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Divalent Metal Ions Mg^{2+} and Ca^{2+} Have Distinct Effects on Protein Kinase A Activity and Regulation

Matthias J. Knape[†], Lalima G. Ahuja[‡], Daniela Bertinetti[†], Nicole C.G. Burghardt[†], Bastian Zimmermann[§], Susan S. Taylor[‡], and Friedrich W. Herberg^{†,*}

[†]Department of Biochemistry, University of Kassel, 34132 Kassel, Germany

[‡]Department of Pharmacology, University of California at San Diego, La Jolla, California 92093, United States

[§]Biaffin GmbH and Co KG, 34132 Kassel, Germany

Abstract

cAMP-dependent protein kinase (PKA) is regulated primarily in response to physiological signals while nucleotides and metals may provide fine-tuning. PKA can use different metal ions for phosphoryl transfer, yet some, like Ca^{2+} , do not support steady-state catalysis. Fluorescence Polarization (FP) and Surface Plasmon Resonance (SPR) were used to study inhibitor and substrate interactions with PKA. The data illustrate how metals can act differentially as a result of their inherent coordination properties. We found that Ca^{2+} , in contrast to Mg^{2+} , does not induce high-affinity binding of PKA to pseudosubstrate inhibitors. However, Ca^{2+} works in a single turnover mode to allow for phosphoryl-transfer. Using a novel SPR approach, we were able to directly monitor the interaction of PKA with a substrate in the presence of Mg^{2+} ATP. This allows us to depict the entire kinase reaction including complex formation as well as release of the phosphorylated substrate. In contrast to Mg^{2+} , Ca^{2+} apparently slows down the enzymatic reaction. A focus on individual reaction steps revealed that Ca^{2+} is not as efficient as Mg^{2+} in stabilizing the enzyme:substrate complex. The opposite holds true for product dissociation where Mg^{2+} easily releases the phospho-substrate while Ca^{2+} traps both reaction products at the active site. This explains the low steady-state activity in the presence of Ca^{2+} . Furthermore, Ca^{2+} is able to modulate kinase activity as well as inhibitor binding even in the presence of Mg^{2+} . We therefore hypothesize that the physiological metal ions Mg^{2+} and Ca^{2+} both play a role in kinase activity and regulation. Since PKA is localized close to calcium channels and may render PKA activity susceptible to Ca^{2+} , our data provide a possible mechanism for novel crosstalk between cAMP and calcium signaling.

*Corresponding Author: herberg@uni-kassel.de.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.5b00271. PKA-C binding profile after on-chip phosphorylation, antibody binding to phosphorylated PKA, differences in complex stability of PKA-C:PKI and PKA-C:PKS in the presence of magnesium and calcium, respectively, and binding of PKA-C to PKA-RI α (92–245) in the presence of magnesium and calcium, respectively (PDF)

Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

Graphical abstract

Protein kinases play a pivotal role in signal transduction since they catalyze the most important signaling reaction by transferring the γ -phosphoryl group of ATP to the hydroxyl group of Ser, Thr, and Tyr residues of signaling proteins.¹ Hence, protein kinases need to be accurately regulated in response to physiological signals and conditions. Because the structure and function of cAMP-dependent protein kinase (PKA) is extremely well characterized, it can serve as a model for studying the effects by factors like nucleotides, metals, and substrates on protein kinase function.² Physiological inhibitors such as the regulatory subunit (R) type I and II of PKA and the heat stable protein kinase inhibitor (PKI) allow us to study inhibitor action at a molecular level. PKI was discovered at about the same time as the catalytic (C) subunit of PKA.^{3,4} It was copurified with the PKA C-subunit and was found to be a high affinity pseudosubstrate inhibitor of the C-subunit.^{3,5-7} While the type II R-subunit is a substrate as well as an inhibitor of the C-subunit, PKI and RI are pseudosubstrate inhibitors, and their interaction with the C-subunit is highly dependent on Mg^{2+} ATP.⁸ The inhibitory sequence of PKI is localized at the N-terminus and includes an amphipathic helix followed by a classic PKA recognition motif with three arginines preceding the P-site, where alanine replaces the phospho-acceptor residue of a true substrate (RXXRRNAI).^{6,7,9} Based on extensive peptide mapping, Glass and co-workers established that a helical motif is needed for high affinity binding while the arginine-rich region is a prerequisite for docking to the active site of the C-subunit.⁹ The first structure of the PKA C-subunit demonstrated how an inhibitor peptide (IP20; PKI 5–24) is docked to the active site of the C-subunit, and the subsequent study of the ternary complex defined how the peptide is anchored to the active site in a conformation that resembles a transition state in the presence of ATP and two magnesium ions.¹⁰⁻¹²

The essential role of metal ions on catalysis was established early on with kinetic as well as magnetic resonance studies, defining an activating and an inhibitory metal binding site.¹³⁻¹⁵ This two-metal mechanism is also employed by other kinases like cyclin-dependent protein kinase 2 (CDK2), and recent results showed both metals are required for optimal phosphotransferase activity.^{16,17} Interestingly, inhibitor binding is also influenced by two metal ions. Direct interaction studies based on SPR measurements showed that the presence of the second Mg^{2+} is correlated with a tremendously slowed dissociation rate of PKI.¹⁵ Moreover, kinases can use various divalent metal ions to facilitate phosphotransferase activity. Magnesium, as the most abundant divalent metal ion in the cell, is believed to be the favored coordinating ion for kinases. Other metals, such as manganese or cobalt are suitable for catalysis; however, calcium does not support reasonable steady-state catalysis in

the case of PKA.^{13,18} Surprisingly, nucleotide binding is enhanced in the presence of calcium.¹⁸ Recently, crystal structures of PKA depicting the product complex after phosphoryl-transfer were obtained with Mg^{2+} and Ca^{2+} .¹⁹ In kinetic experiments, Ca^{2+} can also assist in phosphoryl-transfer when measured under non-Michaelis–Menten conditions.²⁰ Since PKA is located near calcium channels, Ca^{2+} may possibly act as a cofactor in catalysis and inhibitor binding.²¹ This concept may be applicable for many, if not all, protein kinases.

Early studies on metalloenzymes were often done by exchanging the native metal ion for a similar one to examine metalloenzymes biochemically, i.e. by replacing Zn^{2+} with the spectroscopically accessible Co^{2+} in carbonic anhydrase or by using Mn^{2+} for magnetic resonance measurements on the catalytic subunit of PKA.^{14,22} This provided insight into the chemical environment around a metal ion bound to an enzyme and was used to understand the roles of metal ions for nucleotide binding and catalysis. However, other techniques where the influence of a specific metal of interest can be investigated directly would be more favorable. In this study, we combined several experimental strategies to investigate the role of the two major divalent metal ions, Mg^{2+} and Ca^{2+} , on inhibition using the physiological pseudosubstrate inhibitor PKI and on kinase activity. To determine the effects on activity, we used a PKI-derived substrate termed PKS, where the invariant Ala was replaced by a Ser residue (PKI A21S, see Figure 1). In combination with crystal structures available for IP20 and SP20 (PKI 5–24 N20A/A21S) in complex with the C-subunit of PKA and binding studies based on FP and SPR, we illustrate how metal ions can perform distinct roles as a result of their inherent coordination properties. This provides an understanding at the molecular level on the profound differences between two divalent metal ions (Mg^{2+} versus Ca^{2+}) in inhibition and catalysis.

RESULTS AND DISCUSSION

Catalytic activity of protein kinases is highly dependent on divalent metal ions. In contrast to magnesium, other earth alkali metals such as calcium, strontium, or barium were found to be incapable of supporting steady-state catalysis of the PKA catalytic subunit (PKA-C).^{13,18} However, calcium was shown to bind to the active site of the kinase at least in combination with an ADP analog (*lin*-benzo-ADP).¹⁸ Surprisingly, although Ca^{2+} could not support steady-state catalysis, crystallization of the C-subunit with the substrate peptide SP20 and ATP in the presence of Ca^{2+} resulted in a trapped product complex showing SP20 in a phosphorylated state and stably bound to the catalytic subunit in combination with Ca^{2+} and ADP.¹⁹ To unveil the differences between Mg^{2+} and Ca^{2+} in catalysis and inhibition, we examined their contributions to substrate and inhibitor binding as well as ATP and ADP binding.

PKA Phosphorylates Protein Substrates in the Presence of Calcium

Recently, phosphoryl transfer in the presence of calcium was described under non-Michaelis–Menten conditions wherein SP20 was used as an artificial substrate peptide corresponding to amino acids 5–24 of PKI with the P-1 site Asn and P0 site Ala replaced by Ala and Ser, respectively (see Figure 1).²⁰ We tested whether the PKA catalytic subunit (human isoform *Ca1*) could also phosphorylate a real protein substrate in the presence of

calcium. To examine this, we generated a protein substrate, termed PKS, where only the P0 site Ala of full-length PKI is replaced by Ser (see Figure 1). As a first step, the major intracellular divalent metal ions, Mg^{2+} and Ca^{2+} , were studied in a classical way under steady-state conditions. A radioactive assay, where the incorporation of radiolabeled phosphate from $\gamma\text{-}^{32}\text{P}\text{-ATP}$ onto a GST-fusion of PKS was determined, revealed phosphotransferase activity in the presence of 1 mM calcium, although with significantly reduced activity ($17 \pm 2 \text{ nmol min}^{-1} \text{ mg}^{-1}$) as compared to 1 mM Mg^{2+} ($458 \pm 18 \text{ nmol min}^{-1} \text{ mg}^{-1}$; Figure 2, inset). Similar results were obtained at various other metal concentrations.

Calcium Does Not Support High Affinity Binding of PKA-C to Pseudosubstrate Inhibitors

The observation that calcium can assist in phosphoryl transfer, even though Mg^{2+} is more efficient, prompted us to investigate how the two metals behave while mediating catalysis and also during inhibition. Using the heat stable protein kinase inhibitor PKI and a peptide (IP20 or PKI 5–24), we first addressed the initial binding step by direct binding studies. IP20 includes the canonical PKA substrate recognition motif and is thought to bind like a substrate to the C-subunit and thereby resembles the initial state of the catalytic cycle where ATP and two metal ions are bound tightly to the kinase core. Interaction studies based on FP of human PKA-C with the fluorescently labeled inhibitor peptide FAM-IP20 are shown in Figure 3A. In the absence of nucleotide or any metal (100 μM EDTA), a reasonable affinity ($K_D = 279 \text{ nM}$) of the C-subunit to the inhibitor peptide was found; however, supplementing the buffer with 1 mM ATP and 10 mM Mg^{2+} greatly enhanced IP20 binding ($K_D = 1.7 \text{ nM}$) (Table 1). Surprisingly, using Ca^{2+} instead of Mg^{2+} resulted in an even lower affinity (570 nM) as compared to EDTA (Figure 3A).

To test whether complex formation or stabilization is impaired in the presence of Ca^{2+}ATP , we used an SPR-based assay where the respective association and dissociation rates of the C-subunit to immobilized full-length PKI (GST-tagged) were determined. While in the presence of 1 mM ATP and 10 mM Mg^{2+} (Mg^{2+}ATP) a highly stable complex was formed with a slow dissociation rate ($k_{\text{diss}} = 1.3 \text{ s}^{-1}$) and with high affinity ($K_D = 0.5 \text{ nM}$; Figure 3C), Ca^{2+}ATP was again unable to induce high affinity binding of PKA-C to PKI ($K_D = 353 \text{ nM}$; Figure 3D, Table 1). The affinity was reduced by a factor of 2 as compared to conditions without ATP and free metal ions (EDTA, $K_D = 138 \text{ nM}$), thereby demonstrating transient binding (Figure 3B). It is noteworthy that the association rate was fast and almost unchanged ($10^6 \text{ M}^{-1} \text{ s}^{-1}$; Figure 3B, Table 1). Yet, in the case of Ca^{2+}ATP , the dissociation rate constant approached the limit that could be resolved by SPR instrumentation.

ATP is capable of forming stable complexes with Ca^{2+} as well as with Mg^{2+} . To ensure that Ca^{2+} is sufficient to promote binding of ATP to the catalytic subunit, fluorescence titration techniques were used. Figure 3E clearly demonstrates that Ca^{2+} was not only able to promote ATP binding to the active site of PKA-C but also enhanced the affinity by more than 2-fold as compared to Mg^{2+} ($K_{D(\text{Ca})} = 9.2 \pm 0.8 \mu\text{M}$; $K_{D(\text{Mg})} = 22.1 \pm 1.8 \mu\text{M}$).

Calcium Prolongs Binding of PKA to the PKI Derived Substrate Analog PKS

Having shown that Ca^{2+}ATP binds well to the PKA C-subunit but could not induce stable complex formation between PKA and pseudosubstrates, we next asked how calcium influences the binding of a substrate. To test complex formation with a protein substrate, we used PKS (PKI_{A21S} ; see Figure 1). The mutant protein was immobilized via a GST-tag on an SPR sensor chip, and binding kinetics were measured as described above for PKI. In a buffer containing only chelators (EDTA and EGTA), the binding kinetics observed were almost identical to PKI; however, the affinity was slightly increased due to a slightly enhanced association rate (Figure 4A, Table 2).

Generally speaking, SPR is rarely used to analyze enzyme–substrate interactions since the kinetics are too fast and the species of reactants cannot clearly be distinguished. Here, however, for the first time, we were able to directly monitor the interaction of PKA-C with a substrate in the presence of Mg^{2+}ATP by SPR. Injecting low concentrations of PKA-C (3–90 nM) in a buffer containing Mg^{2+}ATP resulted in atypical binding kinetics with a sharp peak in the first few seconds of the association phase followed by a subsequent decrease to the baseline (Figure 4B). At higher concentrations of PKA-C (0.27–2 μM), a binding plateau was rapidly reached after the initial peak and maintained during the association phase (Figure S1). These very unusual binding kinetics can be explained by the phosphoryltransfer on PKS catalyzed by PKA-C on the chip surface during the injection in the presence of Mg^{2+}ATP . Thus, the observed kinetics reflect the complete phosphoryl-transfer reaction including (i) complex formation (binding of PKA-C to PKS on the chip), (ii) phosphoryl-transfer (no direct change of the SPR signal), and (iii) release of PKA-C after phosphoryl-transfer (dissociation also in the association phase; Figure 4D).

To check if PKS could in fact be phosphorylated on the chip surface, we injected an anti-phospho PKA substrate antibody recognizing the sequence RRX(p)S/T . Strong binding of the antibody was observed after the surface-bound PKS was incubated with PKA-C in the presence of Mg^{2+}ATP , while after injection of PKA-C without Mg^{2+}ATP , the antibody injection resulted only in a low binding signal (Figure S2A). In parallel, PKS phosphorylation was demonstrated by Western blot analysis (Figure S2B inset). Since PKA-C indeed phosphorylates PKS directly on the chip surface, the binding profile after the initial peak should reflect the binding of PKA-C to phosphorylated PKS with an equilibrium binding constant in the low micromolar range (Figure S1). In fact, repeating the experiment to analyze binding to the prephosphorylated GST-PKS (pPKS), immobilized on a parallel flow cell, revealed similar low affinities of around 2 μM (Figure S1).

We next asked if calcium can also induce binding and phosphoryl-transfer to PKS directly on the chip surface. Again, in the presence of Ca^{2+}ATP , atypical binding kinetics were observed, yet with very broad peaks (Figure 4C), not only indicating that PKA-C is able to bind to PKS in the presence of Ca^{2+} but also that phosphoryl transfer can take place on the chip surface in the presence of Ca^{2+} . However, the phosphoryl-transfer reaction seems to be considerably slower as implied by the broadened peaks.

Use of an ATP-analog to Study PKA-C:PKS Complex Formation for Mg^{2+} and Ca^{2+}

We hypothesized that Ca^{2+} may prolong the enzymatic reaction by either lowering the phosphoryl-transfer rate or by decreasing the release rate of the phosphorylated substrate and ADP. On the basis of a simplified Michaelis–Menten model, we dissected the reaction into the three steps: (A) complex formation of kinase and substrate, (B) the phosphoryl transfer, and (C) product release. B cannot be observed by SPR, but presteady state kinetic experiments suggested that Ca^{2+} , in contrast to Mg^{2+} , shows no initial burst phase (Ahuja et al., in preparation). However, complex formation (A) can be directly measured by SPR employing non-hydrolyzable ATP analogs that prevent phosphoryl transfer. Along this line, we used AMP-PNP either in the presence of Mg^{2+} or Ca^{2+} to probe substrate binding. AMP-PNP in combination with Mg^{2+} was highly efficient at promoting binding of PKA-C to the substrate PKS (Figure 5A). In the case of 1 mM Ca^{2+} , substrate binding was also observed, again showing that Ca^{2+} does not inhibit substrate binding, but complex stabilization was reduced as compared to Mg^{2+} and resulted in a 7-fold increase in off-rate and a 10-fold increase in K_D (Figure 5B, Table 2). Again, and as shown in Figure 5C, fluorescence titration clearly demonstrates that AMP-PNP can directly bind to PKA-C with both metal ions. Ca^{2+} enhanced the binding of AMP-PNP to the active site of PKA-C when compared to Mg^{2+} by more than 2-fold.

Ca^{2+} Traps the Reaction Products in the Active Site Cleft of the Kinase

Using prephosphorylated PKS and ADP as the reaction products, we were also able to measure the release of PKA-C from pPKS in real-time (reaction step C as seen in Figure 6). Association rate constants were almost identical for Mg^{2+} and Ca^{2+} ; however, dissociation, reflecting product release after phosphoryl transfer, was fast in the case of Mg^{2+} ($k_{diss} = 6.5 \times 10^{-2} \text{ s}^{-1}$) but slowed down by a factor of 14 for Ca^{2+} ($k_{diss} = 4.7 \times 10^{-3} \text{ s}^{-1}$; Table 3).

Hence, while Mg^{2+} binds the substrate more tightly than Ca^{2+} , it releases the product much more effectively after phosphoryl-transfer occurs (Figure 6A and B). However, for high turnover, not only is the release of the phosphorylated substrate important but ADP release is also crucial.²³ Therefore, we tested ADP binding directly in the presence of Mg^{2+} and Ca^{2+} using the fluorescence titration assay. We found that binding of ADP was impaired as compared to AMP-PNP and ATP for both metal ions tested. As compared to Mg^{2+} , ADP binding in the presence of Ca^{2+} was enhanced by approximately 3-fold, again demonstrating that calcium “traps” the reaction products in the active site of the kinase (Figure 6C).

Interestingly, based on SPR, calcium in combination with ADP can also induce stable binding of PKA-C to nonphosphorylated PKS, while interactions with PKI were very transient (Figure S3). On the other hand, PKS only bound weakly in the presence of ADP- Mg^{2+} , while PKI still binds tightly under these conditions. These results indicate the importance of the side chains for P-site residues in metal-substrate interactions.

Ca^{2+} Can Compete with Mg^{2+} and Thus Alters Kinase Activity and Regulation

In a physiological context, at least some subcellular compartments contain both Mg^{2+} and Ca^{2+} which can influence substrate or inhibitor binding. Therefore, we asked whether Ca^{2+} can outcompete Mg^{2+} from the active site, thus lowering complex stabilization with

pseudosubstrate inhibitors. Using SPR, we measured PKA-C (30 nM) binding to GST-PKI in buffer containing a fixed concentration of 1 mM ATP/1 mM Mg^{2+} and various concentrations of Ca^{2+} (ranging from 0–8 mM). With increasing Ca^{2+} concentrations, the dissociation rate of the inhibitor complex was further enhanced and resulted in a reduced affinity (Figure 7A).

The corresponding experiment was also performed with the substrate PKS. In this case, the opposite effect was observed. Elevating Ca^{2+} concentrations caused an increase in the apparent binding of PKA-C to PKS due to the slowed kinetic reaction on the SPR chip (Figure 7B). This strongly demonstrates that Ca^{2+} can reduce the phosphoryl-transfer rate and also traps PKA-C with the product even in the presence of Mg^{2+} . At high ratios of $Ca^{2+}:Mg^{2+}$, PKA-C is likely to be released from pseudosubstrate inhibitors. Under the same conditions, PKA-C can phosphorylate substrates, however, with a drastically decreased turnover. This data emphasize the plausible role of single turnover events.

Discussion

Regulating the activity of a protein kinase is a critical step in signal transduction. Most kinases are regulated by a plethora of factors rather than by a single event switching it on or off.²⁴ These factors comprise (I) regulatory proteins, which bind to and temporarily alter kinase activity, (II) post-translational modifications including phosphorylation to either inhibit (i.e., inhibitory phosphorylation of cyclin-dependent protein kinases) or stabilize a catalytically efficient conformation (i.e., activation loop phosphorylation of kinases), or (III) binding of cofactors like metal ions.^{15,24,25} These regulatory layers interconnected and result in a highly concerted output for a particular signaling system. In the case of catalysis, metal ions are not necessarily considered to be true “regulatory elements,” since enzymes are thought to choose the “right” metal ion by providing a specific binding site. Yet, metal ion concentrations highly depend on the subcellular compartment. Furthermore, although many enzymes appear to prefer a certain metal ion, they are able to use others.²⁶

Kinases are highly dependent on divalent metal ions and, so far, there is only one protein kinase known (Ca^{2+} /calmodulin-dependent Ser-Thr kinase, CASK) that does not require divalent metal ions for catalysis and is even inhibited at high metal ion concentrations.²⁷ The prototypical protein kinase A is able to use various divalent metal ions for phosphoryl transfer including Mn^{2+} , Co^{2+} , or even Ni^{2+} .¹³ Interestingly, some divalent metal ions (i.e., Ca^{2+} or Sr^{2+}) are able to stabilize nucleotide binding with the catalytic subunit, but they do not support steady state catalysis.¹⁸ Furthermore, no direct correlation between nucleotide stabilization or phosphotransferase activity and the ionic radii or the electronic configurations of the metals has been observed.¹⁸ The major role of the metal ions is the compensation of the negative charges associated with ATP binding and also to assist in γ -phosphoryl-transfer.²⁸ Initially, the binding of the nucleotide generates new ionic binding sites. In PKA, one site is directly correlated to the substrate’s P-3 basic residue, and the other sites (named Me1 and Me2, see below) are occupied by metal ions.²⁸ Binding of two Mg^{2+} ions is required to stabilize the PKA-C:substrate complex by closing the active site cleft, and thus the metals act as linchpins to lock the active conformation, and in particular the γ -phosphate, in place and poised for transfer.^{15,28} Historically, due to the decrease in

catalytic activity with increasing metal ion concentrations (mM range), the low affinity binding site (Me1) was termed the inhibitory site, while the tightly bound metal (Me2) was termed the activating metal or activating metal binding site.¹⁴

A recent structure showed PKA bound to IP20 at low Mg concentrations and one that displays the phosphoryl transfer by PKA in a crystal lattice. These structures shed new light on the roles of each metal. Whereas Mg1 was thought to be the activating metal, the PKA-C:MgATP:IP20 structure at low Mg concentrations showed only Mg2 bound to the PKA-C:IP20 complex.²⁹ Furthermore, although Mg1 was thought to remain in the product complex until the phosphorylated substrate was released, Bastidas et al. showed that Mg1 was expelled after phosphoryl transfer.³⁰ In this structure displaying complete phosphoryl-transfer with AMP-PNP, the residual electron density for Mg1 was very low, albeit all six ligands were still available. A closer look at the metal ion modeled in the residual electron density reveals a highly distorted coordination sphere for this metal, while octahedral Mg2 coordination is unaffected due to binding of an additional water molecule at the vacant binding site (Figure 8).³⁰ Interestingly, this is different from the Mg2 coordination in the PKA-C:MgATP:IP20 structure showing an atypical tetrahedral coordination of the magnesium ion.²⁹ These findings led to the conclusion that Mg2 binds with the nucleotide first, while Mg1 is the weakly bound metal ion transiently bound for catalysis, which was also observed for CDK2.¹⁶ The low affinity of the second metal site can also be explained by the fact that this binding site inherently suffers from the use of Asp184 as a bidentate ligand with a predefined coordination angle.^{16,17} Any resulting octahedral coordination of the Mg ion has to be distorted due to an angle of about 54°, while perfect octahedral coordination would instead be 90° (Figure 8B). Nonetheless, both metal ions assist in γ -phosphoryl-transfer to a substrate's P-site acceptor residue.¹⁶

As a consequence of phosphotransfer, the coordination spheres change, resulting in an overall destabilization of the product complex. In the PKA-C:Mg₂ADP:pSP20 structure, this is reflected by increased B-factors for the glycine-rich loop and the phosphorylated substrate (Figure 9).¹⁹ Release of the metal ion (Mg1) is then accompanied by a sequence of several conformational changes involving the glycine-rich loop opening to allow for P-substrate release and a portion of the C-tail becoming disordered. These steps are also crucial for the other rate-limiting step: MgADP release. As long as the phosphoryl group has not been transferred from ATP, Mg²⁺ locks the enzyme:substrate complex in place and then rapidly causes release of the reaction products following transfer of the γ -phosphoryl group. This corresponds to slow dissociation rates for the (pseudo)-Michaelis complex and fast dissociation rates for the enzyme:product complex. Thus, metal ions may “sense” the current status of the reaction progress based on changing coordination geometries. Mg²⁺ and Ca²⁺ act in opposite ways. Ca²⁺ binds in complex with ATP to the catalytic site (Ca2) but is not able to effectively stabilize the [ES] complex. The second Ca²⁺ ion site (Ca1) is not occupied securely, and the substrate is released more easily. However, once the phosphoryl group is transferred, the resulting coordination geometry builds up a highly stable pentagonal bipyramidal complex, which is found in many Ca²⁺ binding proteins such as the EF hand motif of calmodulin.^{31–33} The Me–O distances (between 2.3 and 2.5 Å) together with the ligand angles are an excellent fit to such a heptacoordination with Ca²⁺ as the

central metal ion.^{19,34} The increased stability is further underpinned by the B-factors of structural elements surrounding Me1 in the PKA-C:Ca₂:ADP:pSP20 structure compared to the PKA-C:Mg₂:ADP:pSP20 structure (Figure 9).

Moreover, we demonstrate that ADP is more tightly bound to PKA-C with Ca²⁺ rather than Mg²⁺. Therefore, calcium will trap both the phosphorylated substrate and ADP in the active site cleft. This explains the low activity of PKA-C with calcium under steady-state conditions since phospho-substrate release and ADP release are rate limiting for catalytic turnover.²³ Figure 10 summarizes a hypothetical catalytic cycle depicting in detail critical steps.

To elucidate the effects of both ions, we utilized a substrate analog of the heat-stable protein kinase inhibitor PKI to directly compare a substrate with a corresponding pseudosubstrate. This engineered substrate has an unusually high affinity to the catalytic subunit even in the absence of metal nucleotide (89 nM) due to an amphipathic helix that immediately precedes the arginine-rich peptide-binding site.⁹ As a consequence, the turnover of PKS is slowed significantly, allowing for an SPR-based analysis. In a radioactive kinase assay with PKS, we also found that catalytic activity was notably reduced as compared to peptide substrates.⁷

Our results clearly demonstrate how two prominent divalent metal ions have distinct roles on kinase activity simply due to their preferred coordination. For efficient catalysis, both an ideal confinement and a specific metal seem to be necessary. In the field of bioinorganic chemistry, the term “entatic state” is used to illustrate how an enzyme adapts to its current function. The IUPAC describes an entatic state as “a state of an atom or group which, due to its binding in a protein, has its geometric or electronic condition adapted for function.”^{35,36} In contrast to enzymes, metal ions as coordination centers are static atoms and can only change their electronic state. In the case of alkaline earth metals such as Mg²⁺ and Ca²⁺, oxidation or reduction are unfavorable, and thus different ions must act differently due to their imposed surroundings. This includes the geometry (coordination sphere) as well as the types of ligands chelating the metal ion. Protein kinases have adapted to high intracellular Mg²⁺ concentrations since they use this metal ion very efficiently; however, PKA-C and other kinases can additionally employ different metals. While this can lead to malfunction due to a toxic mismetallation, it is also conceivable that some physiologically relevant metal ions like Ca²⁺, Mn²⁺, or even Zn²⁺ can play a role in the fine-tuning of kinase activity and regulation.³⁷

PKA can be found directly in the vicinity of calcium channels including the ryanodine receptor (RyR) or voltage-dependent calcium channels (VDCCs) where calcium is expected to have high local concentrations (10⁻⁴ M).^{38,39} Along this line, the influence of calcium sparks on PKA activity and regulation should be considered. Furthermore, we found that in the presence of Ca²⁺ the catalytic subunit can phosphorylate substrates but in contrast to Mg²⁺ cannot be inhibited by PKI very well. Interestingly, the binding affinity to PKI was even lower when no metal ions are present (EDTA Figure 3B and D). During the submission of this manuscript, new PKA crystal structures containing Ca²⁺ were published.⁴⁰ These structures reveal a reorientation of the β - and γ -phosphoryl groups of ATP, which may account for the reduced affinity in the presence of Ca²⁺.

A pseudosubstrate inhibition motif is not unique to PKI. Type I PKA regulatory subunits are major pseudosubstrate inhibitors of PKA-C. Therefore, we also tested PKA-C binding to RI α ₉₂₋₂₄₅, a mutant form of RI α lacking the dimerization domain as well as the second cyclic nucleotide binding domain. As found with PKI, calcium resulted in drastically reduced binding to the pseudosubstrate inhibitor as compared to magnesium (Figure S4). In contrast, substrate inhibitors, such as RII regulatory subunits, will bind in the presence of Ca²⁺.⁴¹ This could be an indication why A-Kinase Anchoring Proteins (AKAPs) localize the catalytic subunit via type II R-subunits nearby calcium channels.³⁸ This scenario would make PKA activity prone to Ca²⁺ regulation and provides a novel concept for crosstalk between the second messengers cAMP and calcium.

Conclusion

Our study sheds new light on how metal ions influence protein kinase activity and inhibition. We demonstrate that two prominent divalent metal ions differentially control kinase activity simply by their preferred coordination. For an efficient catalysis, both, an ideal confinement of the metal-nucleotide binding pocket and a specific metal are mandatory. A scheme describing the catalytic cycle is provided. Most protein kinases seem to have adapted to magnesium, since this metal is very effective in assisting substrate binding and at the same time efficient in product release. Calcium behaves in an opposite way by trapping the product at the kinase active site. This explains the low steady-state phosphoryl-transfer activity. In addition, kinase inhibition by pseudosubstrate inhibitors is highly impaired in the case of Ca²⁺. Since PKA can be tethered directly to Ca²⁺ channels, our results strongly suggest that PKA activity is susceptible to Ca²⁺ regulation. This provides a novel link between cAMP- and calcium signaling.

METHODS

Expression and Purification of PKA Catalytic Subunits

The PKA-catalytic (C) subunit (isoform $\alpha 1$) was overexpressed in *E. coli* BL21(DE3) cells after induction with 0.4 mM IPTG for 16 h at RT using the expression vector pRSETb-hPKAC α and then purified by affinity chromatography using an IP20-resin as described earlier.⁴² The purified C-subunit was then stored at 4 °C in elution buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 200 mM L-arginine, 1 mM EDTA), and the buffer was changed to buffer A (20 mM MOPS, pH 7.0, 150 mM NaCl, 2 mM β -mercaptoethanol) by dialysis preceding further use.

His(6x)-tagged PKA C-subunit transformed BL21(DE3) cells were grown at 18 °C for 12–16 h after induction (1 mM IPTG). Cells were lysed in buffer containing 50 mM KH₂PO₄ at pH 8.0, 250 mM NaCl, and 1 mM β -mercaptoethanol. The lysate was then incubated with Ni beads for metal affinity chromatography, and the beads were subsequently washed with a lysis buffer containing 10 mM imidazole and finally eluted in 200 mM imidazole. Eluted fractions were pooled and dialyzed against 25 mM KH₂PO₄ and 25 mM KCl at pH 7.5 and loaded onto a Mono-S column. The (6x)His-tagged PKA C-subunit resolved into three peaks on the cation-exchange column in a gradient of 25 mM–1 M KCl. Peak II with three phosphorylation sites⁴³ was used for all nucleotide binding studies.

Site-directed Mutagenesis, Expression, and Purification of GST Tagged PKI α and PKI α A21S (GST-PKS)

Site-directed mutagenesis of the GST fusion protein GST-PKI α to generate GST-PKI α A21S was done by using the QuickChange mutagenesis kit (Stratagene), and successful mutagenesis was confirmed by sequencing (GATC biotech). After overexpression in *E. coli* BL21(DE3) cells for 16 h at RT, the GST fusion proteins were purified using Protino glutathione agarose 4B according to the manufacturers instructions (Machery-Nagel). For *in vitro* phosphorylated GST-PKS (GST-pPKS), the GST-resin bound protein was incubated with 1 μ M PKA C-subunit in buffer P (20 mM MOPS, pH 7.0, 150 mM NaCl, 10 mM MgCl₂, 1 mM ATP) for 1 h at 30 °C and washed four times with PBS (pH 7.4) containing 1 mM EDTA prior to elution. The purified proteins were stored in PBS (pH 7.4) at -20 °C until further use.

Kinase Assay

Protein kinase activity was measured in principle as described by Kish using a radioisotopic method.⁴⁴ The reaction mixture of 300 μ L contained 20 mM MOPS (pH 7.0), 150 mM NaCl, 100 μ M ATP, app. 550 fmol [γ -³²P]ATP (stock 110 TBq/mmol, Hartmann Analytic), 5 nM purified human PKA C-subunit, 30 μ M purified GST-PKS, and the respective amount of metal (MgCl₂ or CaCl₂) as indicated in the figure. The reaction was started by the addition of C-subunit, incubated at 30 °C, and 50 μ L samples were taken after 10, 20, 45, and 60 min, respectively. The samples were directly added to 500 μ L of ice cold 1 mM ATP (in 20 mM MOPS (pH 7.0), 150 mM NaCl) followed by 550 μ L of ice cold 10% trichloroacetic acid (TCA) containing 3% sodium pyrophosphate and incubated for at least 5 min on ice. The stopped reaction mixture was filtered under a vacuum through mixed cellulose ester membrane filters (MF-Millipore Membrane Filter, 0.45 μ m, 25 mm diameter) presoaked in 1 mM ATP for at least 30 min at RT. Each filter was washed twice with 5 mL of ice cold 5% TCA containing 1.5% sodium pyrophosphate. Radioactivity of each filter was counted in 20 mL of distilled water in a scintillation counter (Hidex 300 SL, for 300 s or until a maximum of 10 000 counts was achieved) by detection of Cerenkov radiation.

For each run, the total amount of radioactivity, the blank (reaction mixture without kinase), as well as the $t = 60$ min of 1 mM MgCl₂ was measured. For evaluation all values (cpm) were blank value subtracted. For calculation of the relative kinase activity in percent the values were normalized to the respective value of 1 mM MgCl₂ at $t = 60$ min (see Figure 2). Data evaluation was carried out using Graph Pad Prism 5. Calculation of free and complexed metals was performed with the software Bound and Determined.⁴⁵

Surface Plasmon Resonance (SPR)

SPR interaction studies were performed using a Biacore T100 instrument (GE Healthcare) according to Zimmermann et al.¹⁵ Briefly, a polyclonal anti-GST antibody (Carl Roth, 3998.1) was immobilized via standard NHS/EDC amine coupling on a S-series CM5 sensorchip (GE Healthcare) to a level of 15 000 response units (RU). At the beginning of each measurement cycle 70–130 RU of each GST fusion protein (GST-PKI, GST-PKS and GST-pPKS) was sequentially captured on separate flow cells. Interaction analysis was then started by the injection of increasing concentrations of PKA C-subunit at a flow rate of 30

$\mu\text{L}/\text{min}$ for 150 or 300 s (association) at 25 °C. Dissociation was then induced by switching to a buffer without C-subunit and was monitored for 150 or 300 s. Running buffer (20 mM MOPS, pH 7.0, 150 mM NaCl, 100 μM EDTA, 100 μM EGTA, 0.005% P20 surfactant) was supplemented either with 10 mM MgCl_2 or 10 mM CaCl_2 and/or 1 mM ADP or 1 mM ATP. Complex formation of C-subunit with GST-PKS was analyzed in running buffer containing 0.2 mM AMP-PNP (Roche Lifescience) and 1 mM of MgCl_2 or CaCl_2 , while measurements of product complex formation and dissociation were performed with 0.2 mM ADP and 1 mM of MgCl_2 or CaCl_2 .

Unspecific binding was removed by subtracting SPR signals from a blank flow cell (without GST-protein) and additional blank runs without C-subunit in each buffer condition (double referencing). After each cycle, the sensorchip was regenerated by injecting 10 mM glycine, pH 2.2, to remove the GST-fusion protein until the baseline level was reached.

On-chip phosphorylation of GST-PKS was investigated by the injection of an anti-PKA-substrate antibody (anti RRXpS/pT, Cell Signaling, #9624). For this, GST-PKS was immobilized to a level of 350 RU, and the C-subunit was either injected in running buffer only or supplemented with 1 mM ATP and 10 mM MgCl_2 for 300 s at a flow rate of 30 $\mu\text{L}/\text{min}$ and 25 °C. Subsequently, 100 nM of the anti-PKA-substrate antibody diluted in TBS-P buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 0.005% P20 surfactant) was injected over the chip surface for 150 s association and 150 s dissociation. Kinetic data analysis using nonlinear regression was carried using the Biacore T100 Evaluation Software 2.0.2 and BIAevaluation 4.1.1 (GE Healthcare) based on a global fit assuming a 1:1 Langmuir binding model.

Fluorescence Polarization (FP)

FP assays were performed using the fluorescein labeled inhibitor peptide PKI 5–24, FAM-IP20 (synthesized by Peps4Life) according to Saldanha et al.⁴⁶ A minimum of 12 protein concentrations was tested for each buffer condition in triplicate in two independent measurements. Measurements were performed in an assay buffer (20 mM MOPS pH 7.0, 150 mM NaCl, 100 μM EDTA, 100 μM EGTA, 0.005% CHAPS) supplemented with ATP, ADP, MgCl_2 , or CaCl_2 , according to each condition at 20 °C using 384 well microtiter plates (PerkinElmer Optiplate, black) and Fusion αFP reader (PerkinElmer) as described by Moll et al.⁴⁷ Binding constants were determined by fitting data to sigmoidal dose response function using the software GraphPad Prism 5.

Nucleotide Binding Assays

Nucleotide binding assays were performed by determination of changes in the intrinsic tryptophan fluorescence of PKA catalytic subunit upon ligand binding. ATP, ADP, and AMP-PNP were obtained from Sigma. Stock concentrations were ascertained by using the extinction coefficient of adenine ($15\,400\,\text{M}^{-1}\,\text{cm}^{-1}$ at 260 nm). Increasing concentrations of nucleotide (0–250 μM) were titrated against a fixed concentration of (6xHis)-PKA-C (3.0 μM) in a buffer containing 25 mM MOPS, 100 mM NaCl, 100 μM EDTA, and 100 μM CaCl_2 at pH 7.0. Buffer was supplemented with 10 mM of free metal concentration for both magnesium (MgCl_2) and calcium (CaCl_2). Changes in intrinsic tryptophan fluorescence

were monitored using a Jobin Yvon Horiba Fluorolog-3 Jobin spectrofluorometer. Samples were excited at 295 nm (3 nm slit width), and fluorescence emission spectra were recorded from 300–400 nm (3 nm slit width). The excitation wavelength of 295 nm was chosen to minimize the inner filter effect of ATP.⁴⁸ A decrease in fluorescence of PKA at 331 nm was fitted to a single-site binding model using the nonlinear regression module of Sigma Plot Software to obtain the dissociation constants (K_D).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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aa	1	10	20	30	40	50	60	70	75
PKI	TDVETTYADFIASGRTGRRNAIHDILVSSASGNSNELALKLAGLDINKTEGEEDAQRSSTEQSGEAQGEAAKSES*								
IP20	TTYADFIASGRTGRRNAIHD								
PKS	TDVETTYADFIASGRTGRRNSIHDILVSSASGNSNELALKLAGLDINKTEGEEDAQRSSTEQSGEAQGEAAKSES*								
SP20	TTYADFIASGRTGRRASIH								

Figure 1.

Sequences of the heat-stable protein kinase inhibitor (PKI), its substrate derivative PKS, and peptides IP20 and SP20. The main recognition motif of the PKA catalytic subunit is shown with a gray background.

Figure 2.

Protein substrate phosphorylation in the presence of Mg^{2+} and Ca^{2+} under steady-state conditions. The phosphotransfer ability of PKA-C, using a substrate derivative of the heat-stable protein kinase inhibitor GST-PKS, was tested in the presence of either Mg^{2+} or Ca^{2+} at various metal concentrations. Relative activities are expressed as a percentage of the amount of incorporated ^{32}P after 60 min in the presence of 1 mM $MgCl_2$ (see inset). All measurements were done at least in triplicate, and values are given as the mean $\pm$ standard deviation (SD).

Figure 3.

Calcium does not support high affinity binding of PKA-C to pseudosubstrate inhibitors. (A) Fluorescence polarization was used to determine binding affinities under various buffer conditions. Increasing concentrations of the PKA-C subunit were incubated with 0.9 nM FAM-IP20. Binding affinities decreased by 3 orders of magnitude when magnesium ($K_D = 1.7$ nM) was substituted with calcium ($K_D = 570$ nM) or in the absence of any metal or nucleotide ($K_D = 279$ nM). The plot shows representative binding experiments in triplicate. Table 1 shows the mean $\pm$ SD values of two independent triplicate experiments. SPR

analyses in (B) demonstrate that binding of PKA-C to full-length PKI without Mg^{2+} ATP leads only to transient binding ($K_D = 197$ nM), while the addition of Mg^{2+} and ATP causes high affinity binding with a decreased off-rate (C, $K_D = 0.5$ nM). In contrast, Ca^{2+} ATP is unable to stabilize the complex and shows a high off-rate (D, $K_D = 300$ nM) that is nearly comparable to the EDTA control. (E) Nucleotide (ATP) binding to PKA-C was probed in the presence of 10 mM Mg^{2+} and 10 mM Ca^{2+} . Both metals induced binding of ATP to PKA-C in the low micromolar range.

Figure 4.

SPR analysis of PKA-C binding to PKS under various buffer conditions. (A) Complex formation in a metal-free buffer (EDTA/EGTA) shows transient binding kinetics with high on and off rates. However, compared to PKI, the on rate is slightly increased, which results in a higher affinity ($K_D = 89$ nM) compared to PKI ($K_D = 197$ nM). (B) In the presence of 1 mM ATP and 10 mM $MgCl_2$, a sharp peak is observed which disappears after several seconds, reflecting the complete phosphoryl-transfer reaction (including binding and dissociation) taking place on the sensor chip surface. (C) Replacing magnesium with calcium changes the observed binding kinetics. An apparent off rate is slowed down compared to Mg^{2+} , thus indicating a delayed phosphoryl-transfer and/or product release. (D) Schematic illustration of the phosphoryl reaction, taking place on the sensor chip surface, including substrate binding and product dissociation. Superposition of the two events results in a multi-phasic SPR curve.

Figure 5.

PKA-C:Substrate complexes are strongly stabilized by Mg^{2+} but not with Ca^{2+} . AMP-PNP (0.2 mM) as a nonhydrolyzable ATP analogue was utilized to compare PKA-C:PKS complex formation by SPR in the presence of either Mg^{2+} (1 mM) or Ca^{2+} (1 mM) prior to phosphoryl transfer. (A) With Mg^{2+} , the catalytic subunit shows stable complex formation with the unphosphorylated substrate ($K_D = 2.9$ nM). In the presence of calcium, the complex formation is hampered, mainly due to a higher off-rate lowering the affinity ($K_D = 29.5$ nM). Rate constants and affinities are summarized in Table 2, which were determined using BIAevaluation (GE Healthcare). (C) Binding of AMP-PNP to PKA-C was checked in the presence of either 10 mM Mg^{2+} or Ca^{2+} . Both metal ions induced binding of AMP-PNP to PKA-C, however, with a slightly higher affinity in the presence of Ca^{2+} compared to Mg^{2+} . (D) Scheme depicting the complex formation.

Figure 6.

Reaction products are trapped at the active site by calcium. SPR was utilized to investigate the product complex of PKA-C composed of the phosphoryl-transfer reaction products phospho-PKS and ADP (0.2 mM) in the presence of either (A) Mg^{2+} (1 mM) or (B) Ca^{2+} (1 mM). Regardless of whether calcium or magnesium was used for complex formation, the association rate constant was unchanged, although the dissociation rate (reflecting phospho-PKS release after phosphoryltransfer) was slowed down with Ca^{2+} in comparison to Mg^{2+} by a factor of 14. Rate constants and affinities are summarized in Table 3, which were determined using Biacore T100 Evaluation Software (GE Healthcare). (C) Binding of the second reaction product ADP to PKA-C was investigated in a nucleotide binding assay. The affinity of ADP was almost 3 times higher in the presence of Ca^{2+} compared to Mg^{2+} , thus showing Ca^{2+} is able to trap both reaction products at the active site. (D) Scheme depicting the release of the phosphorylated substrate.

Figure 7.

Calcium is able to destabilize the inhibitor complex (PKA-C:PKI) even in the presence of Mg^{2+} ATP. (A) Complex formation of PKA-C and full-length PKI was monitored by SPR with fixed concentration of 1 mM Mg^{2+} /1 mM ATP and increasing concentrations of calcium. Calcium competes with magnesium in a concentration dependent manner, resulting in higher off-rates, thus lowering the binding affinity to PKI. (B) In the case of a substrate (PKS), increasing calcium concentrations lead to prolonged reactions, due to product trapping at the active site cleft.

Figure 8.

Differences in the metal coordination of the product complexes C:Mg₂ADP:pSP20 (PDB 4IAD) and C:Ca₂ADP:pSP20 (4IAI). After phosphoryl transfer, both Mg²⁺ ions are coordinated by six ligands (A, B). Since Mg₂ loses its interaction with the transferred γ -phosphate, a water molecule occupies the vacant site. Mg₁ is still coordinated by the transferred phosphate but shows a highly distorted octahedral coordination sphere. (C, D) The calcium bound structure shows additional water molecules surrounding the Ca²⁺ ions, resulting in stable pentagonal bipyramidal structures with seven ligands. All structures were visualized using PyMOL v1.3 (Schrödinger LLC).

Figure 9.

B-Factor visualization of the ternary complexes showing PKA-C: Mg_2 AMPPNP:SP20 (PDB 4DG0), PKA-C: Mg_2 ADP:pSP20 (PDB 4IAD), and C: Ca_2 ADP:pSP20 (PDB 4IAI). Calcium:ADP is able to induce stable active site closing with a phosphorylated substrate-peptide while the comparable Mg^{2+} bound structure shows high B factors indicating a destabilized complex. Structures were aligned and visualized using PyMOL v1.3 (Schrödinger LLC).

Figure 10.

Catalytic cycle for protein kinase A. Substrate binding, phosphoryl transfer, and phospho substrate release could be accessed by the SPR-based strategy described here. ADP binding was determined by fluorescence titration.

Table 1

Interaction Analysis of PKA-C Binding to IP20 and PKI Under Various Buffer Conditions

	cofactors^a	k_a (1/Ms)	k_d (1/s)	K_D (nM)
FP (FAM-IP20)	EDTA			279 ± 40
	Mg ²⁺ ATP			1.7 ± 0.5
	Ca ²⁺ ATP			570 ± 200
SPR (GST-PKI)	EDTA	2.4 × 10 ⁶	3.3 × 10 ⁻¹	138
	Mg ²⁺ ATP	2.5 × 10 ⁶	1.3 × 10 ⁻³	0.5
	Ca ²⁺ ATP	1.5 × 10 ⁶	5.3 × 10 ⁻¹	353

^a 10 mM total metal concentration and 1 mM nucleotide concentration; EDTA/EGTA 100 μM each.

Table 2

SPR Analysis of Pseudo-Michaelis Complex Formation Using the Non-Hydrolyzable ATP Analog AMP-PNP

PKA-C:PKS	k_a (1/Ms)	k_d (1/s)	K_D (nM)
EDTA ^a	3.7×10^6	3.3×10^{-1}	89
Mg ²⁺ AMP-PNP ^b	2.8×10^6	8.1×10^{-3}	2.9
Ca ²⁺ AMP-PNP ^b	2.0×10^6	5.9×10^{-2}	29.5

^a 100 μ M EDTA/EGTA each.^b 1 mM total metal concentration, 0.2 mM nucleotide concentration, and 100 μ M EDTA/EGTA each.

Table 3

SPR Analysis of Product Dissociation After Phosphoryl-Transfer

PKA-C:pPKS	k_a (1/Ms)	k_d (1/s)	K_D (nM)
Mg ²⁺ ADP ^a	1.7×10^6	6.5×10^{-2}	38.2
Ca ²⁺ ADP ^a	2.3×10^6	4.7×10^{-3}	2.0

^a 1 mM total metal concentration and 0.2 mM nucleotide concentration.