

ization. A similar finding was obtained with adrenaline and DNP by Trautwein & Schmidt (1960). These discrepancies might have been caused by the so-called 'secondary effects', due to longer exposures of cardiac tissues to ouabain (Aronson *et al.*, 1973), which include: depressed delayed rectification; blockade of the repolarization phase of the action potential (Isenberg & Trautwein, 1974), suppression of slow responses in guinea-pig heart muscle (Schneider & Sperelakis, 1976) and possibly an increase in passive Na^+ permeability (Robbins, 1977). It should be emphasized that any non-specific decrease in membrane resistance under the influence of ouabain, as observed in rat diaphragm muscle fibres (Kuba & Nohmi, 1987), could concomitantly decrease the measured peak of hyperpolarization, without necessarily reflecting reduction in an outward current. On the other hand, as pointed out by Gadsby (1984), pump stimulation by catecholamines might be indirect, due to extracellular K^+ accumulation caused by the increase in K^+ permeability. This hypothetical mechanism has recently received experimental support in relation to the hyperpolarizing effects of acetylcholine on bull-frog atrial muscle fibres (Hasuo *et al.*, 1988). If we assume

that such a mechanism is also contributing to the Iso-induced hyperpolarization, ouabain would be expected partially to reduce this phenomenon, as observed in the present experiments. The contribution of an augmented electrogenic Na^+ extrusion to the observed hyperpolarization is difficult to imply from the present study.

The experiments in which caesium was used to block Ca^{2+} -dependent K^+ channels (Isenberg, 1976; 1977b; Hille, 1986), reinforce the suggestion that the Iso-induced hyperpolarization observed might be caused by an increase in K^+ permeability secondary to a rise in $[\text{Ca}]_i$.

It would be of interest to test other preparations such as nervous tissues, skeletal muscle etc, in which catecholamines have shown hyperpolarizing effects (Smith, 1984b; Kuba & Nohmi, 1987), to determine whether they are also Ca^{2+} -dependent phenomena.

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