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Molecular modeling of manganese regulation of calmodulin-sensitive adenylyl cyclase from mammalian sperm☆

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Abstract

The soluble calmodulin-sensitive isoform of adenylyl cyclase isolated from equine sperm is unique because it requires Mn^{2+} rather than Mg^{2+} for activity. To gain insight into the molecular action of metals on sperm adenylyl cyclase, the kinetics of Mn^{2+} and ATP effect was examined. A biphasic response to increases in ATP concentration was observed when metal was held constant. When [Mn^{2+}] exceeded [ATP], however, greatly enhanced enzyme activity was observed. The kinetic profiles were consistent with allosteric activation of adenylyl cyclase by Mn^{2+}. Linear transformation of the data yielded an apparent K_m for Mn–ATP of 5.8 mM and calculated V_{max} of 12 nmol cyclic AMP formed/min/mg. Data analysis using calculated equilibrium concentrations of free and complexed reactants provided similar estimates of these kinetic parameters.

Keywords: Cyclic nucleotides; Signal transduction; Metal ions; Molecular modeling; Kinetics; Allostery; Fertility; Sperm motility

It is a privilege to participate in this tribute to Gunny, who had a great impact on my life as a scientist. Gunny has a passion for science, which he imparted to all of his students in a variety of ways. We who worked in his laboratory have many anecdotes that could be related, but I felt that the greatest honor I could pay to my mentor is to describe a biochemical study using some of the kinetic methods I learned while in his laboratory. During that time, we worked with colored proteins, cytochrome P450 (red), putidaredoxin (brown), putidaredoxin reductase (yellow), and azurin (blue). The present study is with an enzyme that has no color, is regulated by a metal, and can be extracted by a freeze–thaw process [1]. Hence, based on these similarities with our old systems, it fits in the sphere of Gunny's broad scientific interest.

Cyclic AMP plays an important, but not completely understood, role in mammalian sperm function [2]. Intracellular cyclic AMP levels are regulated by a unique form of soluble adenylyl cyclase [ATP-pyrophosphate lyase (cyclizing), EC 4.6.1.1], whose activity is mediated by calmodulin [3]. The isozyme found in mammalian sperm is insensitive to the typical regulators of somatic cell adenylyl cyclase including hormones, forskolin, and GTP [1,3] but is similar to the somatic cell isoforms, in sharing regulation by inorganic ions, such as manganese and calcium [3,4]. Cyclic AMP is associated with motility in developing sperm and the acrosome reaction in mature sperm [5]. Metals play an important role in modulating sperm function, and thus fertility in many animal species including humans [6]. Thus as part of our ongoing studies to understand mechanisms of environmental modulation of human reproduction, we examined the kinetic parameters for Mn^{2+} and ATP to gain insight into possible roles that metal ions could play in sperm function.

Materials and methods

Calmodulin-sensitive adenylyl cyclase was prepared from freshly ejaculated equine semen (generous gift from Dr. J. Patel, Tufts University School of Veterinary Medicine) using previously published

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methods [1]. All other reagents used in this study were of the highest grade commercially available. Adenylyl cyclase activity was measured as described earlier [3]. All assays were performed in quadruplicate. The standard error of the mean was typically less than five percent. Protein was estimated using previously published protocols with bovine serum albumin as a standard [7]. Routine calculations and statistical analyses were performed using commercial software packages (Microsoft, Redmond, WA). Equilibrium concentrations of reactants were calculated using the following stability coefficients for metal complexes determined under conditions of pH and ionic strength similar to those employed in the enzyme assay [8,9]: Mn–ATP, $7.08 \times 10^{-4}\,M^{-1}$; Mn-phosphocreatine, $1.0 \times 10^{2}\,M^{-1}$; and Mn–EDTA, $1.43 \times 10^{-11}\,M^{-1}$. Equilibrium equations were solved simultaneously by an iterative Newton–Raphson procedure using a Pascal program.

Results

Equine sperm adenylyl cyclase exhibited complex kinetic interactions with Mn^{2+} and ATP. At fixed Mn^{2+} concentration, enzyme activity was biphasic as a function of ATP (Fig. 1). At each fixed level of Mn^{2+}, [ATP] was varied to delineate the limiting velocity of the enzyme. When both Mn^{2+} and ATP were varied to the point of maximal enzyme activation, the observed V_{max} was 13 nmol cyclic AMP formed/min/mg protein. The apparent inhibition of enzyme activity observed as [ATP] increased relative to [Mn^{2+}] could be a result of allosteric actions of free ATP, free Mn^{2+} or both. When [Mn^{2+}] was in excess of ATP, greatly enhanced enzyme activity was observed (Fig. 2). The apparent increase in V_{max} is consistent with an allosteric site on sperm adenylyl cyclase for Mn^{2+}.

The observed kinetic patterns closely resemble theoretical velocity profiles derived for enzymes that require metal–reactant complex (Mn–ATP) as substrate and that are modified by free metal ion (Mn^{2+}) [10,11]. This kinetic model predicts that the apparent inhibitory actions of ATP result from total complexation of Mn^{2+} by ATP, leaving no free Mn^{2+} to allosterically activate the enzyme. The enzyme would thus be converted from an activated to a non-activated form. The apparent kinetic parameters for an enzyme exhibiting these characteristics can be determined graphically from a double reciprocal plot of [ATP] vs. the apparent limiting velocity observed when [Mn^{2+}] was varied at a constant [ATP] [10,11]. For equine sperm adenylyl cyclase, the calculated K_m for Mn–ATP was 5.8 mM, with a calculated V_{max} of 12 nmol cyclic AMP formed/min/mg, consistent with the limiting velocity of 13 nmol cyclic AMP formed/min/mg protein observed when [ATP] and [Mn^{2+}] were optimal.

It has been proposed that rigorous kinetic analysis of interactions between activators and inhibitors that cannot be independently varied depends on knowing the actual equilibrium concentration of a particular species at each set of assay conditions [12]. The equilibrium concentrations of ATP, free Mn^{2+}, and Mn–ATP were calculated for each data point of Figs. 1 and 2 using published K_{sp} values and an iterative Newton–Raphson approximation. The calculated values were used to generate subsequent kinetic plots.

To determine whether free ATP was an inhibitor, the actions of free ATP were examined under conditions where either $[Mn\text{–}ATP]_{eq}$ or $[Mn^{2+}]_{free}$ was constant.

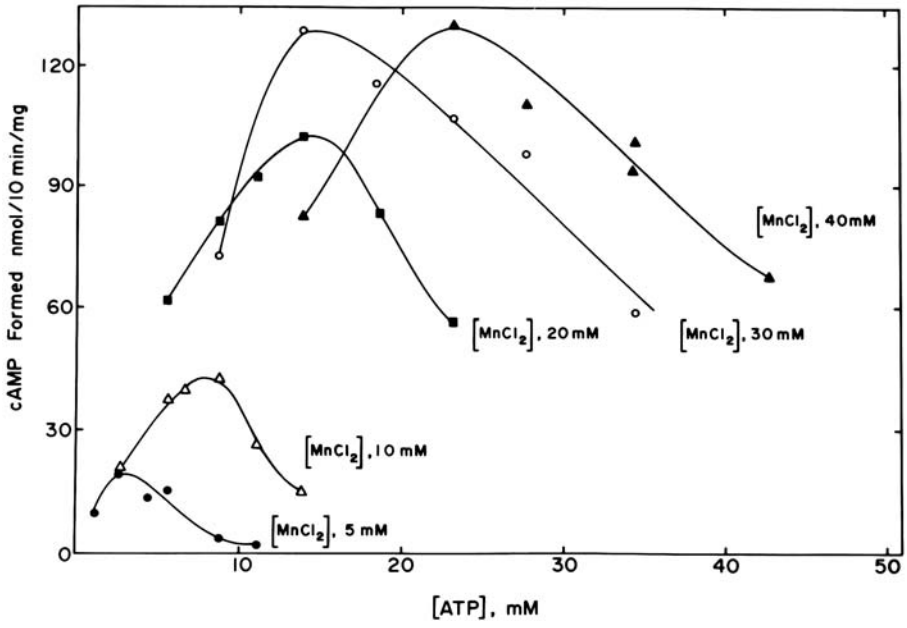

Fig. 1. Equine sperm adenylyl cyclase activity as a function of total ATP concentration. The data shown are means of quadruplicate assays with a standard error of the mean of 5% or less. The final concentrations of $MnCl_2$ in the assays were as denoted in the figure.

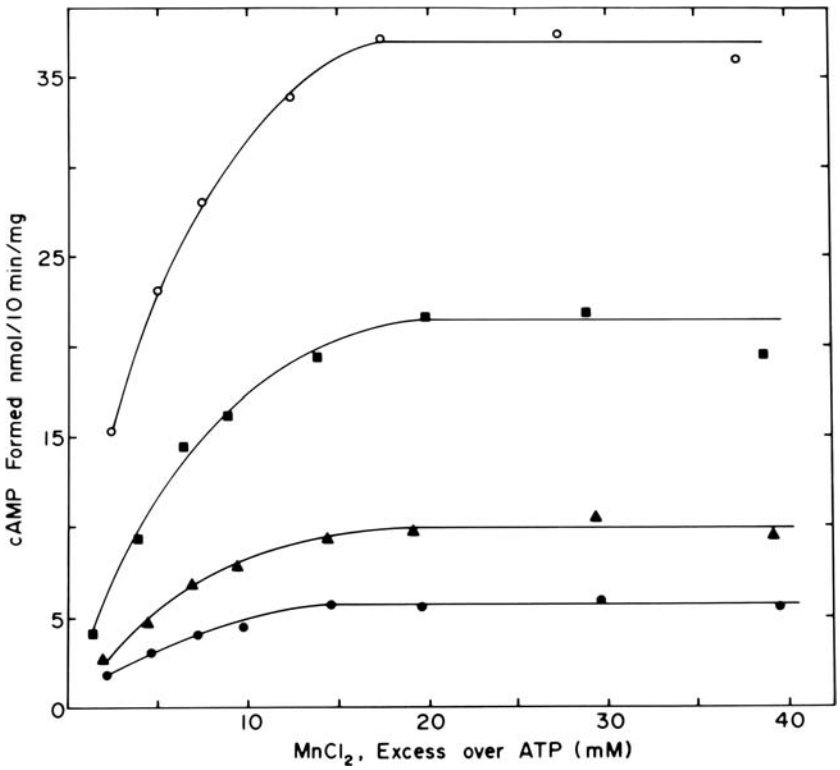

Fig. 2. Equine sperm adenylyl cyclase activity as a function of excess Mn over [ATP]. Conditions used in the assay were as described in the legend to Fig. 1. Final concentrations of ATP used in the assays were: (●) 290 μM, (▲) 570 μM, (■) 1.12 mM, and (○) 2.61 mM.

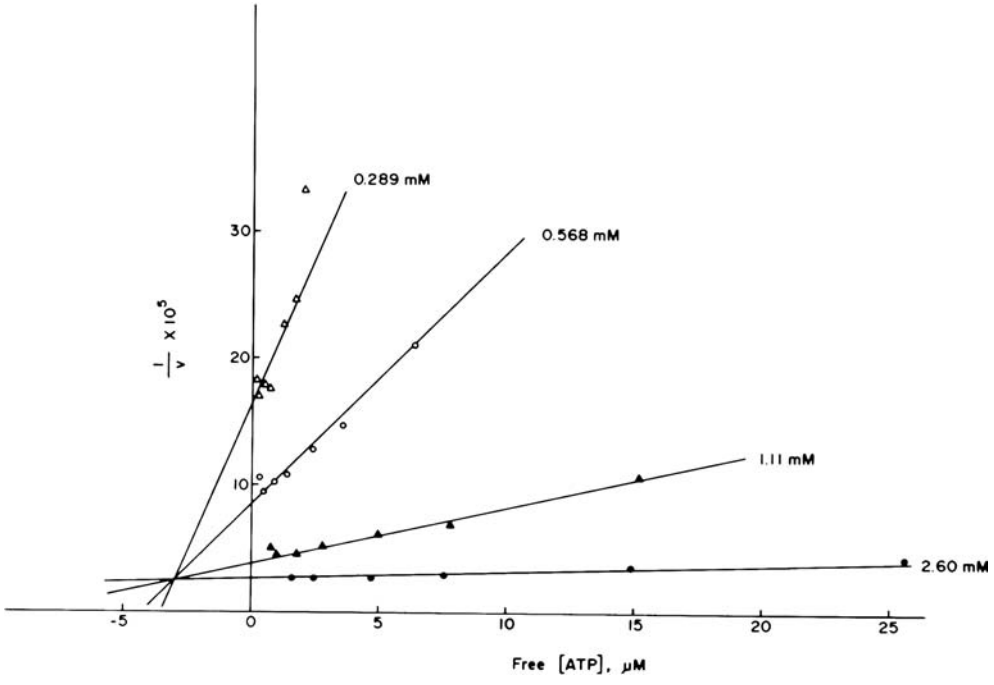

Fig. 3. Dixon's plot assuming free ATP is an inhibitor using calculated equilibrium concentrations of uncomplexed ATP at constant calculated Mn–ATP concentrations of 0.3 (△), 0.6 (○), 1.1 (▲), and 2.6 mM (●), respectively.

The Dixon plot (Fig. 3) indicates that $[ATP]_{free}$ could act as a competitive inhibitor (apparent $K_i = 3\,\mu M$). Because $[ATP]_{free}$ and $[Mn^{2+}]_{free}$ are interdependent, however, these data could not be used to distinguish whether ATP is an independent inhibitor of sperm adenylyl cyclase or acts indirectly by decreasing $[Mn^{2+}]_{free}$. Direct linear

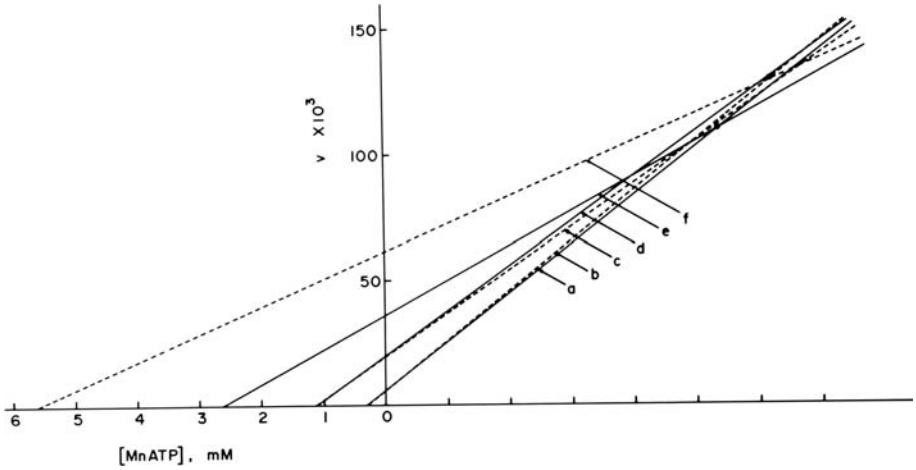

Fig. 4. Direct linear plots [13] obtained when calculated free ATP was varied over a concentration range bracketing its apparent K_i under conditions where the calculated concentration of $[Mn^{2+}]_{free}$ was constant at 5.9 mM (solid lines) or 23.8 mM (broken lines). Calculated $[ATP]_{free}$ were 0.17 (a), 0.67 (b), 2.80 (c), 0.66 (d), 1.63 (e), and 13.3 μM (f).

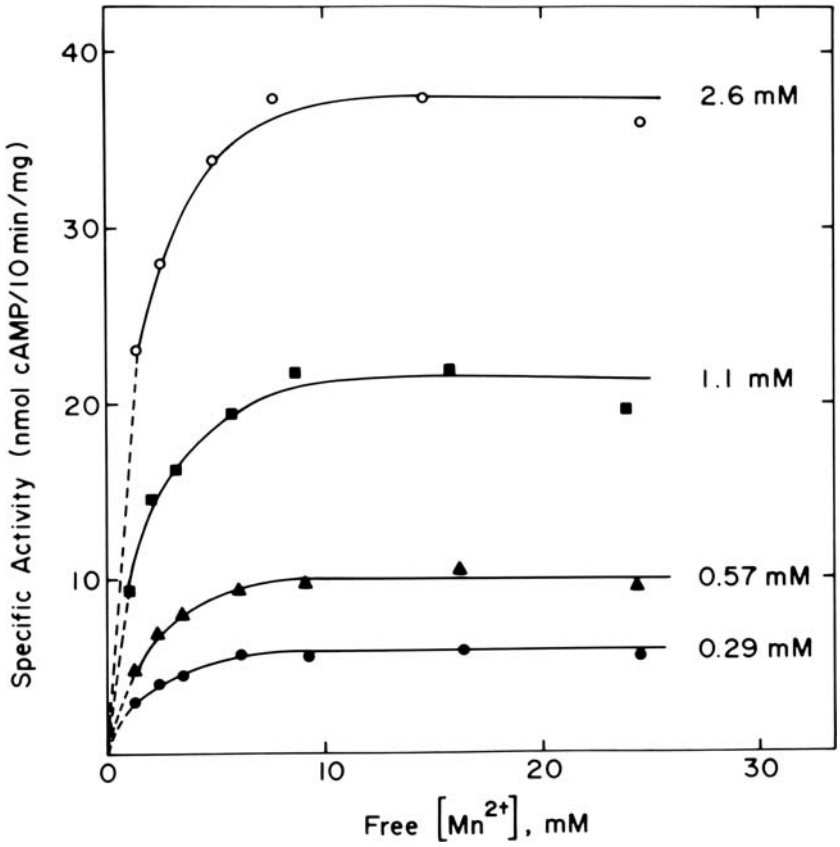

Fig. 5. Equine sperm adenylyl cyclase activity as a function of $[Mn^{2+}]_{free}$ at constant concentrations of substrate. Calculated [Mn–ATP] were: 0.3 (●), 0.6 (▲), 1.1 (■), and 2.6 mM (○).

plots have been used to resolve such ambiguities [13]. When this analysis was carried out for calculated $[ATP]_{free}$ varied over a 10-fold range near the apparent K_i value when calculated $[Mn^{2+}]_{free}$ was essentially constant at two different values of $[Mn^{2+}]_{free}$, the kinetic parameters for the enzyme extrapolated from the plot intersection points were similar (Fig. 4). The apparent $K_m = 6.7$ mM and $V_{max} = 12$ nmol cyclic AMP formed/min/mg protein.

A different kinetic pattern was observed when the action of Mn^{2+} rather than $[ATP]_{free}$ on enzymatic activity was considered. A plot of enzyme activity as a function of calculated $[Mn^{2+}]_{free}$ at constant [ATP] yielded a half-maximal effective concentration (EC_{50}) for

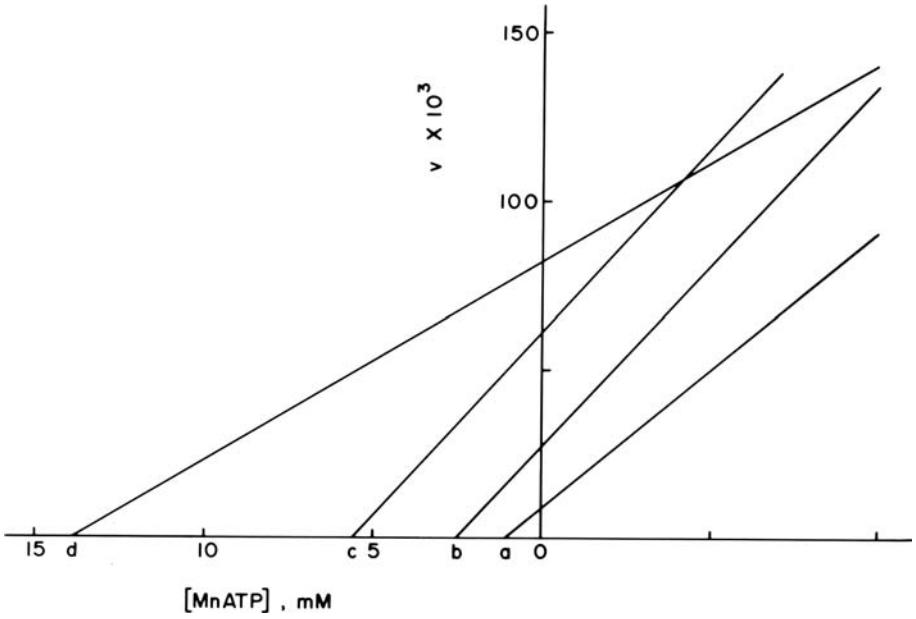

Fig. 6. Direct linear plots obtained when $[ATP]_{free}$ was constant (0.015 μM) and $[Mn^{2+}]_{free}$ was varied over its apparent effective range at calculated concentrations of 1.0 (a), 2.5 (b), 6.0 (c), and 13.6 mM (d). Velocity was expressed as pmol cyclic AMP formed/min/mg protein.

stimulation by uncomplexed Mn^{2+} of 1.3 mM (Fig. 5). This stimulatory effect was saturated at $[Mn^{2+}]_{free}$ greater than 10 mM and the EC_{50} for activation by Mn^{2+} was independent of substrate concentration.

When $[ATP]_{free}$ was constant and $[Mn^{2+}]_{free}$ was varied over its apparent effective concentration range, a direct linear plot of enzyme activity indicated that $[Mn^{2+}]_{free}$ was an independent allosteric activator of the enzyme (Fig. 6).

Discussion

The action of free Mn^{2+} or free ATP on the activity of the soluble isoform of adenylyl cyclase that is regulated by Ca^{2+}-calmodulin supports the hypothesis that this isoform is also subjected to allosteric regulation by $[Mn^{2+}]_{free}$. The substrate-velocity profiles for the enzyme under conditions where whether total Mn^{2+} or total ATP was held constant while the other parameter was varied were similar to theoretical curves for an enzyme that uses a metal–reactant complex as a substrate and is also activated by the free metal [11]. That Mn^{2+} is an allosteric regulator of adenylyl cyclase was first proposed by the late Eva Neer [14]. Confirmation of this hypothesis, however, awaited the demonstration of the utility of re-plotting methods used in this study and in membrane-bound forms of adenylyl cyclase [12]. The EC_{50} for activation of sperm adenylyl cyclase is in close agreement with the levels of manganese found to stimulate motility of sperm in humans [15]. Kinetic curves obtained in the present study are qualitatively similar to those obtained for membrane-bound bovine sperm adenylyl cyclase, [12]; however, significant quantitative differences exist between the two forms of the enzyme. The K_i value for ATP was 10-fold lower in equine sperm than in bovine sperm (3 vs. 50 μM), which may be a reflection of the marked differences in ATP concentrations in sperm of horses and cattle (111 vs. 37 nmol/10^6). Likewise, the EC_{50} value for Mn^{2+} activation of equine isozyme is 7-fold higher than for the membrane-bound bovine enzyme (1.5 vs. 0.2 mM).

It is useful to speculate on the role that Ca^{2+} may play in allosteric regulation by Mn^{2+} under physiological conditions. Although the in vitro actions of free Mn^{2+} on the activity of the soluble sperm isozyme are profound, the physiological relevance of these observations is unclear because Mn^{2+} is present at only micromolar levels in intact cells. It is possible that compartmentalization of the enzyme occurs in regions of sperm where $[Mn^{2+}]$ is high or subject to high variation. Alternatively, it has been previously demonstrated that Ca^{2+} potentiates activation of spermatozoal adenylyl cyclase by Mn^{2+} in a synergistic manner [16,17]. Since the isozyme of adenylyl cyclase used in the present study is Ca^{2+}-sensitive by virtue of its regulation by calmodulin [3], it is possible that Ca^{2+} and Mn^{2+} act coordinately in vivo to modulate adenylyl cyclase at significantly lower concentrations in combination or separately.

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