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Effects of calcium on the gramicidin A single channel in phosphatidylserine membranes

Screening and blocking

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Abstract. In phosphatidylserine membranes the decrease in the conductance of the gramicidin A single channel caused by calcium is attributed to a reduction of surface potential and to a direct blocking of the pore (Apell et al. 1979).

The aim of this paper is to make a quantitative evaluation of these two effects. We recorded the conductance of gramicidin single channels in 100 m*M* KCl in the presence of different amounts of $CaCl_2$, $MgCl_2$ or TEACl.

The ionic activities at the channel mouth were calculated using the Gouy-Chapman-Stern theory. Our experiments showed that even when the K^+ activity at the channel mouth was estimated to be the same, the single channel conductance was lower if divalent cations were present. This effect is attributed to a blocking action of these ions.

Key words: Single-channels, model membranes, gramicidin, surface potential, phosphatidylserine, calcium

Introduction

In natural and model membranes the movement of ions through a pore does not obey the independence principle because of competition of different ions for binding sites within the channel (see Hille 1984 for a review). Particularly, divalent cations modify ionic fluxes either by specific binding to membrane proteins or by changing the effective ionic concentration at the cell surface (Frankenhäuser and Hodgkin 1957; Rose and Loewenstein 1976; Bamberg and Läuger 1977; Apell et al. 1979; Hamill 1983; Hille 1984; Bers et al. 1985).

The existence of membrane surface potentials, caused by negative charges, gives rise to a counterion distribution which is modified by the presence of small concentrations of divalent cations (McLaughlin et al. 1971, 1981; Bers et al. 1985). Therefore, adsorption properties can be relevant in controlling membrane currents in model and in natural systems.

Ionic pores deeply embedded in the lipid matrix, or extending into the aqueous phase for a short distance, will be particularly sensitive to variations of the ionic environment at the membrane-solution interface (Wilson et al. 1983; Bell and Miller 1984; Moczydlowsky et al. 1985).

To verify the possibility of a simultaneous contribution of blocking and screening mechanisms we investigated the potassium transport properties of the gramicidin A single channel in phosphatidylserine model membranes in the presence of impermeant cations. It is well known that in both natural and model membranes gramicidin A forms ionic pores by a head-to-head association of two monomers. This channel is highly permeable to small monovalent cations while it is impermeable to divalent cations (Urry 1971; Haydon and Hladky 1972; Finkelstein and Andersen 1981).

The length of the dimer has been estimated to be of the order of 26 Å (Urry et al. 1971; Koeppe II et al. 1979) and therefore the pore is deeply embedded in the hydrophobic region of the bilayer. In this channel the presence of charged blocking compounds can affect the transport properties of the gramicidin pore either by a direct interaction with the channel interior or by modifications of the ionic environment at the membrane-solution interface.

Materials and methods

Phosphatidylserine (PS) from beef brain was provided by FIDIA Research Laboratories, Abano Terme (Italy), thanks to the courtesy of Dr. Gunther

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Abbreviations: PS: phosphatidylserine; TEA: tetraethylammonium

Kirschner. Gramicidin A was purified from natural gramicidin (SIGMA Chemical Company, St. Louis, MO) following the method of Koeppe II and Weiss (1981).

Artificial planar bilayers were formed by the technique of Montal and Mueller (1972). PS, previously dissolved in *n*-hexane (4 mg/ml), was spread at the air-water interface of the two sides of a cell divided by a teflon septum. Bilayers were formed on a small hole present in the teflon septum ($\cong 200\,\mu m$ in diameter) by the apposition of the two monolayers.

The water level of the two chambers was raised using a micrometer (Mitutoyo 153-201) connected to two plastic syringes filled with the test solution. A small difference in the water level was previously established by addition of slightly different volumes of the solution. Then the water level was raised simultaneously in the two chambers and the volume difference compensated after membrane formation. This device allowed us to avoid any pretreatment of the teflon septum for the membrane formation.

Experiments were performed at room temperature 24–26 °C. If not otherwise indicated, the standard water solution was 100 m*M* KCl, unbuffered at pH $\simeq$ 6. The Ca^{2+} concentration in nominally Ca^{2+}-free solutions was evaluated to be 10^{-5} *M*, because of impurities in the distilled water and in the salts. The presence of calcium, even at this concentration, was always considered (see for example Table 1) to evaluate the surface potential of the membrane.

To perform the experiments in steady state conditions TEA, or divalent cations, were usually already present in the solution in which the membrane was formed. Nevertheless, in some control experiments $CaCl_2$ was added to the KCl solution after membrane formation and modifications of the channel properties were followed as a function of time.

Electrical measurements were made with Ag/AgCl electrodes connected to a voltage source and to an ultra-low-bias current operational amplifier (Burr Brown 3528) in a virtual ground configuration. The feedback resistance of the amplifier could be chosen in the range $10^7 - 10^{10}\,\Omega$. Higher resistance were used to record current fluctuations due to the opening of ionic channels. A lower resistance ($10^7\,\Omega$) was used to control the formation of the membrane which was monitored by applying a triangular waveform and measuring the capacitive current.

In the absence of gramicidin A typical values of the specific resistance of the membrane were of the order of $10^{11}\,\Omega\,mm^2$. Single channels were obtained by adding small amounts of gramicidin A to the aqueous solution.

The signals were stored automatically (Rauch et al. 1986) and transferred via a serial interface RS232-C to an Olivetti M24 computer for subsequent analysis. A suitable program identified single channel events and measured either the mean life-time or the amplitude of the channels.

Theory

Gramicidin A forms channels which are deeply embedded in the lipid bilayer and do not extend into the surrounding aqueous solution. Therefore, we assume that the concentration of ions at the mouth of the pore is equal to the concentration of free ions at the membrane solution interface, which can be different from the value in the bulk solution. As a consequence, a drop in the concentration of the permeant cation at the membrane interface causes a decrease in the channel conductance.

We used the Gouy-Chapman-Stern theory (McLaughlin 1982) which allows one to determine the membrane surface potential in the presence of monovalent and divalent cations. It accounts for the screening of surface charge by various ions and for their adsorption to the phospholipid polar heads.

The Grahame equation gives the relationship between the surface charge density, σ, and the surface potential, $\psi(0)$:

$$\sigma^2 = 2\,\varepsilon_r\,\varepsilon_0\,RT\sum_i C_i\,[\exp(-z_i\,e\,\psi(0)/kT) - 1]\,, \qquad (1)$$

where ε_r is the dielectric constant of water, ε_0 the free-space permittivity, C_i the concentration in the bulk solution and z_i the valency of ion i, e the elementary charge, T the absolute temperature and k the Boltzmann constant.

The adsorption of cations to the phospholipid molecules is described by the Langmuir isotherm. It is assumed that either monovalent or divalent cations form 1 : 1 complexes with phosphatidylserine polar heads with intrinsic association constant K_1 (Eisenberg et al. 1979) and K_2 (McLaughlin 1982), respectively. The surface charge density (in units of charge/Å^2 will be given by:

$$\sigma = -\frac{\{P^-\}^{tot}\,[1 - K_2\,C_2(0)]}{1 + K_1\,C_1(0) + K_2\,C_2(0)}\,, \qquad (2)$$

where $C_i(0)$ indicates the concentration of different ions (1 for monovalent and 2 for divalent ions) at the membrane-solution interface and $\{P^-\}^{tot}$ is the total surface density of PS.

The surface concentrations ($C_i(0)$) are related to the bulk concentrations (C_i) by the Boltzmann relation:

$$C_i(0) = C_i \exp(-z_i\,e\,\psi(0)/kT)\,. \qquad (3)$$

The combination of Eqs. (1), (2) and (3) is defined as the Stern equation (McLaughlin 1982). The numerical solution of this equation allows one to calculate the cation concentrations at the mouth of the pore, $C_i(0)$.

We used the values $K_1 = 0.15\,M^{-1}$ for potassium, $K_1 = 0.03\,M^{-1}$ for tetraethylammonium, $K_2 = 12\,M^{-1}$ for calcium and $K_2 = 8\,M^{-1}$ for magnesium (Eisenberg et al. 1979; McLaughlin 1982). The total surface density of PS, $\{P^-\}^{tot}$, was assumed to be equal to one phospholipid molecule per 60 Å^2.

The values of $\psi(0)$, the ionic activities at the membrane-solution interface and the experimental values of the single channel conductance, g, are shown in Table 1. We have derived the values of the surface activities by using the expression for the activity coefficients of single ion species reported by Pitzer (1979; see Eq. (89)).

Our evaluation of $\psi(0)$ and the corresponding changes in the presence of added calcium are consistent with the data (for PS membranes) obtained by Alvarez et al. (1983) (see Fig. 7 in their article) under similar conditions.

Table 1. Effects of impermeant cations on the gramicidin A single channel conductance

	KCl = 100 mM				
Ca [M]	$a_K(0)$ [M]	$a_{Ca}(0)$ [M]	$\psi(0)$ [mV]	g [pS]	g/g_{max}
10^{-5}	1.90	$6.1 \cdot 10^{-4}$	−96	20.55∓0.02	1
$5 \cdot 10^{-4}$	0.60	$5.4 \cdot 10^{-3}$	−60	8.08∓0.79	0.39∓0.04
10^{-3}	0.46	$6.9 \cdot 10^{-3}$	−52	5.92∓0.30	0.29∓0.02
$5 \cdot 10^{-3}$	0.24	$1.1 \cdot 10^{-2}$	−33	3.06∓0.07*	0.15∓0.01
Mg [M]	$a_K(0)$ [M]	$a_{Mg}(0)$ [M]	$\psi(0)$ [mV]	g [pS]	g/g_{max}
$5 \cdot 10^{-3}$	0.27	$1.1 \cdot 10^{-2}$	−38	5.82∓0.16	0.28∓0.01
TEA [M]	$a_K(0)$ [M]	$a_{TEA}(0)$ [M]	$\psi(0)$ [mV]	g [pS]	g/g_{max}
0.58	0.53	3.1	−69	15.52∓1.02	0.75∓0.05
0.98	0.36	3.5	−60	12.92∓0.02	0.63∓0.01

The single channel conductance, g, and the corresponding normalized value with respect to the maximum conductance, $g_{max} = 20.55$ pS. The first column reports the bulk concentration of the impermeant cations added to 100 mM KCl. $a(0)$ are the calculated surface activities for the different ions (Pitzer 1979). $\psi(0)$ is the surface potential derived from the Stern equation. The conductance values were derived from the slope of the I–V plot in the linear range. Errors were evaluated by the Student t distribution with a confidence coefficient $P = 99\%$. In the case of 100 mM KCl + 5 mM $CaCl_2$ (*) the conductance refers to the value obtained at $V = 180$ mV

Results

Measurements of the gramicidin A single channel conductance were performed in phosphatidylserine membranes bathing in a symmetric solution containing 100 mM KCl. The mean value of the conductance in the linear I–V range is (20.55 ∓ 0.02) pS. The amplitude histogram is shown in Fig. 1. Increasing the potassium concentration in the bulk solution above 100 mM does not result in an increase of the single channel conductance. Indeed, in the presence of 1 M KCl the conductance is (20.90 ∓ 0.28) pS. Probably because of the large accumulation of potassium at the membrane-water interface. In fact, as shown in Table 1, at 100 mM KCl the surface potassium activity is already 1.9 M.

Modifications of the single channel conductance

The effect of Ca^{2+} on the single channel conductance is illustrated in Figs. 2 and 3. As shown in Fig. 2a

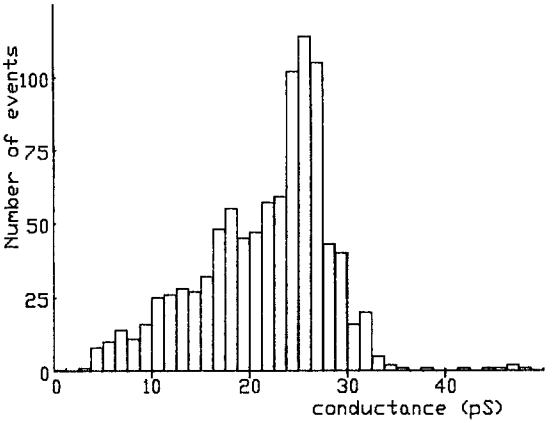

Fig. 1. Conductance histogram of gramicidin A single channels in phosphatidylserine membranes. The distribution (965 events) was obtained in the linear range of the I–V curve. The bathing solution was 100 mM KCl

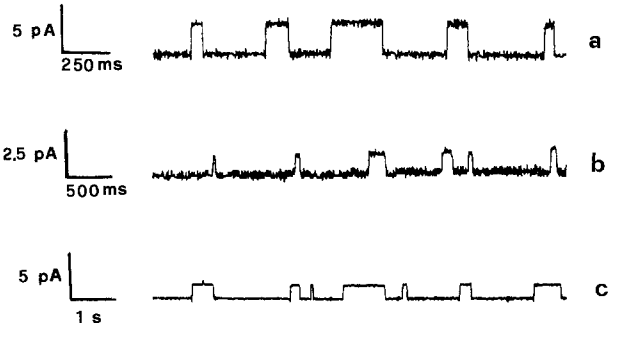

Fig. 2a–c. Typical single channels recorded in: **a** 100 mM KCl solution, **b** 100 mM KCl + 0.5 mM $CaCl_2$ and **c** 100 mM KCl + 980 mM TEACl

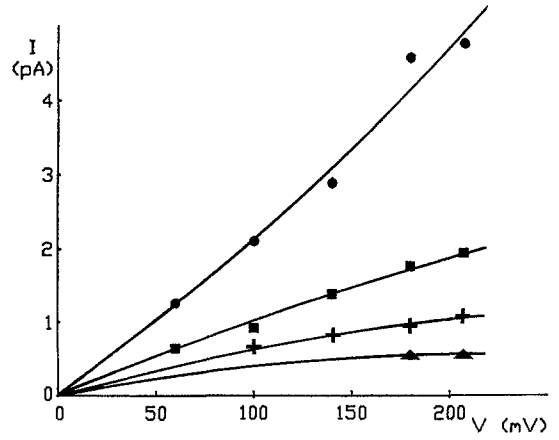

Fig. 3. Current-voltage characteristics for the single channel of gramicidin A in phosphatidylserine membranes. Each *point* is the mean value derived from the current histograms. The aqueous solutions contained: (●) 100 m*M* KCl, 100 m*M* KCl with $CaCl_2$: (■) 0.5 m*M*, (+) 1 m*M*, (▲) 5 m*M*. The *solid curves* were drawn by eye

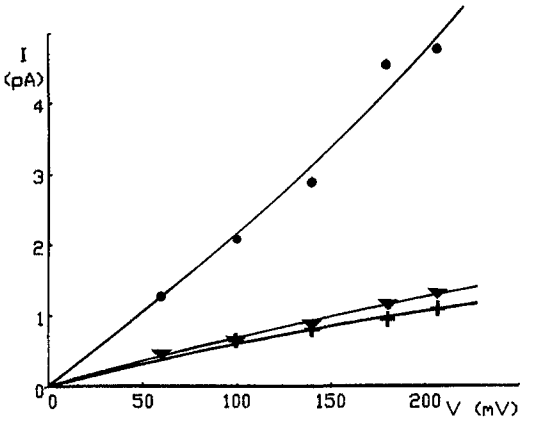

Fig. 4. Current-voltage characteristics for the single channel of gramicidin A in phosphatidylserine membranes. Each *point* is the mean value derived from the current histograms. The aqueous solutions contained: (●) 100 m*M* KCl, 100 m*M* KCl plus (▼) 5 m*M* MgCl or plus (+) 1 m*M* $CaCl_2$. The *solid curves* were drawn by eye

and b the single channel conductance is halved when 0.5 m*M* $CaCl_2$ is added to the bathing solution. Higher concentrations of calcium gave larger reductions (see Fig. 3).

Millimolar concentrations of calcium also modify the shape of the current-voltage characteristics. In 100 m*M* KCl solution the I–V curve is superlinear at higher potentials, whereas the presence of calcium changes the I–V shape from superlinear to linear (0.5 m*M* $CaCl_2$) and sublinear (1 m*M* and 5 m*M* $CaCl_2$) (Fig. 3).

Control experiments were performed by adding calcium to the 100 m*M* KCl solution after membrane formation and in the presence of small amounts of gramicidin A. A progressive reduction in the amplitude and in the frequency of single channel events was clearly observed after the addition of millimolar concentrations of calcium.

To investigate the effect of a different divalent cation on the gramicidin A channel in PS membranes we performed some experiments in a 100 m*M* KCl + 5 m*M* $MgCl_2$ solution. In this case, the conductance reduction is comparable to that obtained in the presence of 1 m*M* $CaCl_2$ while the sublinear trend of the current-voltage characteristic is partially reduced (Fig. 4).

Also the impermeant monovalent cation tetraethylammonium (TEA) causes a decrease in the single channel conductance. The effects due to TEACl (580 m*M* and 980 m*M*) are illustrated in Fig. 5, where we show the current of the single channel as a function of the applied potential (see also Fig. 2c).

The conductance distributions in the presence of TEA (as for Ca and Mg) are always shifted with

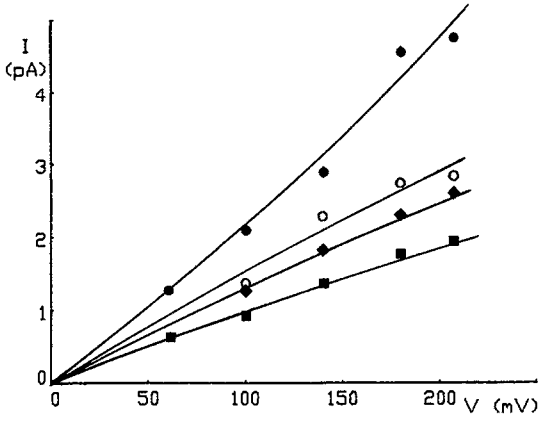

Fig. 5. Current-voltage characteristics for the single channel of gramicidin A in phosphatidylserine membranes. Each *point* is the mean value derived from the current histogram. Aqueous solutions: (●) 100 m*M* KCl, 100 m*M* KCl containing: (■) 0.5 m*M* $CaCl_2$, (○) 580 m*M* TEACl (◆) 980 m*M* TEACl. The *solid curves* were drawn by eye

respect to that obtained in 100 m*M* KCl. In each experiment different populations of normal and reduced channels were not observed.

The mean values of the single channel conductance were derived from the slopes of the I–V characteristics in the linear range (Table 1).

In Fig. 6 we plot the measured single channel conductance as a function of potassium activity at the membrane surface, derived from the Gouy-Chapman-Stern theory (McLaughlin 1982). It is clear that divalent cations give rise to larger reductions of the single channel conductance compared with TEA.

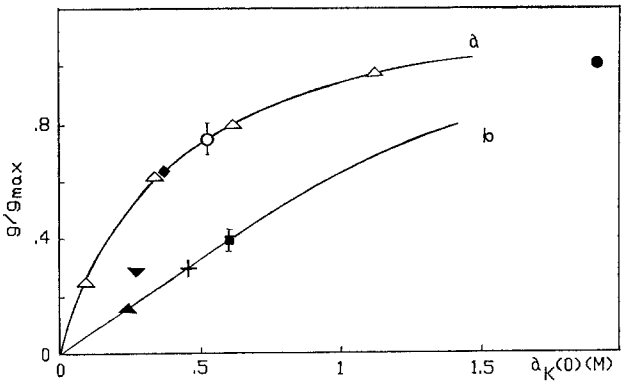

Fig. 6. The single channel conductance versus potassium activity at the membrane-solution interface evaluated by the Gouy-Chapman-Stern theory. The *continuous curve a* was drawn according to Eq. (4) for $K_s = 0.3\,M$. *Curve b* was drawn by eye. *Aqueous solutions:* (●) 100 m*M* KCl, 100 m*M* KCl containing: (○) 580 m*M* TEACl; (◆) 980 m*M* TEACl; (▼) 5 m*M* $MgCl_2$; (■) 0.5 m*M* $CaCl_2$; (+) 1 m*M* $CaCl_2$; (▲) 5 m*M* $CaCl_2$. (△) Experimental data replotted from Hladky and Haydon (1972) and normalized with respect to the maximum values in Fig. 10 of their paper, $g_{max} = 52.1$ pS

Discussion

Small monovalent cations are able to permeate through the pore formed by the gramicidin A dimer (Hladky and Haydon 1972) while the presence of divalent cations such as calcium or magnesium induces a decrease of the single channel current.

In neutral membranes these effects may be explained on the basis of a blocking mechanism of divalent cations (Bamberg and Lauger 1977; Heitz and Gavach 1983).

Nevertheless, mainly in the case of negatively charged membranes, the decrease of the channel current may also be ascribed to a drop in the concentration of the permeant cation at the membrane-solution interface (Apell et al. 1979; Duzgunes and Ohki 1981). In fact, in charged membranes (McLaughlin 1977) the ionic distribution at the membrane-solution interface is different from that in the bulk solution. Different authors (McLaughlin et al. 1971, 1981; Alvarez et al. 1983) report experimental evidence that in PS membranes the addition of divalent cations causes a variation of the surface potential which, according to the Gouy-Chapman-Stern theory (McLaughlin 1982), causes a decrease in the permeant cation concentration at the membrane-solution interface. Since the gramicidin A dimer is deeply embedded in the lipid bilayer, the mouth of the pore experiences ionic concentrations assumed to be the same as that of the non-bound ions at the membrane-solution interface. A decrease in the permeant cation concentration may cause a consequent decrease in the channel conductance.

Therefore in charged membranes a direct interaction of divalent cations with the pore (blocking mechanism) may give only a partial contribution to the observed reduction in the channel conductance.

In order to separate the screening from the blocking effects of divalent cations we screened the surface charge of PS by using another impermeant cation: TEA[1]. Gramicidin single channels were recorded in the presence of 100 m*M* KCl with 580 m*M* or 980 m*M* TEACl. In these conditions the calculated potassium activity at the channel mouth is in the same range as that in the presence of divalent cations. However, the single channel conductance is higher in the presence of TEACl than in the presence of divalent cations. The greater reduction of conductance due to magnesium and calcium is attributed to a blocking action of these ions.

This fact is evident in Fig. 6 where the single channel conductance was plotted as a function of the calculated surface potassium activity. It is also interesting to observe that magnesium is less blocking than calcium.

For potassium activities lower than 1.5 *M*, the conductance of gramicidin A single channels follows a Michaelis-Menten law, i.e.:

$$\frac{g}{g_{max}} = \frac{a_K(0)/K_s}{1 + a_K(0)/K_s} \tag{4}$$

where K_s is the ratio between the dissociation and association constants describing the reaction between the ion and the gramicidin pore, g_{max} is the maximum conductance (20.55 pS in PS membranes) and $a_K(0)$ is the potassium activity at the membrane surface. The continuous curve a in Fig. 6, obtained for $K_s = 0.3\,M$, fits equally well either our data obtained in the presence of TEA or experimental values measured in neutral membranes and replotted from Hladky and Haydon (1972). The reduction in the channel conductance caused by the decrease of the permeant ion activity at the surface of PS membranes is equal to the reduction in the conductance obtained by decreasing the bulk ion activity in neutral membranes. For this reason we consider that under our

1 TEA has long been regarded as being inert for the gramicidin A channel but Eisenman and Sandblom (1983) showed that the effect of this ion depends on its concentration and on the permeant ion concentration. It is possible to consider TEA as "inert" only in certain concentration ranges. As an example in CsCl solution a blocking effect for TEA at 1 *M* is expected only at Cs concentrations lower than 10^{-4} M, while for Cs > 0.3 *M* the conductance of the gramicidin single channel does not change in the presence of 1 *M* TEA.

Since under our experimental conditions the surface concentration of potassium is always > 0.3 *M* we assume that TEA is inert for the gramicidin channel. We will show later that this hypothesis is confirmed by the comparison with experimental data obtained in neutral membranes

experimental conditions the main effect of TEA is to screen the surface charges of the membrane.

At the same potassium activity the lower conductance measured in the presence of millimolar concentrations of calcium is ascribed to a blocking mechanism. As shown in Fig. 6, at $a_K(0) = 0.25\,M$ the conductance is reduced to 15% of the maximum value as a consequence of the addition of calcium (curve b). The screening mechanism (corresponding value in curve a) is responsible for 50% of this effect.

Conclusion

Calcium has a screening and a blocking action on the conductance of the gramicidin channel in PS membranes. We showed the existence of both effects and we describe quantitatively the screening of the surface charge using the Gouy-Chapman-Stern theory.

We think that this analysis may be useful in understanding some mechanisms of ionic transport in biological systems.

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