EFFECTS OF MAGNESIUM AND TEMPERATURE DURING THE RECOVERY PROCESS FROM THE POTASSIUM CONTRACTURE OF THE PREGNANT RAT MYOMETRIUM

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Abstract Effects of external ions (Na, K, Mg) on the recovery from K-contracture of the pregnant rat myometrium were investigated in Ca-free media. Relaxation was faster, when the external concentration of Na and K ions was higher. The time course of relaxation within a limited range of 60–20% of the mechanical output followed a single exponential function. The rate of relaxation, given by the reciprocal of the period during which the contraction decayed to $1/e$, was $0.3\text{--}0.5\text{min}^{-1}$ at $34\text{--}35^\circ\text{C}$ in isotonic Na solution. Low temperature caused prolongation of the relaxation, and the activation energy estimated on the rate of relaxation was $3\text{--}4\text{ kcal}\cdot\text{mol}^{-1}$. Relaxation became faster when Mg ($0.5\text{--}20\text{ mM}$) was added to the relaxation media. Relaxation in isotonic K media was slower than in isotonic Na media, and the addition of Mg caused a slight acceleration of the relaxation. Low temperature caused an acceleration of the relaxation, irrespective of the presence or absence of external Mg ions. In low K media, where K ions were replaced by isosmolar sucrose, addition of Mg caused a significant acceleration of the relaxation. Activation energy ranged between $13\text{--}15\text{ kcal}\cdot\text{mol}^{-1}$ in the solutions containing Mg. This favors the existence of a relaxing system in pregnant rat myometrium that is Mg- and metabolism-linked.

Physiological and pharmacological results reported by previous investigators suggest that the tension development in smooth muscle tissues would be due to the release of Ca, bound to plasma membrane as well as to intracellular organelles, on one hand, and transmembraneous Ca-influx on the other hand. In order to recover from contraction, the operation of the above-mentioned processes in an opposite way may be involved, but little is known about details (DURBIN and

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JENKINSON, 1961; SCHATZMANN, 1961; EDMAN and SCHILD, 1962; URAKAWA and HOLLAND, 1964; HURWITZ *et al.*, 1967; IMAI and TAKEDA, 1969; MAGARIBUCHI *et al.*, 1973; VAN BREEMEN *et al.*, 1973). Na-Ca exchange is a current topic of research (KATASE and TOMITA, 1972; REUTER *et al.*, 1973).

In the present report, effects of external ions, *e.g.*, Na, K, and Mg, on the recovery of the K-contraction of the pregnant rat myometrium were studied. It was found that the presence of Mg ions in the bathing media caused a marked acceleration of the relaxation in a variety of ionic compositions except for high K concentrations. This effect was investigated in some detail with relation to the effects of temperature.

METHODS

Longitudinal strips of about 1 mm in width and 6 mm in length were excised from uteri of about 10–15 days pregnant rats. The preparation was mounted in an organ bath of 0.15 ml capacity. Mechanical activity was transmitted to a mechano-electric transducer (Nihon Kohden, SB-1T), which in turn passed to a potentiometric pen-recorder (National Electronics, VP-652 B). Mechanical records were cut by scissors and superimposed graphically. Intracellular recording of the membrane activity was made by a conventional glass micro-electrode filled with 3 M KCl, and the stimulating apparatus was according to the partition method described by ABE and TOMITA (1968). In these cases, longitudinal strips of 3 mm in width and 7 mm in length were used.

The control bathing solution was a modified Locke Ringer, and other solutions served as contraction-inducing or relaxation-inducing media. The ionic composition chiefly used is given in Table 1. Examples of the abbreviation of contraction and relaxation media are shown in parentheses. All media listed in Table 1 contained 5.5 mM glucose. External Na and K ions were reduced by replacing them with isosmolar sucrose. Reduction of Na less than 8 mM contained as Na-bicarbonate was done by replacement with Tris-Cl. The Ca-free

Table 1. Ionic composition of control Locke solution (1), contraction-inducing media (2), relaxation-inducing media (3; a, b). In parentheses, examples of the abbreviation of the media are illustrated. Concentrations of other constituents are described in METHODS.

	NaCl	NaHCO ₃	KCl	CaCl ₂	KHCO ₃	MgCl ₂ (mM)
1. Control Locke Ringer	154	8	5.6	2.2	—	—
2. Contraction media (K, Ca)	—	—	159.6	2.2	8	—
3. Relaxation media						
a) Na solution	0–161.8	8	—	0–2.2	—	0–20
b) K solution (K, Ca-free)	—	—	0–161.8	—	8	0–20

media were prepared by omitting Ca and adding 1 mM EGTA (GEDTA, Dojin Pharm., Co.), which was titrated by Tris to yield a pH of 7.3. Mg ions were added only to the relaxation media. Because $\log K$ (stability constant) of EGTA is 11.0 for Ca, and 5.2 for Mg, the chelating action should be much smaller on Mg to leave the ion in an ionized form. Solutions were equilibrated with a mixture gas of 3% CO_2 +97% O_2 in reservoirs, and the pH was 7.3. The flow of bathing media was 20 ml/min; therefore, the exchange of the bulk solution in the organ bath should be finished within several sec. The temperature of the bathing media was varied between 36°–21°C by using a thermo-regulating apparatus and was measured in succession by the thermistor thermometer (Nihon Kohden, MGA III). During a set of experiments, the temperature varied within a limit of $\pm 0.3^\circ\text{C}$.

RESULTS

Effects of external Na and K ions on the recovery from the K-contracture

In the isotonic K media containing 2.2 mM Ca, the pregnant rat myometrium undergoes the tonic contraction succeeding the phasic contraction. All of the following results are concerned with the recovery from the K-contracture, which lasted for 5–10 min previously. The tonic contraction was terminated when the preparation was exposed to Ca-free media containing either isotonic Na or isotonic K ions (Fig. 1). When the step of adding Ca was omitted, the contaminating amount of external Ca still affected the results. Therefore, unless otherwise

Fig. 1. The recovery from K contracture in Ca-free (1 mM EGTA added) media. Filled triangle and circle indicate the time when the isotonic Na or isotonic K solutions was applied. Records were superimposed graphically, and the K-contracture in the other trial was omitted. Note that faster sweep of pen-writing paper was started about 20 sec before the exposure to the relaxation media. This applies to the following records. Dashed line represents the resting tension level. Unless otherwise described, temperature was 35°C in the present and following experiments.

described, 1 mM EGTA was further added to Ca-free, K media in the present and following experiments. The time sequence of relaxation in Ca-free isotonic Na solution differed from that in isotonic K solution by the preceding phasic contraction. However, about 3 min after exposing the tissue to Ca-free media, the relaxation occurred at a faster rate in isotonic Na solution. When the time course of the recovery was plotted on a logarithmic scale against the period of the ex-

Fig. 2. Semilogarithmic presentation of the time course of the recovery (ordinate) against the exposure period to Ca-free isotonic K or isotonic Na media (abscissa). The relations were derived from the results shown in Fig. 1. Dashed lines represent the extrapolation of exponentials.

Fig. 3. Effects of external K concentration on the relaxation. Filled circle indicates the time when the application of Ca-free media containing various concentrations of external K was started. Numerical figures to the right represent the K concentrations in mM. Dashed lines from top to bottom are the resting tension levels for the records d to a.

posure to the relaxation media, the relation in the Na solution was linear in the range between 1.5–0.3 g of the mechanical output (Fig. 2). The relation in the Ca-free, K solution was more complicated in the present result, apparently manifesting three exponentials.

The effects of external K concentration on the relaxation was investigated by using the Ca-free solution, in which K concentration was reduced by replacement with isosmolar sucrose (Fig. 3). The effects of lowering K were biphasic, one being the development of phasic contraction during the earlier period of exposure to low K media, the other the prolongation of relaxation.

Effects of Na, Mg ions and temperature

The relaxation in isotonic Na media was remarkably affected by external application of Mg or Ca ions (Fig. 4). The relaxation was the slowest in Ca-free media (a) containing 0 mM Mg, and it was accelerated when those divalent cations were present in the relaxation media. The results are plotted on a semilogarithmic scale and shown in the graph. The curves of the recovery in either medium are not linear in the whole range. They were possibly composed of two exponentials, fast and slow ones during earlier and later periods, respectively. Concerning the fast exponential, which covered the range of 1.9–0.3 g of mechanical output, the rate of relaxation was slower in the following order: Ca-free (a) < 2.2 mM Ca-containing (d) < 9 mM Mg-containing media (c). For other curves, the rate of relaxation in 3 mM Mg-containing media (b) nearly equalled d and that in 2.2 mM

Fig. 4. Effects of Mg and Ca application on the recovery in the isotonic Na solution. For curves a, b, and c, Ca was omitted and 1 mM EGTA was added in relaxation media. 2.2 mM Ca was present in cases of d and e, EGTA being absent. The time course is plotted on a semilogarithmic scale in the right graph. a, relation in Ca-free, Mg-free media; d, in 2.2 mM Ca containing Mg-free media; c, Ca-free, 9 mM Mg containing media. An example of K-contraction is given in d.

Ca plus 9 mM Mg (e), c. It is furthermore indicated that Mg depressed the phasic contraction. In two other experiments, it was found that Mg as low as 0.1 mM was effective in causing the acceleration of the relaxation, and the effect persisted until the Mg concentration was increased up to 20 mM.

The membrane was depolarized to -30 mV in Ca-free isotonic Na solution, and it was repolarized when Mg (0.1–9 mM) was applied (Fig. 5). The amount of repolarization, underlying the decrease in the membrane conductance, was larger when the Mg concentration was higher. Therefore, the present result is in accord with the general concept of Mg action in the membrane stabilization.

The effects of external Na concentrations on the relaxation investigated in the experiment are shown in Fig. 6. The relaxation media contained 2.2 mM Mg in place of Ca. The effects of low Na were again biphasic, *i.e.*, the generation of phasic contraction and the prolongation of the relaxation. As to the phasic contraction, it was preceded by a transient relaxation in a high range of Na concen-

Fig. 5. Effects of Mg application (0.3, 1, 9 mM) to the isotonic Na, Ca-free solution on the membrane potential and the electrotonic potentials produced in the longitudinal muscle cells by the external current application. Microelectrode was located 0.3 mm away from the stimulating electrode, and the records were from an identical cell. Responses were recorded after about 5 min equilibration in the test solutions. The membrane potential in Ca-free, Mg-free media was -30 mV. The graph below shows the V-I relation, the intensity of stimulating current being given in arbitrary units.

Fig. 6. Effects of external Na concentration on the recovery in Ca-free media. 3 mM Mg was contained in the relaxation media. a, response in the isotonic Na solution; b-d, those in lower Na concentration, being shown to the right.

Fig. 7. Semilogarithmic presentation of the effects of external Na concentration on the recovery in Ca-free media plotted against the exposure period to relaxation media. For the linear part of the relation (extrapolated by dashed lines), τ_e , which denotes the time during which the mechanical response decayed to $1/e$, was calculated and rate of relaxation ($1/\tau_e$) was deduced. The rates are plotted against the external Na concentrations (lower graph).

tration (a, b, c). The magnitude as well as the duration of phasic contractions were a function of the external Na concentration. Taking the present results together with those shown in Figs. 1 and 3, it is suggested that the removal of K (and Na) ions was in some way responsible for the generation of the phasic contraction in Ca-free media. The phasic contraction in the present experiment was possibly devoid of spike activity (OSA, 1973).

The results shown in Fig. 6 are plotted in Fig. 7 on a semilogarithmic scale against the exposure period in relaxation media. The relations followed a single exponential function in the range between 2–1.4 g of mechanical responses. The rate of relaxation was tentatively defined by the reciprocal of τ_e , τ_e being the time during which the mechanical response fitting the earlier exponential decayed to $1/e$. The rate of relaxation is definitely related to the external Na concentrations.

The effects of temperature on the relaxation in Ca-free isotonic Na media studied in the experiment are shown in Fig. 8. The rate of the relaxation is shown in the Arrhenius plot, which shows a linear relation against $1/T$. The activation energy is estimated to be $3.2 \text{ kcal} \cdot \text{mol}^{-1}$. Another finding was the enhancement of phasic contraction in the relaxation media with lower temperature. In contrast, temperature affected remarkably the time course of relaxation in isotonic Na media containing 3.0 mM Mg (Fig. 9). The Arrhenius plot (graph) gives an activation energy of $14.3 \text{ kcal} \cdot \text{mol}^{-1}$, Q_{10} being 2.3. The effect of Mg and temperature was essentially the same in the low Na media, where Na ions were replaced by isosmolar sucrose. Activation energy varied between $13\text{--}15 \text{ kcal} \cdot \text{mol}^{-1}$.

Fig. 8. Effects of temperature on the relaxation in Ca-free, isotonic Na solution. The rates of relaxation ($1/\tau_e$) are shown in an Arrhenius plot (lower graph). Ordinate: logarithmic plot of the rate of relaxation. Abscissa: reciprocal of the absolute temperature.

Fig. 9. Effects of temperature on the relaxation in Ca-free media containing isotonic Na and 3 mM Mg. Temperature: a, 35.5; b, 31.2; c, 29.5; d, 25.6; e, 21.7 (°C). Graph to the right shows the Arrhenius plot of the rates of relaxation. Results from the same preparation.

Fig. 10. Effects of temperature on the recovery from K-contracture in the isotonic Na solution containing 2.2 mM Ca (A). Effects of lowering temperature on the spontaneous mechanical activity are shown in lower records (B). Note that the gain was halved in B.

Strong dependency on temperature was also observed in the relaxation process in isotonic Na solution containing 2.2 mM Ca (Fig. 10A). In the Arrhenius plot, the linear relation is disrupted at 22°C, showing a smaller rate against $1/T$. In the linear part of the relation, the activation energy is estimated to be $7.7 \text{ kcal} \cdot \text{mol}^{-1}$. In the same preparation, the spontaneous mechanical activity in the control Locke solution was increased, and the base line tension interpolated between the periodic contractions was elevated when the temperature was lowered (B). When the bathing solution was rewarmed up to 34.5°C, spontaneous activity ceased and it took several tens of min for the tissue to resume spontaneous contractions. Those events in the normal Locke solution were possibly due to the operation of the electrogenic Na pump, which has been proposed to exist in rat myometrium (TAYLOR *et al.*, 1970).

Effects of K, Mg ions and temperature

The relaxation of pregnant rat myometrium in Ca-free isotonic K media manifested a peculiar dependency on temperature (Fig. 11). The effect of Mg application was also different in isotonic K media (b) from that in isotonic Na media. In the first place, lowering the temperature generally caused an acceleration of the relaxation (a). The relation in terms of temperature was essentially the same, when Mg ions were present in the relaxation media (b). The faster relaxation process during the early period was abolished in the presence of Mg ions, which is most typically seen in the cases at 34°C (b vs. a). When plotted in semilogarithmic scale, the rate of relaxation during the later stage was increased only a little when Mg ions were present. The rate of relaxation was the fastest at 22°C in either the absence or presence of Mg ions. Phasic contractions were caused by low temperatures in accordance with the results shown in Fig. 8.

The effect of external K concentrations on the relaxation became complicated when Mg was applied simultaneously (Fig. 12a). The rate of relaxation was the

Fig. 11. Effects of temperature and Mg ions on the relaxation in the Ca-free media containing isotonic K. For records in a, Mg was not contained in the relaxation media, and 3 mM Mg was contained for records in b.

fastest when the K concentration was the lowest (8 mM). However, the situation was different when the relaxation media was cooled down to 22.5°C (b). The relation between the rate of relaxation and the external K concentration is graphically presented in Fig. 13. At 22.5°C, the rate of relaxation showed a smaller dependence on the external K concentration. At 34.5°C, the rate of relaxation was the largest in 8 mM K solution. In contrast, low temperatures caused prolongation of relaxation in low K media when Mg was absent in the bathing media.

Fig. 12. Effect of temperature and external K concentration on the recovery in the Ca-free media containing 3 mM Mg. Results at two different temperatures: 34.5°C for a and 22.5°C for b.

Fig. 13. Relation between the rate of relaxation and the external K concentrations at two different temperatures. Rates of relaxation were derived from the results shown in Fig. 12.

The relations obviously resulted from the combined effects of the relaxation of the accelerating effect of Mg, which was depressed in higher K concentration (Fig. 11) and the accelerating effect of K ions, which became dominant when the temperature was low (Figs. 3, 11). The use of Mg ions in the present experiments was sometimes to eliminate the time, and to prevent a change in the mechanical property during the prolonged exposure to artificial saline media (Fig. 6). Since external ion species possibly interact with each other in the processes of tension development and relaxation, further experiments are required to formulate a quantitative interrelationship.

DISCUSSION

On the assumption that tension development corresponds to an increase in intracellular free Ca ions, the phasic contractions evoked upon withdrawal of external K ions (Figs. 3, 4, 6) were most probably due to the release of Ca bound to the surface membrane. This is because the contractions were produced in Ca-free media and the magnitude was highly dependent on the ionic composition of the bathing media. On the other hand, because the phasic contraction in response to lowering temperature was less dependent on the external ionic composition (Figs. 8, 12), the Ca source responsible for that seems to originate possibly in other cellular structures, *e.g.*, the intracellular organelles. Since detailed analysis was not made in the present experiments, the nature of the phasic contraction in the present context cannot be further examined, and the following discussion will be confined to the relaxation process.

Not only the mobilization of Ca from and to the membrane, but also the movement of the ions across the membrane are subject to the membrane potential (see for review, EBASHI and ENDO, 1968). There is an indication in the present results suggesting that relaxation occurred faster in the external ionic composition, where the membrane potential was more inside-negative. For example, relaxation was faster when divalent cations were added in Na-containing solution (*cf.*, Fig. 8). Faster relaxation is likely a contradictory event from the membrane stabilization, when one assumes that the relaxation is due to diffusion process. Slow relaxation by Mg application as observed for the fast relaxation process in Ca-free isotonic K solution (Fig. 11) was a single exception which favors the above concept. On the other hand, the relaxation also occurred faster in solutions which contained higher K and Na ions (Figs. 2, 6). No doubt, the membrane potential is dependent on the K gradient across the membrane, and the membrane of pregnant rat uterus is more inside-negative when external the Na is reduced (CASTEELS and KURIYAMA, 1965; MARSHALL, 1963). Because the causal relationship between the rate of relaxation and ionic composition which in turn determines membrane potential seems indefinite at present, the effects of ions and temperature on the relaxation is approximated to occur under the direct influence of ions on the intracellular Ca regulation. The relaxation in Ca-free media containing isotonic Na had low

dependency on temperature (Fig. 8), and this indicates the physical process. It is very possible that Na-coupled Ca extrusion operated in the media containing Na ions (Figs. 6, 7). On the other hand, external Na was not a prerequisite for the relaxation (*cf.*, guinea pig taenia coli, KATASE and TOMITA, 1972; rabbit aorta, REUTER *et al.*, 1973). Probably several systems work for the recovery of K-contracture in pregnant myometrium, and they are complementary in bringing about relaxation. It was suggested from the flux measurement that intracellular active-Ca accumulation by certain cellular structures, such as the vesicles close to the membrane, removes Ca from the cytoplasm of rat uterine smooth muscle (VAN BREEMEN *et al.*, 1966). This process is said to be ATP-dependent (VAN BREEMEN *et al.*, 1973), and much morphological background has been offered in a variety of smooth muscle tissues (DEVINE *et al.*, 1972). The loss of ^{45}Ca out of the ileal longitudinal muscle of guinea pig became faster in the media containing higher Ca concentrations. Furthermore, it was shown that the exchangeability of intracellular Ca was lower when external Mg was omitted (LÜLLMANN and SIEGFRIEDT, 1968). It would be of interest if these events signify that the Ca extrusion was under the control of the relaxing system coupled with external divalent cations.

Temperature mainly affects the enzymatic activity related to the contractile activity. It was shown in the present experiment that the accelerating effect of Mg ions on the relaxation is characterized by strong dependency on the temperature (Figs. 9, 12), the activation energy being estimated to be 13–15 kcal·mol⁻¹, a value close to that reported for the binding of Ca ions by isolated sarcoplasmic reticulum (INESI and WATANABE, 1967). It has been demonstrated that the Ca binding to sarcoplasmic vesicles, which utilizes ATP, needs Mg ions contained in incubation media (EBASHI, 1960; HASSELBACH and MAKINOSE, 1961). Therefore, it is highly probable that Mg ions were involved in a relaxing system of pregnant rat myometrium linked to the metabolism. However, it seems peculiar that the system was easily affected by external application of Mg ions. The change in the ionic composition of the bathing media may cause an alteration of the intracellular ionic composition in a parallel way, possibly because of the small surface-volume ratio of smooth muscle cells, hence an amount of intracellular Mg concentration was attained. On the other hand, external K ions appear to depress the accelerating effect of Mg (Fig. 12). Therefore, an alternative site of Mg action would be, strange as it may seem, at the outer surface of a cell membrane. In line with this speculation, the interference between Mg and K ions may be parallel to the competition between the ionic species in the Mg activated ATP-ase of skeletal microsomal vesicle (CARVALHO and LEO, 1967). Concerning this, the relative affinities such as $\text{Ca} \lesssim \text{Mg} \gg \text{K} > \text{Na}$ for the membrane sites have been determined in the guinea pig taenia coli, which led GOODFORD and WOLOWYK (1972) to emphasize a counter-cation hypothesis for the plasma membrane surface.

Low temperature accelerated the relaxation in Ca-free isotonic K-media (Fig.

12), whereas it caused prolongation of the relaxation in low K media. Therefore, it should be said that an amount of external K ions or the consequent change in membrane potential is a prerequisite for the acceleration of the relaxation by lowering the temperature. It might be speculated that the effect of low temperature in high K media is due to the removal of metabolic support in maintaining the role of membrane as the diffusion barrier to ions.

In line with the above speculations, processes related to the elimination of intracellular-free Ca could be distinguished as follows: 1, Na-coupled Ca extrusion, which is not linked to metabolism; 2, Mg-linked Ca extrusion, linked to metabolism; 3, membrane-limited diffusion when external K concentration is high and the external Ca concentration is extremely low (Fig. 11), which may be affected by metabolic change. The former two would be physiologically significant. Of the artificial saline media for mammals, the lack of Mg ions in the Locke Ringer is one of the characteristics of this solution. It is very probable that the Mg action in the relaxation process and in the membrane stabilization can partially or wholly be taking place by Ca ions which are normally contained (Figs. 4, 5). When an experimental condition such as the use of Ca-free media in the mechanical investigation is considered, the neglect of the role of Mg ions leads to an omission of a factor among relaxing systems.

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