AN ANALYSIS OF THE INOTROPIC EFFECT OF CALCIUM AND MANGANESE IONS ON GUINEA-PIG ATRIUM

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It is well known that in the cardiac muscle the amount of uptake of Ca ions depends on external Ca concentration and on the electrically driven rate of the preparation (1-5). These Ca ions taken up are considered to play an important role as a direct or indirect activator of the contractile system of the muscle (6, 7).

According to Lüttgau and Niedergerke (8) and Niedergerke (2, 3), the external Ca ions may be carried into the heart cells in combination with a hypothetical receptor site or molecule, R. This RCa complex, which may be in close equilibrium with external Ca concentration, appears to move inward, when the membrane is depolarized, so as to initiate the contraction.

In the present report, the relationship between external Ca concentration and contractile force in guinea-pig atria driven electrically was analyzed with the formulation of the above scheme in the kinetic equations originated by Michaelis and Menten (9).

Furthermore, the mode of the modification of the above relationship by Mn ions was analyzed in terms of the same kinetic models.

METHODS

Guinea-pigs weighing 250 to 350 g were sacrificed by a blow on the head. The left atrium was isolated and mounted between a pair of silverchlorided silver wire electrodes of 7 mm length, a part of which was attached lightly to the preparation. The atrium with its mounting assembly was placed in a 15 ml bath containing the standard Tyrode solution from which phosphate buffer was excluded to avoid precipitation of Mn ions.

Composition of the solution had the following figures in mM: NaCl, 137; KCl, 2.7; MgCl2, 1.0; CaCl2, 1.8; NaHCO3, 14.9 and glucose, 5.6. The solution was aerated with a mixture of 95% O2 and 5% CO2, and maintained at 30°C. One of the tissue-mounting wires was pulled to give a resting tension of approx. 0.75 g to the tissue and was connected to a force-displacement transducer (Nihon-koden), the output of which was displayed on an ink-writing oscillograph for recording of isometric contraction. Preparations were equilibrated in the bath for 30-60 min.

During this period, stimulation was given at the rate of either 1, 2 or 3/sec by means

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of rectangular pulses of 3 msec duration of about twice threshold intensity.

Following this period, the standard Tyrode solution was replaced with a low Ca Tyrode solution (0.6 or 0.9 mM), so as to begin a series of experiments.

The concentrated solution of CaCl₂ was added to the bath at 10-min intervals, so as to increase stepwise the final Ca concentration in the bath at the following levels (mM): 0.6, (0.9), 1.8, (2.4), 3.3, 4.5, 5.4, 6.6, 7.6 and 8.6. The concentrated solution of MnCl₂ was added in the same way as the above to the bathing solution with 0.6, 1.8, 3.3, 5.4 or 7.6 mM Ca ions, making the final Mn concentrations (mM) of 0.1, 0.3, 0.6, 1.0, (3.0, 6.0, 10.0). Figures in parenthesis were adopted only for a minority. No correction was made for the changes of osmotic pressure. The measurement of contractile magnitude was made 10 min after the change of Ca and Mn concentrations, i.e., immediately before the subsequent introduction of the test solution. These can be regarded as approx. their steady-state values, since in preliminary experiments, the difference in contractile magnitude 15 to 20 min after the application of the test solution relative to that 10 min after the application was found to be insignificant (mean value ± S.E.: 0.997 ± 0.048 at the 3/sec and 1.003 ± 0.014 at the 1/sec rates of stimulation with a change of Ca concentration, and 0.998 ± 0.048 at the 3/sec and 0.905 ± 0.048 at the 1/sec rates of stimulation with a change of Mn concentration (each 10–20 preparations)).

The contractile magnitude in the test solution of Ca ions was expressed as a relative value to that in the standard Tyrode solution, and the magnitude in the test solution of Mn ions, as a relative value to the solution without Mn ions. These relative values were statistically more significant than the absolute values and were preferred to the latter.

Since all test solutions of either Ca or Mn ions were not tried on a single preparation, the analysis was carried out on curves obtained from the average response by a given dose on different preparations, but when necessary, also carried out on the curves obtained from the responses of the individual preparation. Inasmuch as the magnitude of the response of individual preparation was similar to that of the average, both series of the values later estimated did not greatly differ from each other. This can be theoretically predicted by the procedure of Taylor expansion.

The stimulation rate was kept constant throughout one series of the experiments. The average contractile forces in the standard Tyrode solution at the 3/sec, 2/sec and 1/sec rates of stimulation were respectively 585.0 ± 90.5, 585.4 ± 46.4 and 578.3 ± 41.0 mg. The rate of stimulation within such a range exhibited no significant differences in the force.

The effect of Mn ions was usually reversible when the preparation was rinsed using standard Tyrode solution.

**RESULTS**

1. **Inotropic effect of Ca ions alone**

In the left atrium, electrically driven, the contractile force was enhanced in accordance with the increase in the external Ca concentration up to a certain extent of the concentration. This can be seen in Fig. 1, where the average contractile force was plotted against the
external Ca concentration. As shown here, the dose-response curves of Ca ions flattened out in accordance with a raise of the electrically driven rate from 1 to 3/sec. A maximal response, seen already in 3.3 mM Ca ions at the 3/sec rate of stimulation was not obtained, or only obtained at the maximal concentration of Ca ions at the 1/sec and 2/sec rates of stimulation.

As shown in Fig. 2, the plot of the relative contractile force against Ca concentration, both on reciprocal scale at the 1 and 2/sec rates of stimulation, was found to approximate a straight line. This indicates the fitting of the relation to the kinetic equation of Michaelis and Menten (10, 11).

If it is assumed that the effect of Ca ions, $E_{ca}$, is proportional to the concentration of the Ca-receptor complex, $RCa$, whose dissociation constant (Michaelis constant) is $K_m$, then the following well-known equation is obtained.

$$E_{ca} = k (RCa) = E_m / (1 + K_m/Ca)$$

or

$$1/E_{ca} = K_m/(CaE_m) + 1/E_m$$

where $k$, Ca and $E_m$ represent respectively the proportionality constant (intrinsic activity)
TABLE 1. Kinetic equations of the inotropic effects of Ca, $E_m$, $K_m$ and $K_m'$ values estimated and the F test for a good application to the equations.

<table>
<thead>
<tr>
<th>Rate of stimulation</th>
<th>Kinetic equations</th>
<th>$E_m$</th>
<th>$K_m \times 10^5$</th>
<th>$K_m' \times 10^5$</th>
<th>$F_{k-1, s-k(t)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/sec</td>
<td>$E_{Ca}=1.441/Ca - 0.258$</td>
<td>3.872</td>
<td>5.579</td>
<td>$F_5,F_{1,5} (0.001)$</td>
<td></td>
</tr>
<tr>
<td>2/sec</td>
<td>$E_{Ca}=1.049/Ca - 0.436$</td>
<td>2.295</td>
<td>2.408</td>
<td>$F_5,F_{1,5} (0.001)$</td>
<td></td>
</tr>
<tr>
<td>3/sec</td>
<td>$E_{Ca}=0.084/Ca + 0.198Ca + 1.115$</td>
<td>5.062</td>
<td>5.641</td>
<td>$F_5,F_{1,5} (0.001)$</td>
<td></td>
</tr>
</tbody>
</table>

(1) $k$ : the number of variates, $N$ : the number of data points. Same as in Table 2.

(12, 13), the external concentration of Ca ions and the maximal effect ($E_m=k(R_T)$, where ($R_T$) represents the total concentration of the receptor, $R$).

$K_m$ and $E_m$, estimated from the lines in Fig. 2, are shown in Table 1.

The different values of $E_m$ here obtained at the different rates of stimulation may indicate either the alteration of the proportionality constant, $k$, or that of the total receptor concentration, ($R_T$).

Briggs and Holland (14) obtained a $K_m$ value of $RCa$ complex, of the same order as the $K_m$ values shown here, in rabbit atria driven at the rate of 200/min.

On the other hand, inhibition of the response by excess of the agonist, as seen in the dose-response curve of Ca ions at the 3/sec rate of stimulation, is known as an autoinhibition or autoantagonism, which is classified as uncompetitive and noncompetitive (12). The former includes what is called a competitive substrate inhibition in enzymological terms (11, 15).

In the uncompetitive type of inhibition, the agonist Ca ions are supposed to form not only $RCa$ complex, active for the development of the response, but also inactive or at least less active $RCa_2$ complex, combining with the receptor site $R$. Assuming the inactivity of the complex $RCa_2$, the effect of Ca ions is:

$$E_{Ca}=k(RCa) =E_m/(1+K_m/Ca+Ca/K_m')$$

(3)

where $K_m'$ represents the dissociation constant of the $RCa_2$ complex. Here again the maximal effect $E_m=k(R_T)$.

In the noncompetitive type of inhibition, the agonist Ca ions are supposed to have an affinity to both interdependent receptor systems, $R$ and $R'$, forming an active complex, $RCa$, as well as an inactive or at least less active complex, $R'Ca$. Assuming the inactivity of $R'Ca$ complex, the effect of Ca ions is:

$$E_{Ca}=E_m/(1+K_m/Ca+Ca/K_m')$$

(4)

where $K_m'$ represents the dissociation constant of the complex, $R'Ca$, and $E_m=k(R_T)$.

The equations (3) and (4) can be respectively transformed into the following second order equations of Ca concentration.

$$Ca/E_{Ca}=Ca^2/(E_mK_m') + Ca/E_m + K_m'/E_m$$

(5)

$$Ca/E_{Ca}=Ca^2/(E_mK_m') + (K_m + K_m')Ca/(E_mK_m') + K_m/E_m$$

(6)

From

$$dE_{Ca}/dCa=0, E_{max}=E_m/(1+2\sqrt{K_m/K_m'})$$

(7)

for the uncompetitive autoinhibition and
FIG. 3. Parabolic relationship between the reciprocal of the effect of Ca inos multiplied by Ca concentration (mM) \((\text{Ca/E}_{\text{ca}})\) and Ca concentrations (mM) at 3/sec rate of stimulation. The line was drawn by the application of least square method to the data in Fig. 1 \((p<0.001)\).

\[
E_{\text{max}} = E_m / (1 + \sqrt{K_m/K_{m'}})^2
\]

for the noncompetitive autoinhibition (15).

A parabolic form of the relation is to be expected, irrespective of the mode of autoinhibition, when \(\text{Ca/E}_{\text{ca}}\) is plotted against Ca concentration. Such was the case with the present experimental data at the 3/sec rate of stimulation in Fig. 1, as shown in Fig. 3.

The line here was drawn by the application of the least square method.

\(K_m, K_{m'}\) and \(E_m\) thus estimated for the uncompetitive type of autoinhibition are shown in Table 1. \(E_{\text{max}}\) calculated by the equation (7), was 1.24, quite close to the maximal response obtained at 3.3 mM Ca ions.

Values for the noncompetitive autoinhibition could not be estimated in real numbers either from the lines obtained from the average response or from the lines obtained from the response in individual preparation in 7 cases out of 9. Thus, the uncompetitive autoinhibition is a more probable mode of action than the noncompetitive one.

2. Inotropic effect of Ca ions in combination with Mn ions

Mn ions applied in a cumulative fashion, to the muscle bath of various Ca concentrations manifested dose-dependently a negative inotropic effect as shown in Fig. 4A, B and C.

Such curative procedures were preferred to preventive ones, because the low solubility of Ca ions in high concentration may impede observation of a shift of the dose-response curve of Ca ions in the whole range of concentrations of the ions by the latter procedure.

On the whole, the negative inotropic effect of the same dose of Mn ions was manifested more predominantly in the lower concentration range of Ca ions than in the higher, as seen from these figures. This is in accord with the reports of Meinertz and Scholz (16).
When the effect of Ca ions (E_{Ca}) alone follows the equation (1), as in the cases at the 1/sec and 2/sec rates of stimulation, the effect of Ca ions in the presence of Mn ions (E_{Ca}') will be described by either form of the following, where Mn indicates the external concentration of Mn ions:

$$E_{Ca}' = E_m / \left\{ 1 + K_m \left( 1 + Mn / K_i \right) / Ca \right\}$$

for the competitive type, and

$$E_{Ca}' - E_m / \left\{ (1 + Mn / K_i) \left( 1 + K_m / Ca \right) \right\}$$

for the noncompetitive type, assuming the inactivity of the complex, RMn (competitive type) and R'Mn (noncompetitive type) (11). The inhibitor constants of these complexes are $K_i$ and $K_i'$ respectively.
Hence, with the equation (1),
\[ E_{c_a'} / (E_{c_a} - E_{c_a'}) = K_t (C_a/K_m + 1) / M_n \] (9)
for the competitive type, and
\[ E_{c_a'} / (E_{c_a} - E_{c_a'}) = K_t' / M_n \] (10)
for the noncompetitive one.

One the other hand, when the effect of Ca ions alone \((E_{c_a})\) follows the equation (3), as in the case at the 3/sec rate of stimulation, the effect of Ca ions in the presence of Mn ions will be describable by either form of the following equations according to the type of inhibition of Mn ions:
\[ E_{c_a'} = E_m / \{ 1 + K_m / C_a + C_a / K_m' + K_m M_n / (K_t' C_a) \} \] (19)
for the competitive type, and
\[ E_{c_a'} = E_m / \{ 1 + K_m / C_a + C_a / K_m' + K_m M_n / (K_t' C_a) + M_n / K_t' + C_a M_n / (K_m' K_t) \} \] (20)
for the noncompetitive type.

Here it is assumed that Mn ions would compete for the receptor site, \(R\), with Ca ions to form the inactive complex, \(R M_n\) (inhibitor constant: \(K_t\)) in the former type and would bind to the receptor site, \(R'\), to form the inactive complexes, \(R R' M_n\), \(R C_a R' M_n\) and \(R C_a^2 R' M_n\) (inhibitor constant: \(K_t'\) in common with all these complexes).

Hence, with the equation (3),
\[ E_{c_a'} / (E_{c_a} - E_{c_a'}) = K_t (C_a / (K_m K_m') + C_a / K_m + 1) / M_n \] (11)
for the competitive type of inhibition, and
\[ E_{c_a'} / (E_{c_a} - E_{c_a'}) = K_t' / M_n \] (12)
for the noncompetitive type of inhibition.

Taking \(1 / M_n\) in the equations (10) and (12), \((C_a / (K_m M_n) + 1 / M_n)\) in the equation (9), or \(C_a / (K_m K_m') + C_a / K_m + 1\) in the equation (11), as an independent variate \((X)\) and \(Y = E_{c_a'} / (E_{c_a} - E_{c_a'})\) as a dependent variate, estimation of \(K_t\) or \(K_t'\) values from the regression lines of the \(X\) and \(Y\) values can be recalculated from the data shown in Figs. 4A, B and C. Here, \(K_m\) and \(K_m'\) take the same values as those obtained in the previous section.

An alternative method is to take \(C_a / M_n\) and \(1 / M_n\) as two independent variates in the equation (9) and also to take \(C_a^2 / M_n\), \(C_a / M_n\) as well as \(1 / M_n\) as three independent variates in the equation (11). . .

The F test carried out on both of the procedures indicated the greater significance of the regression equations in the competitive type of inhibition than in the noncompetitive type throughout the whole series of experiments.

The regression lines were considered to pass through the origin within the limits of sampling error (5%) except the case at the 3/sec rate of stimulation in the competitive type of inhibition. These showed a tendency however, to deviate farther away from the origin in the competitive type of inhibition than in the noncompetitive one, contrary to theoretical requirements.

Results by the former procedure are shown in Figs. 5A, B and C, and in Table 2.

Noteworthy is the reduction of both inhibitor constants, \(K_t\) and \(K_t'\), in accordance with the increase in rate of stimulation.
The latter procedure yielded more or less different values of \(K_m\) and \(K_m'\) from those estimated in the previous section (\(K_m \times 10^3 = 0.907\) at the 1/sec, \(0.463\) at the 2/sec and \(0.004\) at the 3/sec rates of stimulation and \(K_m' \times 10^3 = 14.465\) at the last one).

Difference in the values was greatest in experiments at the 3/sec rate of stimulation.

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**Fig. 5.** Linear relationship between \(X\) (1/Mn for the noncompetitive type of inhibition (---) and \((Ca/(K_mM_n) + 1/M_n)\) (A and B) and \((Ca^2/K_mK_m' + Ca/K_m + 1)/M_n\) (C) for the competitive type of inhibition (-----)) and \(Y = E_{ca}/(E_{ca} - E_{ca'})\). A, at 1/sec, B, at 2/sec and C, at 3/sec rates of stimulation. Solid lines on both sides of the above lines indicate the 95% confidence limits.
Thus, the results of the present analysis are not really favorable for regarding Mn ions, as a typical competitive inhibitor of Ca ions.

**DISCUSSION**

In the present experiments with guinea-pig atrial preparations driven electrically the concentration-tension curves of Ca ions declined progressively in their slope as the rate of stimulation was increased from 1/sec to 3/sec.

At the 1/sec and 2/sec rates of stimulation, these curves were shown here to be describable by the classical Michaelis-Menten equation. At the 3/sec rate of stimulation, however, the concentration-tension curve presented a convex form with a maximally-effective concentration of Ca ions approx. twice that in the standard Tyrode solution.

Similar, more or less convex forms of the curves of the steady-state contractile force plotted against Ca concentration were observed by several authors (17–21) in frog as well as in mammalian hearts, particularly when the heart was stimulated at a higher rate or in the solution with reduced external Na concentration or both.

It has been demonstrated by many authors (1–5) that the increment of Ca uptake per beat varied proportionately with the contractile force developed in a certain range of the stimulation rate and in that of the external Ca and Na concentration.

Although this may be true with a certain component of Ca ions which contributes to the development of the contractile force, there may be another component of Ca ions, which when taken up leads to the reduction of the force in another range of high stimulation rate and in that of the excessive external Ca concentration.

From the point of view of the receptor theory developed here, it can be presumed that the former component is taken up into the cells by combining with the hypothetical receptor site, R, while the latter component is taken up by combining uncompetitively with the receptor site, R_Ca, i.e., the receptor already occupied by one molecule of a Ca ion. Possibility of operation of the noncompetitive autoinhibition of Ca ions is less in this case.

Thus, the present results may provide a kinetic basis for the existence of two opposing processes, suggested by Niedergerke (21), i.e., a facilitatory one and an inhibitory one, re-
suiting in modification of the contractility.

It is noteworthy that the $K_m$ and $E_m$ values of the 1/sec and 3/sec rates of stimulation were respectively the same order in magnitude, whereas those at the 2/sec rate of stimulation were rather less than those at the other rates as shown in Table 1. One possible explanation is that the $RCa_2$ complex is formed not only at the 3/sec rate of stimulation but also at the 2/sec rate although the extent of the formation is less at the latter rate than that at the former. It may then yield the $K_m$ and $E_m$ values of the same order at the 2/sec rate as those at the other rates.

Mn ions have been considered to compete with Ca ions for the inflow of membrane currents in various tissues, including cardiac cells (22, 23) as well as for the activation of the contractile system in the heart (16, 24). Mn ions do not appear however, to act always as a competitive inhibitor but rather as a synergist with Ca ions in some cases, as reported by Shibata (25).

The present analysis revealed that, in the negative inotropic action of Mn ions added in different concentrations of Ca ions, noncompetitive antagonism may be involved, in spite of a more predominant competitive antagonism of the ions with Ca ions, particularly when the heart was driven at the higher rate of 3/sec. It is also very significant for further research that the dependency of the inhibitory action of Mn ions on the rate of stimulation is expressed as the changes of the inhibitor constants, $K_1$ or $K_1^*$, in terms of the receptor theory.

Though it is possible that the dose response curves are secondarily distorted by many factors, such as the influence of these ions on the effects of the autonomic transmitters released by the electrical stimulation (26), the statistical evaluation of fitting to the theoretical equations, as presented here, may provide a critical way for objective presumption of a more probable mode of action of agents.

SUMMARY

Statistical evaluation of fitting to several kinetic equations was made on the positive and negative inotropic effects of Ca and Mn ions in the electrically driven left atrium of guinea-pigs.

The positive inotropic effect of Ca ions (0.6–8.6 mM) on the atrium driven at the rate of 1/sec and 2/sec was found to approximate the equation of Michaelis and Menten. The positive as well as negative inotropic effects were exerted by Ca ions in the atrium driven at the rate of 3/sec, according to the external concentration of Ca ions. The equations of uncompetitive autoinhibition were consistent with this dual mode of action of Ca ions. As to the negative inotropic effect of Mn ions (0.1–10 mM) added in various concentrations of Ca ions, the regression lines tended to deviate further away from the origin, in spite of the greater significance of regression coefficients in the equations of the competitive type of inhibition than in those of the noncompetitive type of inhibition. The results were interpreted as doubtful regarding Mn ions as a typical competitive inhibitor of Ca ions on inotropic effects.
REFERENCES