Subcellular Distribution of High-Affinity Type IV Cyclic AMP Phosphodiesterase Activity in Rabbit Ventricular Myocardium: Relations to the Effects of Cardiotonic Drugs

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Rabbit ventricular myocardium contains distinct cytosolic and particulate cyclic AMP (cAMP) phosphodiesterase activities that exhibit characteristics ascribed to a high-affinity type IV cAMP phosphodiesterase activity found in several tissues. The particulate activity associated with sarcoplasmic reticulum vesicles has an apparent $K_m$ for cAMP of about 0.3 μM and a $V_{max}$ of 2.45 ± 0.55 nmol/min/mg. Cyclic GMP (cGMP) inhibits hydrolysis measured at 0.25 μM cAMP with an IC₅₀ value of 0.28 μM. In comparison, a ventricular cytosolic high-affinity cAMP phosphodiesterase activity obtained by anion exchange chromatography (Peak III) has an apparent $K_m$ of 0.93 μM and a $V_{max}$ of 17 ± 1 nmol/min/mg. Hydrolysis of 0.25 μM cAMP by this cytosolic activity is weakly inhibited by cGMP with an IC₅₀ value of 142 μM. Particulate enzyme activity is 60-fold more sensitive to inhibition by milrinone than is the cytosolic form ($K_m$ = 0.18 versus 11 μM, respectively); the high-affinity cytosolic form is also 12-fold more potent inhibitor of the particulate activity than of the cytosolic form ($K_m$ = 1.5 versus 18 μM, respectively). Inhibition of both cytosolic and particulate enzyme activities appears competitive in nature. Solubilization of particulate activity did not significantly alter its affinity for substrate or sensitivity to inhibition by cGMP. In the presence of a submaximally activating concentration of forskolin (0.4 μM), selective phosphodiesterase inhibitors potentiated the activation of protein kinase in isolated ventricular septal slices. Under these conditions, changes in cAMP-dependent protein kinase activity ratios correlated more closely with contractile responses than did changes in intracellular content of cAMP. Our findings in the intact tissue are consistent with the hypothesis that selective inhibitors of cardiac type IV phosphodiesterase activity exert their contractile effects through subtle alterations in the metabolism of cAMP. An important subcellular site of action of these drugs appears to be the particulate, rather than soluble, high-affinity cAMP phosphodiesterase activity. (Circulation Research 1988;62:782-789)

Cardiotonic agents of the bipyridine, imidazole, benzimidazole, and pyridazinone chemical classes combine positive inotropism with vasodilating properties. Investigations into the mechanism of action of these drugs have shown that many are relatively selective inhibitors of a cytosolic form of cardiac cyclic nucleotide phosphodiesterase (PDE) activity.1-5 As a result of a high affinity for cyclic AMP (cAMP) as substrate ($K_m$ ≤1 μM) and a lack of stimulation by calmodulin or cyclic GMP (cGMP), this form of activity is defined as a type IV PDE, according to current guidelines for PDE nomenclature.4 More commonly, this cytosolic activity is referred to as Peak III PDE based on the elution profile from an anion exchange column. Selective inhibition of cytosolic Peak III activity has been proposed to enhance contractile function by increasing cAMP levels and consequent activation of cAMP-dependent protein kinase.5-7

The myocardium also contains PDE activity of a particulate nature as first shown by Butcher and Sutherland.8 Recently, a particulate, high-affinity type IV cAMP-PDE activity has been shown to exist in association with sarcoplasmic reticulum (SR) vesicles isolated from canine ventricular muscle.9 This SR-PDE exhibits linear kinetics for cAMP hydrolysis with an apparent $K_m$ of 0.46 μM and a $V_{max}$ of 700 pmol/min/mg. The type IV PDE inhibitory potencies of LY 195115 and a series of structural analogues correlate with their ability to enhance contractile function in the anesthetized dog.

A high-affinity cytosolic PDE activity has also been observed in bovine heart.10,11 This activity, isolated by immunoprecipitation, has kinetic properties similar to the SR-PDE and is also inhibited by certain cardiotonic agents (milrinone, enoximone, and amrinone)10 and by cGMP.12

In our studies, we have further compared cytosolic and particulate type IV PDE activities, not only with regard to inhibition by several newer cardiotonic drugs,
but with an additional emphasis on the role of cGMP as a potential regulator of this activity. These data, obtained with several different chemical classes of cardiotonic drugs, were correlated with effects on contractility, cAMP-dependent protein kinase activation ratios, and cAMP content in the intact tissue.

**Materials and Methods**

**Preparation of Cardiac Membrane Vesicles**

Enriched cardiac SR vesicles from adult rabbit ventricular tissue were prepared and subfractionated by the methods of Jones et al.\(^1\) and Jones and Cala.\(^2\) The steps involved in the preparation of these SR vesicles are shown in Figure 1. The vesicles were further subfractionated by discontinuous sucrose density gradient centrifugation, and 1-ml fractions were collected. Cyclic nucleotide PDE activities were measured with the two-step method of Thompson et al.\(^3\) with 0.25 μM cAMP as substrate in the presence or absence of 5 μM cGMP. Fractions constituting density interfaces of 0.25–0.6, 0.6–0.8, 0.8–1, and 1–1.5 M and the sedimenting pellet were assayed for marker enzymes. Ouabain-inhibitable Na\(^+\),K\(^+\)-ATPase (sarcolemmal marker), Ca\(^2+\),K\(^+\)-ATPase (SR marker), and cytochrome c oxidase (mitochondrial marker) activities were determined according to standard techniques.\(^4,5\)

**Resolution of Cyclic Nucleotide Phosphodiesterase Activities From Rabbit Heart**

Cytosolic cardiac cyclic nucleotide PDEs were resolved by anion exchange chromatography (4°C) on DEAE-cellulose (DE-52, Whatman, Clifton, New Jersey) according to previously described methods.\(^6\)

Approximately 15 g rabbit ventricular tissue (wet weight) was used for each preparation. Hearts were minced and added to 10 volumes of 4 mM EDTA (pH 7). The tissue was homogenized in a Waring blender with three 5-second bursts at maximal setting. The homogenate was filtered with cheese cloth and then centrifuged at 20,000g for 20 minutes at 30,000g. The resulting supernatant was decanted through glass wool and applied to a 30-ml (1.6x20 cm) Whatman DE-52 column equilibrated in 70 mM sodium acetate and 30% ethylene glycol (pH 6.5). Enzyme activity was eluted with a linear, 0.07–1 M sodium acetate gradient (400 ml) with a flow rate of 0.6 ml/min. PDE activities in column fractions (5 ml) were determined with 0.25 μM cAMP as substrate in the presence or absence of 5 μM cGMP or with 1 μM cGMP as substrate.\(^7\)

**Drug IC\(_{50}\)** and **K\(_i\)** Determinations

Cardiotonic drugs of the bipyridine, pyridazinone, imidazole, and imidazopyridine chemical classes were examined for their ability to selectively inhibit the resolved forms of cardiac PDE activities. All drugs were dissolved in 100% dimethyl sulfoxide (DMSO). For determination of IC\(_{50}\) values, the drug concentrations ranged from 0.1 μM to 1 mM, with a final DMSO concentration of 2.5% in the assay. This amount of DMSO decreased basal enzyme activity by 5–10%.

Drug IC\(_{50}\) values were calculated from inhibition curves with 12 to 15 concentrations from a four-parameter logistic function using 2 + 2 linear regression as developed by Rodbard and Hutt and adapted for an IBM PC. K\(_i\) values were determined for each drug with four substrate concentrations and at least five inhibitor concentrations and analysis by the methods of Dixon and Cornish-Bowden.\(^7,5\)

**cAMP-Dependent Protein Kinase Activation**

Interventricular septae were obtained from adult rabbits and sliced to a thickness of 1 mm or less. Slices were individually incubated at 30°C in 20 ml oxygenated Krebs bicarbonate buffer (pH 7.35). After an equilibration period of 30–45 minutes, each slice was treated with a single drug (or the drug plus 0.4 μM forskolin) for 10 and 15 minutes, frozen in liquid nitrogen, and stored at −70°C. Protein kinase activity was determined by a modification of the method of Roskoski.\(^8\) Briefly, slices were homogenized (10:1, vol/wt) with ground glass homogenizers in buffer containing (mM) KH\(_2\)PO\(_4\) 10, EDTA 10, 1-methyl-3-isobutylxanthine 0.5, and β-mercaptoethanol 15, pH 6.8. The homogenate was centrifuged at 27,000g for 20 minutes, and the supernatant was assayed immediately for protein kinase activity.

cAMP-dependent protein kinase activity was measured by 32P incorporation into (1 mg/ml) histone

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**Figure 1. Diagram of procedure for isolating crude cardiac sarcoplasmic reticulum vesicles.** P-4 was applied to discontinuous sucrose density gradient and centrifuged for 2 hours at 140,000g. Gradients were fractionated as described in "Materials and Methods:” P-3b, before calcium loading. P-3a, after calcium loading.
Isolated Papillary Muscles

Right ventricular papillary muscles were isolated from the hearts of adult New Zealand white rabbits. The tendinous end of each muscle was attached to a force transducer (UC-2, Statham, Cleveland, Ohio), and the base was clamped to two platinum electrode plates. Each muscle was placed in a 25-ml bath (pH 7.35, 30°C), which was continuously bubbled with 95% O2-5% CO2, containing a modified Krebs bicarbonate buffer of the following composition (mM): NaCl 127, KCl 2.3, CaCl2 2.5, NaHCO3 25, glucose 5.6, KH2PO4 1.3, and MgSO4 0.6. Each muscle was stimulated to contract isometrically at the maximum length that was optimum for tension development. Square-wave pulses (3-msec duration) at a voltage 10% above threshold were delivered at a frequency of 0.5 Hz. Data were transferred to a computer for storage and analysis. Each data point was the average of six successive contractions for each muscle under any condition. Only muscles with a cross-sectional area less than 1 mm2 were included.

After stabilization, cumulative dose-response relations were obtained for each of the cardiotonic drugs. Results represent the mean of from five to seven different muscles. Drug solutions were prepared fresh in 100% DMSO, and the final DMSO content in the different muscles. Drug solutions were prepared fresh in 100% DMSO, and the final DMSO content in the absence of exogenous cAMP compared with that measured in the presence of a maximally stimulating concentration of cAMP (10 μM). Assays were linear for 20 minutes with 12.5-50 μg supernatant protein. Myocardial protein kinase activity was optimized for homogenization buffer ionic strength, which was determined to be inhibited by protein kinase inhibitor, found unaffected by charcoal treatment, and shown to be activated by forskolin and isoproterenol. Protein was determined by the method of Bradford.22

Materials

The cardiotonic drugs were generously supplied as follows: milrinone (WIN 47203) 1,6-dihydro-2-methyl-6-oxo(3,4'-bipyridine)-5-carbonitrile (Sterling-Winthrop, Rensselaer, New York); imazodan (CI-914) 4,5-dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazine (Warner Lambert, Ann Arbor, Michigan); piroximone (MDL19205) 4-ethyl-1,3-dihydro-5,6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone (Warner Lambert, Ann Arbor, Michigan); IBMX (MIX), 1-methyl-3-isobutylxanthine (Aldrich Chemical, Milwaukee, Wisconsin); densipraz (MDL19216) 4-ethyl-1,3-dihydro-5,6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone (Warner Lambert, Ann Arbor, Michigan); PDE activity had a slightly higher affinity for cAMP, inhibition by cardiotonic drugs (Table 3).

Results

Localization and Characterization of Sarcoplasmic Reticulum-Associated Phosphodiesterase Activity

Preparation of enriched cardiac SR vesicles resulted in an enrichment of a type IV PDE activity inhibited by cGMP (fraction P4, Table 1). At this stage of the membrane preparation, there was minimal enrichment for cytochrome c oxidase, no enrichment of ouabain-inhibitable Na+,K+-ATPase, and a 15-fold enrichment of Ca2+,K+-ATPase. These enriched calcium-loaded SR vesicles were then subfractionated by discontinuous sucrose density gradient centrifugation, revealing five peaks of PDE activity at the four sucrose interfaces and in the sedimenting pellet. As shown in Table 2, sarcolemmal Na+,K+-ATPase activity corresponded to the 0.6-0.8 M sucrose interface; mitochondrial activity was associated with the 0.6-0.8 and 0.8-1 M sucrose interfaces; and SR Ca2+,K+-ATPase activity was highest in the 1.0-1.5 M sucrose interface (subfraction D) and sedimenting pellet (subfraction E).

All five PDE activities showed inhibition by cGMP greater than 90% with 0.25 μM cAMP as substrate and 5 μM cGMP as inhibitor. Hydrolysis of 1.0 μM cGMP as substrate was fourfold to sevenfold less than that seen with 0.25 μM cAMP for each peak. In addition, all five PDE activity peaks displayed a similar pattern of inhibition by cardiotonic drugs (Table 3).

Particulate, type IV PDE activity associated with subfractions D and E and that of Peak III cytosolic PDE activity obtained by anion exchange chromatography were compared with respect to substrate affinity and inhibition by cGMP (Table 4). Although the particulate PDE activity had a slightly higher affinity for cAMP, it was much more sensitive to inhibition by cGMP (IC50 = 0.28 vs. 142 μM). Solubilization of greater than 90% of the particulate activity by freeze-thawing and 0.1% Triton X-100 did not alter the Ks for cAMP, decreased the Vmax twofold to threefold, and resulted in a preparation that was slightly more sensitive to inhibition by cGMP than was the original particulate form (IC50 = 0.1 μM). cGMP inhibition of PDE activity was associated with the 0.6-0.8 and 0.8-1 M sucrose interfaces and in the sedimenting pellet (subfraction D). Table 5 shows effects of cardiotonic drugs.

Effects of cardiotonic drugs are expressed as changes (HF2B, Cooper Biomedical, Freehold, New Jersey).

Effects of cardiotonic drugs are expressed as changes in the protein kinase activity ratio, that is, the amount of [32P]incorporated into histone after 10 minutes in the absence of exogenous cAMP compared with that measured in the presence of a maximally stimulating concentration of cAMP (10 μM). Assays were linear for 20 minutes with 12.5-50 μg supernatant protein. Myocardial protein kinase activity was optimized for homogenization buffer ionic strength, which was determined to be inhibited by protein kinase inhibitor, found unaffected by charcoal treatment, and shown to be activated by forskolin and isoproterenol. Protein was determined by the method of Bradford.22

Designations under sample column correspond to those used in Figure 1. Data are representative of five different preparations.
TABLE 2. Na\(^+\), K\(^+\)-ATPase, Cytochrome c Oxidase, Ca\(^{2+}\), K\(^+\)-ATPase, and Phosphodiesterase Activities Associated With Subfractions of Sarcoplasmic Reticulum Vesicles

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ouabain-inhibitable Na(^+), K(^+)-ATPase ((\mu)mol P/mg/hr)</th>
<th>Cytochrome c oxidase ((\mu)mol/mg/hr)</th>
<th>Ca(^{2+}), K(^+)-ATPase ((\mu)mol P/mg/hr)</th>
<th>High-affinity type IV PDE activity (pmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.9</td>
<td>2.04</td>
<td>0</td>
<td>266</td>
</tr>
<tr>
<td>P(_4)</td>
<td>0.6</td>
<td>3.12</td>
<td>15.2</td>
<td>255</td>
</tr>
<tr>
<td>A (0.25–0.6 M)</td>
<td>0</td>
<td>8.70</td>
<td>1.2</td>
<td>2.13</td>
</tr>
<tr>
<td>B (0.6–0.8 M)</td>
<td>2.8</td>
<td>20.9</td>
<td>27.4</td>
<td>211</td>
</tr>
<tr>
<td>C (0.8–1.0 M)</td>
<td>0</td>
<td>26.4</td>
<td>28.7</td>
<td>232</td>
</tr>
<tr>
<td>D (1–1.5 M)</td>
<td>0</td>
<td>12.2</td>
<td>86.1</td>
<td>800</td>
</tr>
<tr>
<td>E (pellet)</td>
<td>0</td>
<td>6.4</td>
<td>105.3</td>
<td>1260</td>
</tr>
</tbody>
</table>

Correlation of PDE activity with marker enzyme activity in fractions A–E: 0.313, 0.514, 0.984.

Cytochrome c oxidase activity present in fractions D and E represented less than 0.2% of the total activity in the homogenate and 11% of the activity present in P\(_4\), respectively. Total Ca\(^{2+}\), K\(^+\)-ATPase activity in fraction D was 19%, and that in fraction E was 23% of that in P\(_4\). Correlation coefficient was determined by linear regression analysis.

PDE, phosphodiesterase.

Resolution of Cytosolic Cyclic Nucleotide Phosphodiesterase Activities From Rabbit Ventricular Myocardium

DEAE anion exchange chromatography resolved cardiac cytosolic phosphodiesterase into three peaks of activity (Figure 3) as observed in cardiac tissue from other species. Peak I preferred cGMP as substrate and could be stimulated fourfold by the addition of Ca\(^{2+}\)-calmodulin. Peak II also preferred cGMP as substrate. However, if assayed with cAMP as substrate, the addition of cGMP stimulated cAMP hydrolysis 2-2.5-fold. Peak II had anomalous kinetics with cAMP alone as substrate but exhibited linear kinetics and an apparent K\(_m\) for cAMP of 6.7 \(\mu\)M in the presence of stimulating concentrations of cGMP (data not shown).

Peak III prefers cAMP as substrate and has a high affinity for this cyclic nucleotide (apparent K\(_m\) = 0.93 \(\mu\)M; V\(_{max}\) = 17 nmol/min/mg). This activity exhibits partial sensitivity to inhibition by 5 \(\mu\)M cGMP with 0.25 \(\mu\)M cAMP as substrate; inhibition of cAMP hydrolysis by cGMP is approximately 50% across the peak (Figure 3).

Effects of Newer Cardiotonic Drugs on Various Forms of Rabbit Cardiac PDE Activities

With respect to the cytosolic forms of PDE activity resolved by anion exchange chromatography, the majority of cardiotonic drugs are potent, selective inhibitors of the Peak III activity (Figure 3) when compared with other enzyme forms (Table 5). However, when the cytosolic Peak III activity is compared with the particulate SR activity in subfractions D and E (Table 6), the particulate PDE activity is much more sensitive to inhibition by these agents. The magnitude of the difference varies with the class of cardiotonic drugs tested. All of the compounds are competitive inhibitors of both particulate and cytosolic PDE activities as determined by Dixon and Cornish-Bowden plots (data not shown).

Effects on cAMP-Dependent Protein Kinase Activation

cAMP-dependent protein kinase activity in supernatants from homogenized interventricular septal slices is activated in a dose-dependent manner after incubation with 0.25 \(\mu\)M cAMP as substrate; inhibition of cAMP hydrolysis by cGMP is approximately 50% across the peak (Figure 3).

TABLE 3. IC\(_{50}\) Values of Cardiotonic Drugs for cAMP-Phosphodiesterase Activity From Interfaces of Discontinuous Sucrose Density Gradient

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Milrinone</td>
<td>1.26</td>
</tr>
<tr>
<td>Amrinone</td>
<td>22</td>
</tr>
<tr>
<td>Sulmazole</td>
<td>...</td>
</tr>
<tr>
<td>Isomazole</td>
<td>...</td>
</tr>
<tr>
<td>Imaizoden</td>
<td>...</td>
</tr>
<tr>
<td>Piroximone</td>
<td>...</td>
</tr>
</tbody>
</table>

IC\(_{50}\) values determined at 0.25 \(\mu\)M cAMP as substrate. A, 0.25–0.6 M sucrose; B, 0.6–0.8 M sucrose; C, 0.8–1.0 M sucrose; D, 1.0–1.5 M sucrose; E, pellet.

TABLE 4. Kinetic Characteristics of Cytosolic Particulate and Solubilized Rabbit Cardiac Phosphodiesterase

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K(_{m}), cAMP(^*) ((\mu)M)</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>0.97 ± 0.10</td>
</tr>
<tr>
<td>Particulate (SR)</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>Solubilized SR-PDE</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

*Measured in duplicate assays with 26 different cAMP concentrations. Kinetic variables were determined by statistical analysis according to the method of Cleland.32

*Measured at 0.25 \(\mu\)M cAMP with 13 concentrations of cGMP. Particulate (SR) activity was that contained in subfractions D and E.

PDE, phosphodiesterase.
FIGURE 2. Inhibition of solubilized particulate cardiac cAMP-phosphodiesterase activity by cGMP. Particulate activity was released by a combination of freeze-thawing and 0.1% Triton X-100. Over 90% of the total activity was recovered by this procedure. At cAMP concentrations from 0.4 to 2.5 μM, inhibition was competitive with a \( K_i \) of 0.075 μM. From 0.03 to 0.125 μM inhibition appeared noncompetitive with a \( K_i \) of 0.15 μM. Solubilization did not alter the \( K_m \) for cAMP but did decrease the \( K_i \) approximately threefold. Kinetic analysis was according to Dixon with the data replotted according to Cornish-Bowden.

Addition of exogenous cAMP (0.5 μM) to the protein kinase assay fully activates the enzyme with an \( EC_{50} \) value of 0.05 μM. Agonist-stimulated activity is inhibited to basal levels by the addition of cAMP-dependent protein kinase inhibitor. Because the activity of protein kinase in the 27,000 g supernatant is fully stimulated by homogenization in high ionic strength buffer (0.4 M NaCl), it is classified as a type I enzyme.

The effects of cardiotonic PDE inhibitors (500 μM) on the activation state of cAMP-dependent protein kinase were determined as an index of alterations in cAMP metabolism in the intact tissue. Except for MIX, none of the drugs incubated with the slices increases the P-K activity ratio above control values (Figure 5, upper panel). However, in the presence of a submaximal dose of forskolin (0.4 μM), activation by the drugs is evident (lower panel). The rank order of potency with these conditions is MIX > milrinone > imazodan = isomazole > piroximone > sulmazole.

All of the cardiotonic drugs tested slightly increased the endogenous levels of cAMP in the absence of forskolin (upper panel). The addition of 0.4 μM forskolin accentuates the differences between the cardiotonic drugs. The order of potency is MIX > milrinone > sulmazole = piroximone = imazodan > isomazole. Except for sulmazole, the order is similar to that observed for activation of cAMP-dependent protein kinase and inhibition of SR-PDE activity.

Contractile Studies

To further assess drug effects in the intact tissue, contractile responses to the newer cardiotonic drugs were tested in isolated right ventricular papillary muscles. Changes in the maximal rate of tension development (DT/dt) were used as an index of the effects on contractility. All drugs, except amrinone, show dose-dependent increases in contractility (Table 7). Except for sulmazole, the order of potency is as observed for increases in protein kinase activation and inhibition of SR-PDE activity.

Discussion

These results demonstrate that adult rabbit ventricular myocardium contains at least two forms of high-affinity or type IV cAMP PDE activity. Although their apparent affinities for cAMP as substrate are similar, differences exist in the sensitivity to inhibition by cGMP and by selective cardiotonic drugs. The type IV PDE activity most sensitive to cGMP and cardio-

FIGURE 3. Separation of cytosolic cardiac cyclic nucleotide phosphodiesterase activities from rabbit myocardium by DEAE-cellulose anion exchange chromatography. Phosphodiesterase activity was eluted with a linear 70 mM-1 M sodium acetate gradient in the presence of 30% ethylene glycol as described in “Materials and Methods.” Five-milliliter fractions were collected. Phosphodiesterase activity was assayed in alternate fractions with 0.25 μM cAMP, 0.25 μM cAMP + 5 μM cGMP, and 1 μM cGMP as substrate.
TABLE 5. Specificity of Cardiotonic Drugs for Cytosolic Forms of Rabbit Cardiac Phosphodiesterase

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀ (µM mean ± SEM)</th>
<th>PDE I*</th>
<th>PDE II†</th>
<th>PDE III‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milrinone</td>
<td>264 ± 15</td>
<td>225 ± 14</td>
<td>12 ± 1</td>
<td></td>
</tr>
<tr>
<td>Amrinone</td>
<td>500</td>
<td>295 ± 25</td>
<td>206 ± 20</td>
<td></td>
</tr>
<tr>
<td>Sulmazole</td>
<td>500</td>
<td>120 ± 23</td>
<td>87 ± 14</td>
<td></td>
</tr>
<tr>
<td>Isomazole</td>
<td>1,000</td>
<td>230 ± 15</td>
<td>97 ± 7</td>
<td></td>
</tr>
<tr>
<td>Imazodan</td>
<td>306 ± 38</td>
<td>430 ± 25</td>
<td>4 ± 1</td>
<td></td>
</tr>
<tr>
<td>Piroximone</td>
<td>115 ± 12</td>
<td>400 ± 19</td>
<td>7 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

*Measured at 1 µM cGMP, 1 mM Ca²⁺, and 0.15 µM calmodulin.
†Measured at 2 µM cAMP as substrate, plus 1 µM cGMP.
‡Measured at 0.25 µM cAMP as substrate.

Cardiotonic drug inhibition is a particulate enzyme. Based on marker enzyme assays, the particulate type IV PDE activity coenriches with the SR. The portion of this activity that resides in the SR is almost totally inhabitable by 5 µM cGMP. The particulate activity is much more sensitive to inhibition by selective cardiotonic agents than is the cytosolic Peak III activity.

Differences between cytosolic and particulate activities may be partially attributable to contamination of Peak III by other cytosolic activities. Such contamination might be expected to alter the kinetics observed by decreasing the affinity for cAMP and by reducing the sensitivity to inhibition by cGMP and by cardiotonic drugs. However, unlike Peaks I and II, which gradually lose their response to calmodulin and cGMP, respectively, upon storage after anion exchange resolution, Peak III appears to be stable for at least 6 weeks. Further evidence against significant Peak III contamination is provided by the observation that this activity is not stimulated by calmodulin or inhibited by EGTA.

Another possibility is that the enzyme only appears to be cytosolic because of solubilization during tissue homogenization. Our study of solubilization of the particulate SR activity, however, does not support this contention. Solubilization of the particulate activity does not alter its affinity for cAMP as substrate. In addition, the solubilized activity appears more sensitive to inhibition by cGMP than do either the membrane bound or cytosolic forms.

These data support the conclusion that in the rabbit, particulate (SR-PDE) and cytosolic (Peak III) PDE are distinct forms of high-affinity cAMP-PDE activity. These results confirm and extend those described by Kaufman et al as our studies characterize the kinetic and regulatory properties of cytosolic and particulate type IV PDE activities resolved from a single species. The observation that the cardiotonic drugs are competitive inhibitors of both cytosolic and particulate activities suggests that they may be interrelated. This concept is consistent with a specific pooling or compartmentalization of cAMP and the suggestion that certain drugs may activate distinct pools of cAMP-dependent protein kinase. Alterations in cAMP metabolism at the SR membrane system may be important for affecting Ca²⁺ uptake and release. This SR-associated PDE activity is especially sensitive to inhibition by cGMP. The observation that cGMP-inhibited type IV PDE coexists with a cGMP-stimulated type II PDE activity within the same tissue suggests that cGMP may be involved in the regulation of myocardial contractile function.

When tested alone, the cardiotonic drugs do not alter the activation state of cAMP-dependent protein kinase, although changes in cAMP levels are observed to varying degrees. However, the majority of the drugs do increase protein kinase activation ratios in the presence of a submaximal concentration of forskolin. In this instance, the changes in cAMP levels appear to be a less sensitive indicator of the drug effects. Measurement of the total kinase activity in the supernatant or total cAMP content in the tissue may not reflect drug modifications of these variables at specific subcellular locales. Alternatively, the increases in cAMP due to type IV PDE inhibition may be insufficient to mediate the drug effect but sufficient to potentiate the response when raised slightly by agents such as forskolin. Potentiation of the protein kinase response to forskolin

![Figure 4](http://circres.ahajournals.org/)

FIGURE 4. Activation of cAMP-dependent protein kinase in septal slices by forskolin and isoproterenol. Individual slices were incubated in the presence of drug for 15 minutes and frozen at −70°C until assayed for protein kinase activity as described in "Materials and Methods." The EC₅₀ values were 0.7 µM for forskolin (●) and 1.0 µM for isoproterenol (▲).
TABLE 6. Cardiotonic Drug Selectivity for Particulate (SR-Associated) Phosphodiesterase Activity

<table>
<thead>
<tr>
<th>Drug</th>
<th>PDE III</th>
<th>SR-PDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milrinone</td>
<td>11</td>
<td>0.18</td>
</tr>
<tr>
<td>Amrinone</td>
<td>280</td>
<td>8.5</td>
</tr>
<tr>
<td>Sulmazole</td>
<td>155</td>
<td>...</td>
</tr>
<tr>
<td>Isomazole</td>
<td>18</td>
<td>1.5</td>
</tr>
<tr>
<td>Piroximone</td>
<td>15</td>
<td>6</td>
</tr>
</tbody>
</table>

*K_i values determined by Dixon analysis at four different substrate concentrations and six inhibitor concentrations. Competitive inhibition for both activities was confirmed by Cornish-Bowden plots. Particulate (SR) PDE activity was that contained in subfractions D and E.

PDE, phosphodiesterase; SR, sarcoplasmic reticulum.

could also reflect changes in the activation constant of the enzyme.25

Excluding sulmazole, the order of contractile potency for the cardiotonic drugs (obtained from dose-response curves) is the same as that observed for protein kinase activation in the presence of forskolin. The similarity of the inhibitory potency of the cardiotonic drugs for particulate SR-PDE activity is consistent with the hypothesis that alterations in the metabolism of cAMP mediate the positive inotropic action of these drugs.

Sulmazole has the highest intrinsic activity of the cardiotonic drugs when tested for effects on contractility in isolated papillary muscles. Although this drug slightly increases CAMP levels alone or in the presence of forskolin, it does not potentiate forskolin activation of protein kinase and is a weak inhibitor of type IV PDE activity. These data are consistent with the observations of Solaro and Rüegg,26 suggesting that a significant portion of the contractile response to sulmazole results from an increase in the Ca^{2+} sensitivity of the contractile elements and that activation of cAMP-dependent protein kinase assumes a minor role. Isomazole, a close structural analogue of sulmazole (differing only by a shift in the position of the pyridine nitrogen), is an effective inotropic agent that has been proposed to act by increases in both intracellular Ca^{2+} and cAMP content.27,28 This drug is also a weak inhibitor of SR type IV PDE activity.

Several investigators have found positive correlations with the inhibitory potency of different cardiotonic drugs on cyclic nucleotide PDE activity and changes in CAMP levels and/or inotropic potency in certain species.9,29 These types of correlative studies may present several problems. Many of these agents (e.g., carbaszeran, milrinone, and papavarine) also increase tissue cGMP levels. Moreover, the time course for the increase in cAMP seen with certain cardiotonic drugs often lags behind the time course for the increase in contractility.30-' In accordance with the hypothesis that distinct pools of cAMP and protein kinase exist, the inotropic response to cardiotonic PDE inhibitors may be a result of subtle changes in cAMP turnover.

FIGURE 5. Correlative effects of cardiotonic drugs on protein kinase activity ratio and on CAMP concentrations in septal slices. Panel A, effect of cardiotonic drugs on protein kinase activity and CAMP in the absence of 0.4 \( \mu \text{M} \) forskolin. All drugs were tested at 500 \( \mu \text{M} \) in the tissue bath. Control CAMP level was 12.3 pmol/mg protein. Con, control; SUL, sulmazole; PIR, piroximone; ISO, isomazole; IMA, imazodan; MIL, milrinone; MIX, isobutyl methyl-xanthine. Panel B, all drugs were tested at 500 \( \mu \text{M} \) in the presence of a submaximal concentration of forskolin (0.4 \( \mu \text{M} \)). The rank order of potency was MIX > MIL > IMA = ISO > PIR > SUL. Control CAMP level was 17.1 pmol/mg protein. Each drug group is the mean ± SEM of six separate experiments.

TABLE 7. Cardiotonic Drug Effects on Contractility (DT/dt) in Right Ventricular Papillary Muscles

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (( \mu \text{M} ))</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>240</th>
<th>700</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulmazole</td>
<td></td>
<td>107±2</td>
<td>115±5</td>
<td>133±8</td>
<td>156±11</td>
<td>210±21</td>
</tr>
<tr>
<td>Milrinone</td>
<td></td>
<td>111±5</td>
<td>122±9</td>
<td>141±15</td>
<td>159±21</td>
<td>171±21</td>
</tr>
<tr>
<td>Imazodan</td>
<td></td>
<td>107±1</td>
<td>109±2</td>
<td>114±4</td>
<td>123±3</td>
<td>151±7</td>
</tr>
<tr>
<td>Isomazole</td>
<td></td>
<td>102±2</td>
<td>106±2</td>
<td>111±1</td>
<td>117±2</td>
<td>134±3</td>
</tr>
<tr>
<td>Piroximone</td>
<td></td>
<td>105±2</td>
<td>112±5</td>
<td>114±5</td>
<td>116±3</td>
<td>123±4</td>
</tr>
<tr>
<td>Amrinone</td>
<td></td>
<td>99±2</td>
<td>98±1</td>
<td>100±6</td>
<td>97±6</td>
<td>101±12</td>
</tr>
</tbody>
</table>

Each data point is the mean ± SEM of 5-7 determinations. DT/dt values are expressed as a percentage of pretreatment control.
rather than absolute increases in the level of the cyclic nucleotide.

In summary, our findings, in both the intact tissue and isolated forms of cardiac PDE after subcellular fractionation, support the hypothesis that the inotropic effect of cardiotonic inhibitors of type IV PDE activity results from changes in cAMP metabolism. Localization of the particulate activity to SR membranes supports the hypothesis that SR-PDE activity may be the major site of action for these drugs, particularly at low concentrations. Inhibition of cytosolic enzyme activity might assume greater importance at higher drug concentrations.

References


Key Words • cardiotonic drugs • type IV cAMP phosphodiesterase • cAMP-dependent protein kinase • subcellular distribution
Subcellular distribution of high-affinity type IV cyclic AMP phosphodiesterase activity in rabbit ventricular myocardium: relations to the effects of cardiotonic drugs.

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