Structural Characteristics That Govern Binding to, and Modulation through, the Cardiac Ryanodine Receptor Nucleotide Binding Site

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ABSTRACT

Comparative molecular field analysis (CoMFA) predicts that the large electrostatic field around the phosphate groups of ATP plays a crucial role in stabilizing the open state of the cardiac ryanodine receptor (RyR) channel. We therefore investigated the effects of adenosine-5'-[(β,γ-methylenetriphosphate) (AMP-PCP), an ATP analog with lower negative charge in this region, on the gating of the cardiac RyR channel. In the presence of 10 μM cytosolic Ca2+, AMP-PCP exhibited approximately 50% of the efficacy of ATP and optimal doses increased open probability (P_o) to only 0.441 ± 0.156 (n = 4), thus confirming the predictive ability of our preliminary CoMFA model. We also reveal that AMP-PCP has a higher affinity than ATP for the cardiac RyR, demonstrating that the structural properties required for tight binding to RyR differ from those necessary for recruiting long open states and high P_o values. CoMFA identified very strong correlations between the structures of adenine-based ligands and their affinity for RyR and different (but also highly significant) correlations between structure and the ability to activate the channel. Analysis indicates that ATP may be more effective than other adenine nucleotides because it can convert the greatest amount of binding energy into conformational changes that stabilize the open channel state.

ATP and related adenine nucleotides present in cardiac cells have an important function as regulators of RyR channel gating. ATP can induce long open events and a high open probability (P_o) in the presence of micromolar cytosolic Ca2+ (Kermode et al., 1998). High levels of adenine nucleotides and Ca2+ tend to inactivate the channel (Kermode et al., 1998; Ching et al., 1999); therefore, the effects of this group of ligands are complex and are likely to shape both the activation and inactivation processes of intracellular Ca2+ release in cardiac cells. Understanding the underlying molecular nature of the binding of adenine nucleotides to RyR channels and the structural features of ATP that produce open RyR channels is therefore important for a greater understanding of how RyR channels are regulated during the process of excitation-contraction coupling. In a preliminary CoMFA study, we previously correlated the structure of ATP and other adenine-based ligands with the ability to modulate the gating of native sheep cardiac RyR incorporated into planar phospholipid bilayers (Chan et al., 2000). With millimolar luminal [Ca2+] and a maintained cytosolic [Ca2+] of 10 μM, ATP induced P_o levels of approximately 0.9. The maximum P_o levels produced by the other adenine-based ligands investigated (ADP, AMP, adenosine, adenosine) were much lower. CoMFA demonstrated a high correlation between ligand structure and maximum P_o induced. Although it is now well established that the purine ring is important for agonist activity (Morii and Tonomura, 1983; Meissner, 1984; Chan et al., 2000), we demonstrated that the phosphate groups are essential to the high efficacy of ATP. Our model predicts that the charge produced by the phosphate groups is the single most important factor that enables bound ATP molecules to activate the cardiac RyR channel to a greater extent than ligands with fewer phosphate groups (for example, ADP and AMP). To investigate the significance of the charge on the phosphate groups of ATP, we examined how reducing charge in this region would affect the gating of RyR channels. AMP-PCP is a nonhydrolysable analog of ATP in which a methyl group substitutes for the oxygen between the β- and γ-phosphate groups. The methyl substitution leads to a decrease in negative charge around the phosphate groups. We would predict a diminished ability of such a ligand to fully open the channel in the presence of 10 μM cytosolic Ca2+. This pre-

ABBREVIATIONS: RyR, ryanodine receptor; CoMFA, comparative molecular field; CoMSIA, comparative similarity index analysis; AMP-PCP, adenosine-5'-(β,γ-methylenetriphosphate); SR, sarcoplasmic reticulum; EMD 41000, 2-(2-methoxy-4-methyl sulfonylphenyl)-3a,4,5,6,7,7a-hexahydro-1H-imidazo[4,5-c]pyridine.

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diction, however, runs counter to accepted ideas about the effectiveness of AMP-PCP as an activator of RyR channels in that it is reported to be at least as effective as ATP (Smith et al., 1985). In fact, the stable nature of AMP-PCP has led to the routine use of this analog as a replacement for ATP in functional studies of RyR (for example, see Xu et al., 1996; Fruen et al., 2002).

Our single-channel experiments show that AMP-PCP is, as predicted from our initial model, a partial agonist at the ATP sites on the cardiac RyR, thus confirming the validity of our model. Importantly, we also show that AMP-PCP has a higher affinity for RyR than ATP, demonstrating that the affinity and efficacy of ligands at the ATP sites on RyR are dependent on different structural characteristics. Using the new information obtained from observing the effects of AMP-PCP on channel gating, we now present a more detailed description of the correlations between adenine nucleotide structure and the ability to open (efficacy) the sheep cardiac RyR channel. For the first time, we describe the structural characteristics of adenine nucleotides that are associated with high affinity for the ATP sites on the cardiac RyR. Electrostatic interactions are critically involved in the process of nucleotide binding to RyR, although steric factors also play a role. Use of comparative similarity index analysis (CoMSIA) demonstrates that although changes in hydrogen-bond acceptor ability of the adenine nucleotides are strongly correlated with changes in affinity of the ligand for RyR, it does not seem to influence the subsequent ability of the ligand to increase $P_o$.

**Materials and Methods**

**Preparation of SR Membrane Vesicles and Planar Lipid Bilayer Methods.** Heavy SR membrane vesicles were prepared from sheep hearts as described previously (Sitsapesan et al., 1991) and rapidly frozen and stored in liquid nitrogen. Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as described previously (Sitsapesan et al., 1991). The vesicles were fused in a fixed orientation such that the cis chamber corresponded to the cytosolic space and the trans chamber to the SR lumen. The trans chamber was held at ground and the cis chamber was perfused with potentials, Ca$^{2+}$/H$^{1}$0.6

**Data Acquisition and Analysis.** Single channel recordings were displayed on an oscilloscope and recorded on digital audio tape. Steady-state recordings were carried out at 0 mV. At this holding potential, Ca$^{2+}$

**Materials.** ATP, adenosine, and adenosine were 99% pure (Sigma, Poole, UK). EMD 41000 was a gift from Merck (64271 Darmstadt, Germany). Solutions were prepared using MilliQ de-ionized water (Millipore, Harrow, UK) and filtered through a Millipore membrane filter (pore size, 0.45 μm) before use. Other chemicals were AnalR or the best equivalent grade from BDH or Sigma (Poole, UK).
The Effects of AMP-PCP on RyR Channel Gating.

Figure 1 illustrates how AMP-PCP activates single sheep cardiac RyR channels incorporated into planar lipid bilayers. RyR channel openings are typically brief when activated solely by 10 μM cytosolic Ca^{2+} as demonstrated in the top trace. AMP-PCP caused a dose-dependent increase in $P_o$, but even the most effective concentrations (500 μM–2 mM) could not fully activate the channels. Inspection of the traces shows that AMP-PCP produced large increases in the frequency of channel opening. At the maximum level of activation (illustrated in Fig. 1 and occurring at approximately 1 mM AMP-PCP), very brief open and closed events occurred, giving rise to a flickery appearance of gating. This flickery type of gating is characteristic of agents that activate the channel via the ATP sites (Kermode et al., 1998). ATP, itself, also causes flickery gating but at the most effective concentrations produces long events in addition to bursts of short open and closed events. Equivalent long open events were not observed with AMP-PCP under the controlled cytosolic [Ca^{2+}] and pH of the experiments. Figure 2 illustrates the typical gating behavior of a sheep cardiac RyR channel activated by 1 mM ATP. Consecutive single-channel recordings demonstrate the spontaneous changes in gating from high $P_o$ levels characterized by long open times to lower $P_o$ levels characterized by brief open and closed events. Over 3 min of recording, $P_o$ averaged 0.89. This is approximately double the average maximum $P_o$ obtained with AMP-PCP under the same experimental conditions (see Fig. 1). No measurable change in single-channel conductance was observed after addition of AMP-PCP to the cytosolic chamber (results not shown).

The relationship between $P_o$ and [AMP-PCP] is shown in Fig. 3. The maximum $P_o$ obtained was 0.441 ± 0.156 at 1 mM AMP-PCP (SEM; $n = 4$), well below the maximum $P_o$ level that could be achieved with ATP (~0.9) under identical experimental conditions. The results highlight the partial agonist nature of AMP-PCP. The EC_{50} value for channel activation by AMP-PCP was 164 μM. This is lower than the EC_{50} for channel activation by ATP which is 220 μM (Kermode et al., 1998) indicating that AMP-PCP has a higher affinity for the cardiac RyR than ATP. The Hill coefficient for channel activation by AMP-PCP was 2.1 indicating that multiple AMP-PCP molecules must bind to the channel to produce the maximum effect. Concentrations of AMP-PCP above the optimum doses produced channel inactivation, as observed for other adenine nucleotides (Kermode et al., 1998; Ching et al., 1999).

The modulation of $P_o$ by AMP-PCP resulted from changes in both the open and closed lifetime durations. Channel activation was caused mainly by dose-dependent reductions in the mean closed times. For example, in a typical channel activated by 10 μM cytosolic Ca^{2+}, 500 μM AMP-PCP reduced the mean closed time from 101 to 1.63 ms, whereas the mean open time was increased only from 0.51 to 2.62 ms. High [AMP-PCP], which produced channel inactivation rejection by AMP-PCP was 164 μM. This is lower than the EC_{50} for channel activation by ATP which is 220 μM (Kermode et al., 1998) indicating that AMP-PCP has a higher affinity for the cardiac RyR than ATP. The Hill coefficient for channel activation by AMP-PCP was 2.1 indicating that multiple AMP-PCP molecules must bind to the channel to produce the maximum effect. Concentrations of AMP-PCP above the optimum doses produced channel inactivation, as observed for other adenine nucleotides (Kermode et al., 1998; Ching et al., 1999).

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versed the changes in mean open and closed time durations: 2 mM AMP-PCP produced an increase in the mean closed time to 7.31 ms and a decrease in mean open time to 0.8 ms.

How Does AMP-PCP Activation of RyR Compare with the Effects of Other Adenine Nucleotides? In Fig. 4, we compare the affinity, efficacy, and Hill coefficient for the activation of the cardiac RyR by AMP-PCP and other adenine nucleotides (Chan et al., 2000). The properties of AMP seem to deviate significantly from those of the other ligands shown. AMP has a very low affinity for RyR, a low ability to increase $P_o$, and also activates the channel with a Hill coefficient <1. ADP, although it has a low affinity and shows no evidence for a positively co-operative action, still has a greater ability to open the channel if present in sufficient quantities ($\geq 10$ mM). In the presence of 10 $\mu$M cytosolic $\text{Ca}^{2+}$, AMP-PCP is not able to produce the same high level of activation as ATP because it does not induce long enough open events. AMP-PCP, however, has the highest affinity for RyR and the highest Hill coefficient of all the adenine nucleotides studied.

CoMFA of $P_o$. The CoMFA values of $P_o$ described in this article are similar to those obtained earlier with the more limited data set (Chan et al., 2000). In the previous report, electrostatic factors accounted for 64% of the observed correlation between structure and $P_o$. Steric factors accounted for the remaining 36%. In this report, we have used $P_o$ to calculate the increased free energy required to raise the $P_o$ above that seen in the presence of $\text{Ca}^{2+}$ and zero ATP analog. This is calculated as $\Delta \Delta G = (1.987 \text{ cal/mol K}) (296 \text{ K}) \ln(P_o \text{ in the presence of ATP analog})/P_o \text{ in the presence of Ca}^{2+}$ only). The bilayer experiments were done at 23°C (296 K). The cross-validated correlation coefficient (a measure of predictive ability) for $P_o$ is 0.307 with a final correlation coefficient ($r^2$) of 0.994.

Interestingly, AMP is the greatest outlier, not AMP-PCP. The model seems reliable for analysis of the relationship between structure and activity of this group of compounds because of A) the extreme stability of the model in bootstrapping (extremely low S.E.E., < 0.1%, data not shown), B) the linearity of the correlation between predicted and experimental values ($r^2$); and C) the close agreement between predicted and experimental values (9% error or less). Omission of AMP-PCP from the data set increases the cross-validated correlation coefficient to 0.558 (final correlation coefficient, 0.998). In CoMFA, the correlation between structure and activity is based on the steric and electrostatic properties of the compounds. If a correlation exists, as in this case, the CoMFA can assign how much of the structure-activity relationship can be explained by steric or electrostatic differences between the compounds. For the groups of compounds re-

**Fig. 3.** Relationship between $P_o$ and AMP-PCP concentration in the presence of 10 $\mu$M free cytosolic $\text{Ca}^{2+}$. S.E.M. for $n \geq 4$ is shown.

**Fig. 4.** Comparison of the actions of AMP-PCP with ATP, ADP, AMP, adenosine, and adenosine on cardiac RyR channel gating in the presence of 10 $\mu$M free cytosolic $\text{Ca}^{2+}$. Top, maximum $P_o$ that can be achieved with each ligand. Center, comparison of EC$_{50}$ values. Bottom, Hill coefficient for RyR channel activation for each ligand. The dose ranges used to obtain the above information were: AMP-PCP, 10 $\mu$M–10 mM; ATP, 10 $\mu$M–20 mM; ADP, 100 $\mu$M–50 mM; AMP, 100 $\mu$M–10 mM; adenosine, 10 $\mu$M–5 mM; adenosine, 20 $\mu$M–5 mM. For AMP-PCP, ATP, ADP, AMP, adenosine, and adenosine, maximum $P_o$ was observed at 1, 2, 20, 20, 1, and 2 mM, respectively.
ported here, 69% of the correlation is attributable to differences in electrostatic fields of the compounds and 31% is attributable to differences in the steric (van der Waals) properties of the compounds. Electrostatic factors dominate steric factors. In the previous report, we contoured the relationship between changes in the steric and electrostatic fields at the nucleotide binding site and the ability of the adenine nucleotides to increase $P_o$. In Fig. 5, we show a similar diagram illustrating the relationships between changes in structure and changes in the maximum $P_o$ attainable after inclusion of AMP-PCP into the data set. Such diagrams are useful in visualizing how physicochemical factors modulate channel function.

An important property of a CoMFA is the ability to predict the properties of novel compounds. The CoMFA presented earlier (Chan et al., 2000) predicted the free energy of the missing AMP-PCP well. It correctly predicted that AMP-PCP would be less effective than ATP in promoting $P_o$. For comparison, the experimental $P_o$ of ATP is 0.90, the predicted $P_o$ of AMP-PCP is 0.78, and the experimentally determined $P_o$ of AMP-PCP is 0.44. The predicted value of AMP-PCP is a reasonable extrapolation of the CoMFA from the oxygen bridging the terminal phosphorus atoms in ATP to the carbon bridge in AMP-PCP. The error of the estimate is 909 cal/mol compared with the experimentally determined free energy change of 1993 cal/mol (a 46% error). When AMP-PCP is included in the CoMFA basis set, the error decreases to 24 cal/mol (a 1% error). Unless otherwise noted, the full basis set (including AMP-PCP) will be used for all following analyses.

**CoMSIA of $P_o$** Using CoMSIA, no correlations were found between changes in steric bulk, hydrogen bonding, or hydrophobicity and the ability to modulate $P_o$. None of these properties, when tested alone, was sufficient to explain the differences in biological activities. The lack of correlation was seen regardless of inclusion of AMP-PCP in the test group of compounds. Only electrostatic CoMSIA showed strong correlations between changes in structure and changes in $P_o$ ($q^2 = 0.624$, $r^2 = 0.992$). Of the properties tested, only the electrostatic field is sufficient by itself to predict $P_o$.

**CoMFA of $EC_{50}$** We have expressed $EC_{50}$ values as the apparent free energy of binding. This is calculated as $\Delta G = (1.987 \text{ cal/mol K})(296 \text{ K})[\ln (EC_{50})]$. The cross-validated correlation coefficient ($q^2$) for $EC_{50}$ is 0.583 ($r^2 = 1.00$). As for $P_o$, most of the correlation (66%) is between changes in electrostatic field and changes in $EC_{50}$. The remainder of the correlation is with changes in steric bulk. Figure 6 illustrates the locations in which changes in structure are most highly correlated with changes in $EC_{50}$, and compares the regions in which steric and electrostatic factors play the greatest role.
CoMSIA of EC$_{50}$. In contrast to the correlation with $P_o$, additional analysis by CoMSIA found a correlation between hydrogen-bond acceptor ability and changes in EC$_{50}$ (cross-validated correlation coefficient, 0.33; final correlation coefficient, 1.0). Figure 7 details the regions in which hydrogen-bond accepting properties are correlated most strongly with the affinity of the adenine nucleotides for RyR. As was the case with $P_o$, a correlation was seen with changes in electrostatic CoMSIA but not hydrogen-bond donating, hydrophobic potential, or steric bulk. The results demonstrate that at least part of the difference between the ligand-receptor interactions leading to changes in $P_o$ and EC$_{50}$ is the importance of hydrogen-bond accepting ability of the modulators.

**Structure-Activity Relationships and Energetic Considerations.** By correlating the single-channel effects of ATP and related ligands with their structural characteristics, we can distinguish between the structural properties required to produce strong binding to RyR and those required to drive the channel into the open state. Interactions between a ligand and a receptor can be divided into two broad types: A) those that exist in the ligand-receptor complex only and B) those that relate the relative solvation of the ligand by the receptor to that of the bulk medium (including counter ions, detergents, and so forth). In this report, the properties of AMP-PCP as an agonist at ATP sites on RyR have been analyzed in the context of other nucleotides and analogs. The properties compared include the maximum $P_o$, the concentration of ligand required to produce half-maximum $P_o$ (EC$_{50}$), the Hill coefficient, and the mean open and closed lifetimes. Of these, the $P_o$ and any altered open and closed life times are properties of the ligand-receptor complex only. These quantities are often obtained at saturating levels of ligand (e.g., $P_o$). They depend not on affinity of ligand for the binding site but on complementary interactions in the ligand-receptor complex that can provide energy to alter the equilibrium between open and closed states or to lower the energy barrier between open and closed states. In the following analysis, the focus will be on the energetic relationships measured by $P_o$ and EC$_{50}$.

Ultimately, the energy to modulate the channel comes from the binding energy. To the extent that binding energy will be used to favor channel opening, alter the state of allosteric sites, or lower transition state energies, the apparent binding energies will be lessened. For every calorie used to push the channel to the open state, one less calorie will be available to increase the affinity constant. These relationships will be reflected in the EC$_{50}$ of the various effectors. Therefore, correlations between EC$_{50}$ and structure reflect both the intrinsic affinity of receptor for ligand and the conversion of binding energy into conformational energy (e.g., the total energy of binding less the energy required to increase $P_o$).

One can gain a quantitative insight into the structural features of ATP that produce the experimentally observed $P_o$ and EC$_{50}$ by creating imaginary molecules and using the CoMFA to predict the $P_o$ and EC$_{50}$. This type of analysis has been used to predict the effects of the imaginary compounds listed in Table 1. In Table 2, the calculated free energy of binding and free energy used to increase $P_o$ are shown for the different ligands. A comparison of the percentage of the total energy used by each ligand to activate RyR is shown.

**Distribution of Energies Responsible for ATP Modulation of $P_o$ and EC$_{50}$**. Addition of ligands such as ATP increases the value of $P_o$ above that seen with Ca$^{2+}$ alone. This difference in $P_o$ can be related to the additional free energy ($\Delta G$) required to push the equilibrium toward the open states of the RyR. In the case of ATP, the shift in $P_o$ requires the input of 3459 cal/mol of free energy (Table 1). We used CoMFA to show quantitatively the distribution of this energy among the components of the most effective ligand, ATP. To do this, we have created imaginary molecules by removing fragments of the ATP and used CoMFA to predict the properties of these constructs. In the first column of Table 1, the missing elements are indicated by a space. The indicated fragments of the molecule remaining are left with the same charge and atomic positions as the intact ATP. In columns 2 and 3, we have calculated the free energies of these virtual molecules.

From Tables 1 and 2, it is apparent that the $\beta$ and $\gamma$ phosphates contribute most of the interactions that drive the RyR to an open state. In this regard, it is interesting to note that the predicted value of row two (Table 1) (an imaginary compound equivalent to ADP) has a predicted free energy of 

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Delta G$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine-ribose-O-P-O-P-O-P-O</td>
<td>3451 (3459)</td>
</tr>
<tr>
<td>Adenine-ribose-O-P-O</td>
<td>2115</td>
</tr>
<tr>
<td>Adenine-ribose-O-P-O-P-O</td>
<td>2399</td>
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<td>Adenine-ribose-O-P-O-P-O-P-O</td>
<td>3452</td>
</tr>
<tr>
<td>Adenine-ribose-O-P-O-P-O-P-O</td>
<td>4988</td>
</tr>
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The imaginary compounds are indicated by the missing parts of the linear formula. The first row is the complete ATP molecule. Row two contains an imaginary molecule obtained by omitting the terminal (\(\gamma\)) PO$_2$; the atoms remaining have exactly the same position and charge as in ATP. Row 2 is similar, but not identical to, the ADP used in the experimental data. Row 3 is obtained from ATP (row 1) by removal of the \(\beta\) PO$_2$ group. This is similar (but not identical) to an AMP plus a phosphate. In row 4, the \(\alpha\) PO$_2$ group is removed. This is similar to an adenosine plus a pyrophosphate. In row 5, the ribose is omitted. In row 6, the adenine base is omitted. The values in columns 2 and 3 are calculated with the indicated atoms missing while the remaining atoms retain the charge, position, and other properties found in ATP. The experimentally determined values are shown in parentheses for comparison with the predicted values of ATP.
2115 cal/mol compared with the experimentally determined value for ADP of 2318 cal/mol (Table 2, column 2). The imaginary ADP-like fragment is much less effective than ATP in promoting the open state of the channel (only providing 61% of the free energy of ATP, in agreement with the experiment). One might surmise that ADP + Pi would be more effective than ADP alone. However, we have found that addition of Pi to ADP does not enhance ryanodine binding above that seen in the presence of ADP alone (W. M. Chan, W. Welch, R. Sitsapesan, manuscript in preparation). Therefore, the Pi does not simply play a passive role (e.g., to neutralize a charge on the receptor to allow a higher $P_o$). The physical relationship between the $\gamma$-phosphate and the remainder of the nucleotide is important: they form a device to coerce the RyR into a conformation with a high value of $P_o$. In the context of ATP, the adenyl fragment contributes only a small part of the energy driving the increase in $P_o$ ([predicted value for ATP] – [predicted value for ribose triphosphate] = 99 cal/mol) and contributes essentially nothing to binding (EC$_{50}$). According to this prediction, at saturation, a ribose triphosphate should be nearly as effective as ATP in promoting high $P_o$. It would be interesting to conduct this experiment to determine the importance of the nucleotide base in the determination of $P_o$ and EC$_{50}$.

These predictions are of interest because of the relationship with the experimental data. First, although the base contributes little in the context of ATP, the RyR is specific for the base. The ATP analog GTP is incapable of increasing $P_o$, even at concentrations well beyond those that fully saturate the ATP effect. Second, adenine alone can promote $P_o$ by 1287 cal/mol (Table 2, column 2) and has apparent free energy of binding (obtained from the EC$_{50}$ value) of 4523 cal/mol (Table 2, column 3). The result is not a fluke of the CoMFA. The predicted contribution of an imaginary adenyl fragment [formed by removing all atoms from ATP except those of the adenyl group (i.e., the negative of row 6 of Table 1)] was calculated. The predicted free energy to increase $P_o$ is 1464 cal/mol, near the experimentally determined value for adenine (1287 cal/mol; Table 2, column 2). Likewise, the predicted free energy of binding (from EC$_{50}$) is 4447 cal/mol, near the experimentally determined value of 4523 cal/mol (Table 2, column 3). Therefore, the interactions between receptor and ligand are complex and depend upon the context in which the components of the ligand are presented to the receptor, including any ligand-induced changes in receptor conformation. If the charges are removed from ATP (i.e., the charge on all atoms is set to zero, all other atomic properties remain), the predicted energy drops from 3451 cal/mol to 1151 cal/mol. The latter number reflects the steric contribution to the enhanced $P_o$.

**Conversion of Binding Energy to Modulation Energy.** The total binding energy of ATP (Table 2, column 4; 8415 cal/mol) can be estimated from the sum of the experimental energies from the EC$_{50}$ (4956 cal/mol) and the incremental increase in the $P_o$ (3459 cal/mol, see Table 2). Forty percent of the total binding energy is used to modulate channel function. In the case of ADP, the total binding energy is 6276 cal/mol as estimated from the sum of the experimental energies from the EC$_{50}$ (3958 cal/mol) and the incremental increase in $P_o$ (2318 cal/mol). Again, about 40% of the binding energy is used to increase the open probability of the channel. Therefore, the difference between the $P_o$ induced by ATP and ADP seems to be caused by the difference in total energy available.

In contrast, the total binding energy of adenosine is 5810 cal/mol (4523 cal/mol from EC$_{50}$ and 1287 cal/mol from the incremental increase in $P_o$). Only 22% of this smaller binding energy is converted to modulation of the channel. Interestingly, the total binding energy of the larger molecule adenosine 5’-(5654 cal/mol, 4619 cal/mol from EC$_{50}$ and 1035 cal/mol from $P_o$) is slightly lower than that of adenosine. This reflects the antagonism that the sugar ring exerts on channel modulation (see Table 1). Similarly to adenosine, 18% of the total binding energy is diverted to modulate the channel $P_o$. In summary, the base and sugar provide binding energy and position the $\beta$- and $\gamma$-phosphates to promote channel opening.

**Interaction Energy.** As stated previously, the energy to modulate $P_o$ and mean open and closed times comes from the energy of nucleotide-receptor interactions. Unfortunately, at this time, no atomic level model of the nucleotide-binding site of the RyR exists. However, nucleotide-binding sites are generally considered to have a common structural motif. To investigate the effects of nucleotide analog structure on ligand-receptor interactions, we used molecular dynamics to estimate changes in interaction energy using adenylate kinase (Protein Data Base code 1AKY) as a surrogate for the nucleotide-binding site on the RyR. The interaction energy of ADP is 80% of that of ATP (molecular dynamics data not shown). This value compares well with those in the paragraph above. The incremental increase in $P_o$ induced by ADP (2318 cal/mol) is 67% of that of ATP (Table 2, column 2). The total binding energy of ADP (6276 cal/mol) is 74% of that of ATP (Table 2, column 3). In the present study, therefore, a surrogate nucleotide-binding site reasonably parallels the experimentally observed nucleotide-RyR interactions.

**Discussion**

Our preliminary CoMFA model (Chan et al., 2000) predicted that, in the presence of 10 μM cytosolic Ca$^{2+}$, AMP-PCP would be significantly less effective than ATP as an activator of the cardiac RyR channel. Our results demonstrated that the maximum $P_o$ achieved by AMP-PCP was approximately half that obtained with ATP (Figs. 1 and 3). Although these results provided conformation of the validity of our model, they were still surprising in light of previous
reports of the effectiveness of AMP-PCP to activate RyR channels. The literature contains no hint that AMP-PCP would lack efficacy, in fact quite the reverse. There are, however, a number of possible reasons to explain these discrepancies. First, in early reports, only a single dose of AMP-PCP had been compared with a single dose of ATP (Smith et al., 1985) and subsequent investigators did not examine the full dose-response relationship of these ligands. Second, because the effects of adenine nucleotides are strongly Ca\(^{2+}\)-dependent, slight differences in free [Ca\(^{2+}\)] could lead to large changes in AMP-PCP-induced effects. Third, it is possible that AMP-PCP has a higher efficacy at the skeletal isoform of RyR than at the cardiac isoform. Finally, we have demonstrated in the present study that AMP-PCP has a higher affinity for the cardiac RyR than ATP. The relatively high affinity of AMP-PCP for RyR will have masked its low efficacy in studies such as \([\text{H}]\)ryanodine binding studies or Ca\(^{2+}\) flux experiments. This type of experiment is often designed to produce the maximum possible measurable response; therefore, relatively low concentrations of a nucleotide can produce the maximum level of \([\text{H}]\)ryanodine binding or the maximum increase in the rate of Ca\(^{2+}\) efflux from SR membrane vesicles. Thus, partial agonists and full agonists will induce the same maximum level of \([\text{H}]\)ryanodine binding although a high-affinity ligand may seem to be more effective at opening RyR at low concentrations. It is impossible, therefore, to distinguish between the efficacy and affinity of a ligand under these conditions.

In this study, not only did we find that AMP-PCP has a higher affinity for the cardiac RyR than ATP, we also demonstrated that AMP-PCP activates the channel with a higher degree of positive cooperativity. The Hill coefficient for AMP-PCP was 2.1, whereas for ATP, the Hill coefficient was 1.5 (Kermode et al., 1998). An increase in cytosolic [Ca\(^{2+}\)] produced a marked increase in the Hill slope for activation of the cardiac RyR by AMP (Ching et al., 1999). If this is true of adenine nucleotide channel activation in general, then the effects of AMP-PCP may be much more sensitive than ATP to changes in cytosolic [Ca\(^{2+}\)] because of its apparently greater degree of positive cooperativity. These subtle differences in the characteristics of AMP-PCP and ATP may produce marked differences in RyR channel gating. Use of AMP-PCP as a nonhydrolysable “physiological replacement of ATP” may not, therefore, produce reliable results.

The structure-activity relationship reported here demonstrates that the interactions between ATP and related compounds are complex, consistent with a mechanism whereby at least part of the binding energy is converted into conformational changes in the receptor. Adenine triphosphate is the most effective ligand because, at least in part, it has the highest binding energy and because it converts the greatest percentage of its binding energy into increased P\(_o\). Another interesting observation is the relationship of the nucleotide base with P\(_o\), EC\(_{50}\), and ligand specificity. The data in Table 1 imply that, in the context of ATP, the base contributes an unimportant amount of energy to the increase in P\(_o\) or to binding (EC\(_{50}\)), yet the RyR is specific for the adenine fragment. Replacement of the adenine with a guanine completely destroys the ability of the nucleotide to modulate RyR. This fact suggests a tight complementarity of the base sub-binding site such that the additional steric bulk (and/or polar interaction) at the 2-poistion of the base blocks nucleotide binding. In addition, the unligated adenine binds with reasonable strength and promotes a significant increase in P\(_o\) (Table 2). We suggest that interactions between adenine and receptor are weakened in the context of ATP because of binding-induced conformational changes in receptor, ligand, or both. To maximize interactions between receptor and the triphosphate, interactions between receptor and base are relaxed (but not to the point of loss of specificity). We suggest that the ligands bound to the nucleotide site do not serve a permissive role (for example, shielding unfavorable interactions); instead, they are active participants in the functional state of the RyR and different ligands induce or stabilize unique conformers of the RyR. The conformers are manifested not in the magnitude of the conductance but in the equilibrium between open and close channel states and the energy barrier between them. To make an analogy with enzyme-catalyzed reactions, the bound ligand may stabilize one or more transition states between conducting and nonconducting forms of the channel and the ligand may bind somewhat more tightly to the conducting form of the RyR.

Hill coefficients derived from the experimental data indicate that there may be more that one nucleotide binding site per RyR channel. Because RyR is a homotetramer, there may be four nucleotide binding sites per tetramer. However, the three-dimensional structure-activity relationships described in the present study are based on EC\(_{50}\) values and maximum P\(_o\) levels induced. The relationships are therefore not dependent upon assumptions about the number of binding sites or interactions between sites and cannot be used to explain any cooperativity observed. An important future investigation would be to examine the effect of nucleotide structure on the cooperativity of adenine-nucleotide–induced stimulation of P\(_o\).

With increasing knowledge of the effects of adenine nucleotides on RyR gating, the more obvious the high efficacy of ATP becomes and the greater our certainty that the γ-phosphate group of ATP plays a unique role in allowing ATP to induce long open states and high P\(_o\) values. These results prompt the question of whether some of the effects of ATP could be caused by phosphorylation of the channel. Indeed, a recently published report suggests that part of the effects of ATP on the skeletal isoform of RyR results from phosphorylation of the channel by CaMKII (Dulhunty et al., 2001). However, we have no evidence that this is the case in our experiments. Unlike the report by Dulhunty et al. (2001), the effects of ATP that we observe are completely reversible; after 25–30 min in the presence of cytosolic ATP (1–5 mM), perfusion of the cytosolic chamber completely reverses the effects of ATP. Moreover, we have no evidence for any time-dependent increases in P\(_o\), as would be expected to occur if phosphorylation of RyR were producing a change in gating. Compelling evidence that phosphorylation is not producing our ATP-dependent changes in gating comes from the CoMFA itself. Cross-validation of the molecules used in the CoMFA analysis demonstrates that ATP is not an outlier, as would be expected if some of the effects of ATP were caused by phosphorylation. We therefore have strong evidence that our reported effects of ATP on the gating of cardiac RyR channels are caused by ligand-receptor interactions that do not involve phosphorylation. Possibly, the differences between our results and those of Dulhunty et al. (2001) reflect differences in the effects of ATP on skeletal and cardiac
channels. Alternatively, the differences may be caused by variations in the preparation of the heavy SR membrane vesicles, which could alter the phosphorylation state of RyR or the attachment of associated proteins to RyR.

In this study, the effects of adenine nucleotides have been examined in the presence of only one other modulator of RyR channel activity, cytosolic Ca$^{2+}$, and the concentration of Ca$^{2+}$ has been maintained at 10 μM so that the effects of the nucleotides rather than the effects of changes in [Ca$^{2+}$] can be monitored. During EC-coupling, the RyR will experience cyclical changes in cytosolic [Ca$^{2+}$] from 0.1 to >10 μM, so it is important that future studies investigate the ability of adenine nucleotides to modulate RyR activity at a range of cytosolic [Ca$^{2+}$]. Moreover, approximately 0.5 mM Mg$^{2+}$ is present in cardiac cells and it is not known how the effects of the Mg$^{2+}$-bound form of ATP differ from those of the Ca$^{2+}$-bound form. Future experiments must address this by comparing the actions of nucleotides in the presence and absence of physiological levels of Mg$^{2+}$, although this is complicated because Mg$^{2+}$ itself also modulates RyR $P_o$ directly.

In summary, we have demonstrated the reliability of our CoMFA model to predict the ability of ATP analogs to open the cardiac RyR channel. We also report on a new CoMFA model that describes the correlation between adenine nucleotide structure and affinity for RyR. The structural features responsible for high affinity differ from those responsible for high efficacy. The results of this study provide insight into the molecular mechanisms by which adenine nucleotides open the cardiac RyR and should provide a greater understanding of how adenine nucleotides regulate SR Ca$^{2+}$ release in cardiac cells.

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