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SELECTIVE INHIBITION OF CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASES OF CARDIAC NUCLEAR FRACTION*

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Abstract—Approximately 60% of the total particulate phosphodiesterase activity occurring in cardiac tissue was associated with the nuclear fraction. Cyclic GMP phosphodiesterase activity of the purified cardiac nuclear fraction was selectively inhibited by trifluoperazine (I_{50} = 19 μM) with negligible inhibition (<15%) of cyclic AMP phosphodiesterase activity. Inhibition of cyclic GMP phosphodiesterase by trifluoperazine was calcium-dependent and suppressed by ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). The inhibitory response of both phosphodiesterases to papaverine was similar in the presence of calcium. However, in the presence of EGTA, papaverine inhibition of cyclic GMP but not cyclic AMP phosphodiesterase was reduced significantly. Calmodulin (1–5 μg/ml) readily reversed the inhibition by 25 μM trifluoperazine of membranous cyclic GMP phosphodiesterase, but had no effect on inhibition by papaverine. With imidazolidinone analogues (Ro 7-2956 and Ro 20-1724), EGTA enhanced the inhibition of cyclic GMP phosphodiesterase without significantly altering the inhibition of cyclic AMP phosphodiesterase. Inhibition of cyclic AMP or cyclic GMP phosphodiesterase activity by 1-methyl-3-isobutylxanthine, quinidine, or compound SQ 20,009 was not affected appreciably by calcium or EGTA. The selective inhibitory action of certain pharmacological agents on phosphodiesterases of cardiac nuclear fraction and the modulation of the inhibitory response by calcium suggest an intrinsic and predominant association of calmodulin with cyclic GMP phosphodiesterase activity of these membranes.

Cyclic nucleotide phosphodiesterases with specificity toward cyclic AMP and cyclic GMP are distributed widely in both the particulate and soluble portions of the cell [1, 2]. Although the relationship between particulate and soluble forms of the enzyme remains unknown, cyclic GMP- and cyclic AMP-specific enzymes appear to be genetically distinct [3], differentially activated by calmodulin [4–6], individually responsive to hormonal regulation [7], and selectively inhibited by nucleotide analogs and pharmacological agents [8–12]. Previous investigations have focused on the inhibitory and physicochemical properties of the cytosolic enzymes. Only a few studies have characterized the membranous forms of the enzyme [13, 14] or examined their response to potent and selective inhibitors [15]. Since we and others [16] have found that a large percentage of the particulate phosphodiesterase activity of cardiac ventricle resides in the nuclear fraction, the inhibitory response of the phosphodiesterases from the purified nuclear fraction was investigated. A spectrum of pharmacological inhibitors was chosen to determine the inhibitory characteristics of the membranous enzymes and their calmodulin dependence. Several pharmacological agents were highly selective and calcium-dependent in their inhibitory action against the particulate cyclic GMP phosphodiesterase of cardiac muscle.

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MATERIALS AND METHODS

Materials. Cyclic [8-^{3}H]AMP (21 Ci/mmole) and cyclic [8-^{3}H]GMP (8.8 Ci/mmole) were purchased from Schwarz Mann, Orangeburg, NY. 1-Ethyl-4-(isopropylidine-hydrazino) 1 H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester hydrochloride (SQ 20,009) was obtained from Squibb & Sons, Inc., Princeton, NJ. *dl*-4-(3-Butoxy-4-methoxybenzyl) 2-imidazolidinone (Ro 20-1724) and *dl*-4-(3,4-dimethoxybenzyl)-2-imidazolidinone (Ro 7-2956) were obtained from Hoffmann–LaRoche, Nutley, NJ. 1-Methyl-3-isobutylxanthine was from the Aldrich Chemical Co., Milwaukee, WI, and trifluoperazine was supplied by Smith, Kline & French Laboratories, Philadelphia, PA. All other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO, and were the highest grade available.

Preparation of nuclear fraction. Fresh bovine heart was obtained from a local slaughterhouse and rinsed in cold 0.25 M sucrose solution. All of the following procedures were performed at 4°. The isolation procedure was a modification of the method of Jaqua-Stewart *et al.* [17]. The ventricles were dissected from fat and rinsed in homogenizing medium containing 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM $CaCl_2$ and 0.3 mM phenylmethylsulfonyl fluoride. The tis-

sue was minced and homogenized in 3 vol. of the homogenizing medium using a Brinkmann Polytron homogenizer. The time and speed of homogenization were adjusted by following the maximum release of cardiac lactic acid dehydrogenase into the soluble fraction (105,000 *g* for 60 min). Excessive homogenization was avoided.

The homogenate was filtered through cheesecloth and centrifuged at 105,000 *g* for 60 min in a Beckman L2-65 ultracentrifuge using a type 40 rotor. The supernatant fraction from the centrifugation was discarded and the pellet was washed with homogenizing buffer and recentrifuged as above. This particulate fraction was resuspended in homogenizing buffer and subcellular fractions were isolated as described [17]. The nuclear pellet obtained by this procedure was further purified by resuspending this pellet in 1.5 M buffered sucrose containing 20 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.1 mM $CaCl_2$ which was overlaid on 2 ml of 2.2 M buffered sucrose. After centrifugation at 91,772 *g* for 105 min using a Beckman SW 27.1 rotor, the resulting nuclear pellet was resuspended in 0.25 M buffered sucrose and used for enzyme assays. The purity of this nuclear fraction was verified by enzyme markers [17] and light microscopy at 1200-fold magnification. Contamination by sarcolemma, sarcoplasmic reticulum, or mitochondria was less than 3% based on recoverable marker activities.

Phosphodiesterase assay. The radioactive assay of Thompson and Appleman [18] was used to measure cyclic AMP and cyclic GMP phosphodiesterase activity at 1 μM substrate concentrations. Initial velocity measurements at 1 μM were chosen to selectively measure the activity of each high affinity enzyme. The assay mixture contained 40 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, substrate and enzyme in a total reaction volume of 500 μl. Additions included $CaCl_2$ (250 μM), EGTA (1 mM) and specific inhibitors. The reaction was initiated by the addition of [8-^{3}H]cyclic 3′,5′-nucleotide (0.21 μCi) and unlabeled nucleotide and incubated for 10 min at 30°. The reaction was terminated by heating at 100° for 2 min and the 5′-nucleotide product was then converted to the corresponding nucleoside by a second incubation with 5′-nucleotidase of *Crotalus atrox* venom for 10 min. The procedure for the isolation of product and other conditions were as previously described [19]. The mean recoveries of tritiated adenosine and guanosine were 82 and 63% respectively. None of the inhibitors of this study was found to influence the activity of the 5′-nucleotidase used in the enzyme-coupled assay procedure. Recovery of the labeled nucleosides was determined by incubation of the corresponding [^{14}C]-5′-nucleotide with the 5′-nucleotidase and inclusion of the highest employed concentration of each inhibitor.

Calmodulin was purified from bovine cerebral cortex by the procedure of Watterson *et al.* [20] and judged homogeneous by electrophoresis on discontinuous polyacrylamide gels at pH 8.9 [21] and pH 4.3 [22] and sodium dodecylsulfate-containing gels [23]. Protein was determined by the procedure of Lowry *et al.* [24] using crystalline bovine serum albumin as standard.

RESULTS

Nuclear preparations studied by phase contrast microscopy were found to be mainly undamaged and negligibly contaminated with myofibrils, red cells and intact myocytes. The estimated purity of the preparation by microscopy was greater than 95%. Of the applied particulate activity, 61% of the cyclic AMP and 59% of the cyclic GMP phosphodiesterase activity were associated with the cardiac nuclear fraction. This value is in agreement with Moffet *et al.* [16]. After lysis of nuclei in hypotonic buffer, all phosphodiesterase activity sedimented with the membrane fraction containing approximately 18% of the total DNA of the nuclear fraction. At 1 μM substrate and 250 μM calcium, the mean specific activities of cyclic AMP and cyclic GMP phosphodiesterase associated with the nuclear fraction were 1.2 and 0.7 pmoles·min^{-1}·(mg protein)$^{-1}$, respectively.

Trifluoperazine (TFP) inhibited cyclic GMP phosphodiesterase of nuclear fractions in a calcium-dependent and selective manner (Fig. 1A). Trifluoperazine inhibition of Ca^{2+}-dependent cyclic

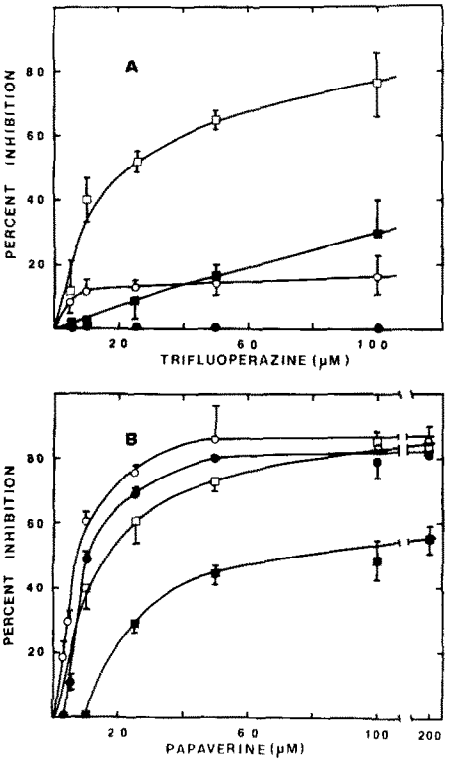

Fig. 1. Trifluoperazine and papaverine inhibition of phosphodiesterases of the nuclear fraction. The mean percent inhibition ± S.D. of cyclic AMP (circles) or cyclic GMP phosphodiesterases (squares) in the presence of calcium (open symbols) or EGTA (solid symbols) is plotted at different concentrations of each inhibitor. Membrane protein, 65 and 130 μg, was used to assay for cyclic AMP and cyclic GMP activity respectively. The inhibition at each concentration was determined in triplicate at 1 μM substrate concentration. Control activity was measured in the presence of either 250 μM $CaCl_2$ or 1.0 mM EGTA and is given in the text.

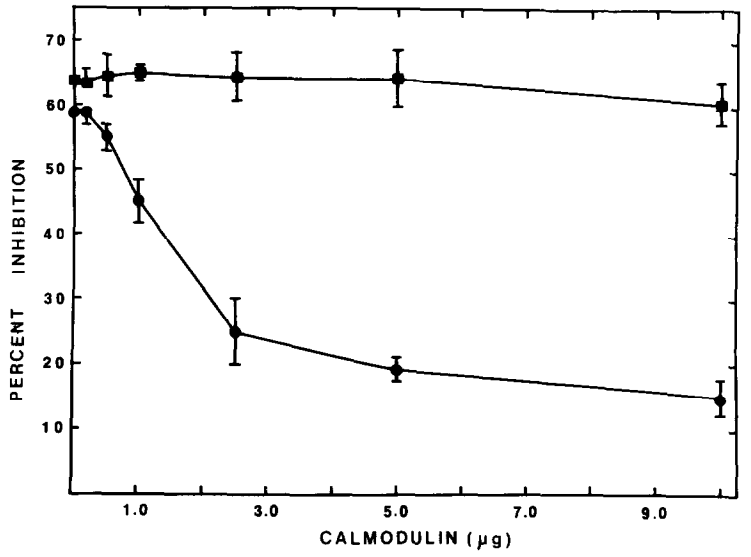

Fig. 2. Effect of increasing calmodulin concentration on inhibition of cyclic GMP phosphodiesterase of the nuclear fraction. Inhibition of cyclic GMP phosphodiesterase by 25 μM trifluoperazine (●–●–●) or 50 μM papaverine (■–■–■) is plotted as a function of exogenous calmodulin added to the reaction mixture. Protein (130 μg) was added to assay mixtures containing 1 μM cyclic GMP. Results are expressed as the mean of triplicate determinations ± the standard deviation at each calmodulin concentration. The total volume of the reaction mixture was 0.5 ml. Assay mixtures contained 250 μM calcium. Control activities were the same as for Fig. 1.

GMP hydrolytic activity appeared maximal at 25 μM since inhibition at higher concentrations was similar to the response of basal activity measured in the presence of EGTA (1 mM). The small amount of inhibition of cyclic AMP phosphodiesterase activity by trifluoperazine (<15%) was completely suppressed by EGTA. EGTA, alone, had a selective action on cyclic GMP phosphodiesterase activity causing 50–55% inhibition when compared to activity measured in the presence of calcium (not shown). Under similar conditions, EGTA inhibited cyclic AMP phosphodiesterase activity from 17 to 19%.

The inhibition of nuclear cyclic AMP and cyclic GMP phosphodiesterase activities by papaverine is shown in Fig. 1B. In contrast to inhibition of TFP, papaverine exhibited more inhibition of cyclic AMP hydrolyzing activity (I_{50} = 4.7 μM) than cyclic GMP activity (I_{50} = 15 μM) in the presence of added calcium. The addition of EGTA again had a much more pronounced effect on cyclic GMP phosphodiesterase causing an increase in I_{50} to 155 μM and substantially decreasing maximal inhibition. EGTA had no effect on maximal inhibition of cyclic AMP phosphodiesterase by papaverine, but the I_{50} value was increased.

The inhibition of nuclear cyclic GMP phosphodiesterase activity produced by 25 μM TFP could be reversed by addition of brain calmodulin in the presence of calcium (Fig. 2). Increasing the concentration of calmodulin from 0.2 to 5.0 μg/ml in the assay mixture reversed the inhibition from 60 to 15%. This reversal of the phenothiazine-induced inhibition of phosphodiesterase by calmodulin has been observed previously in soluble preparations from bovine brain [25]. Since the site of action of trifluoperazine on phosphodiesterase involves binding to calmodulin [26–28], cyclic GMP phosphodiesterase of the cardiac nuclear fraction appears associated with calmodulin. The same concentration range of calmodulin was ineffective in reversing the inhibition of cyclic GMP phosphodiesterase produced by 50 μM papaverine.

In contrast to the effect of EGTA on inhibition by TFP and papaverine, addition of EGTA enhanced the inhibition of nuclear cyclic GMP phosphodiesterase by imidazolidinone analogs, Ro 20-1724 and Ro 7-2956 (Fig. 3, panels A and B). Inhibition of cyclic AMP phosphodiesterase by imidazolidinones was also increased by the presence of 1 mM EGTA in the assay mixture. At 100 μM inhibitor concentration, the presence of EGTA increased the inhibition of cyclic GMP phosphodiesterase by Ro 20-1724 from 13 to 58% and by Ro 7-2956 from 15 to 49% compared to calcium-supplemented assays. This would suggest that the calmodulin-deficient form is more responsive to inhibition by imidazolidinones.

Several other pharmacological agents were examined for their inhibitory effects on the nuclear phosphodiesterases, and the I_{50} values determined in the presence of EGTA or calcium are reported in Table 1. Chlorpromazine inhibited the cyclic GMP phosphodiesterase in a highly selective and calcium-dependent manner analogous to TFP. Chlorpromazine was approximately 10-fold less effective than trifluoperazine as an inhibitor of cyclic GMP phosphodiesterase activity in the presence of calcium. Addition of EGTA dramatically decreased the efficacy of both phenothiazine analogs. In contrast, chlorpromazine inhibition of cyclic AMP phosphodiesterase in the presence of calcium was less than 10% at concentrations of the inhibitor as high as 500 μM.

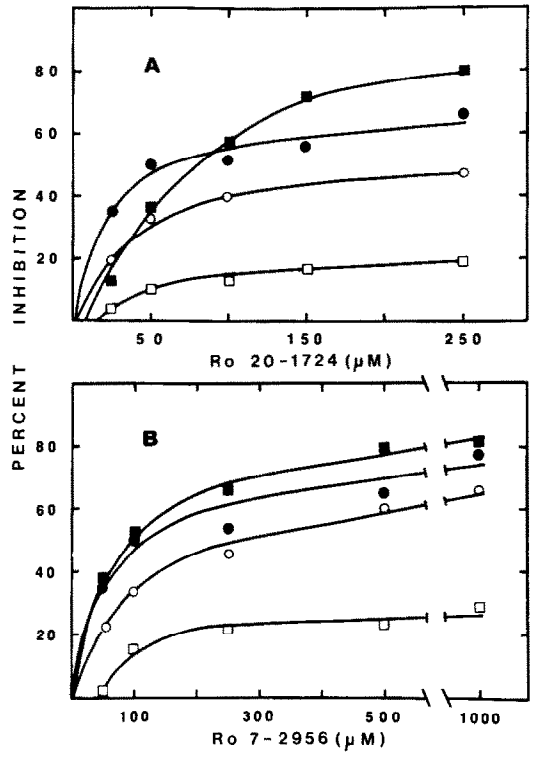

Fig. 3. Effect of imidazolidinone analogs on cyclic AMP and cyclic GMP phosphodiesterases of the nuclear fraction. Percent inhibition of cyclic AMP (circles) and cyclic GMP phosphodiesterase (squares) is plotted versus increasing concentration of Ro 20-1724 (panel A) and Ro 7-2956 (panel B). Inhibition was measured in the presence of 250 μM calcium (open symbols) or 1 mM EGTA (closed symbols). Other conditions are as described in the legend of Fig. 2.

Papaverine, unlike the phenothiazine analogs, was more effective as an inhibitor of cyclic AMP, than of cyclic GMP, hydrolytic activity but, like the phenothiazines, the I_{50} value was increased when measured in the presence of EGTA. This effect was not due to interaction with calmodulin, as with TFP, since exogenous calmodulin could not reverse papaverine-induced inhibition. Whereas 1-methyl-3-isobutylxanthine inhibited both cyclic AMP and cyclic GMP with nearly the same effectiveness, SQ 20,009 and papaverine were more effective inhibitors of cyclic AMP phosphodiesterase as shown in Table 1.

Quinidine and 1-methyl-3-isobutylxanthine inhibition of cyclic AMP and cyclic GMP hydrolytic activity was not affected by EGTA. Exogenous calmodulin had no effect on the inhibition of cyclic GMP phosphodiesterase produced by 100 μM 1-methyl-3-isobutylxanthine (not shown). Although the potency of SQ 20,009 on cyclic AMP activity was unchanged by EGTA, a slight decrease in the I_{50} for inhibition of cyclic GMP hydrolysis was observed. SQ 20,009 was the most potent of all the inhibitors tested for cyclic AMP hydrolysis and more potent than the phenothiazine, imidazolidinones analogs or quinidine for cyclic GMP hydrolysis. With the exception of quinidine or 1-methyl-3-isobutylxanthine, the observed maximum inhibition for cyclic GMP phosphodiesterase was changed by the presence of EGTA. Thus, calcium through its interaction with calmodulin appears to modulate inhibitor affinity as well as the extent of maximal inhibition for most of the pharmacological agents tested.

DISCUSSION

Based on their selective inhibition by a broad chemical spectrum of pharmacological compounds and the modulation of the inhibitory response by calcium, discrete cyclic AMP and cyclic GMP phosphodiesterases appear to be present in the nuclear fraction of cardiac ventricle. Differential effects of pharmacological agents on cyclic GMP and cyclic AMP phosphodiesterases have been observed previously for soluble forms from lung tissue [9–11] and the membrane-bound forms of renal cortex [15]. The

Table 1. Inhibitory constants of cardiac cyclic nucleotide phosphodiesterase activities of the nuclear fraction

	I_{50}* (μM)			
	Cyclic AMP		Cyclic GMP	
Inhibitor	$+Ca^{2+}$	+EGTA	$+Ca^{2+}$	+EGTA
Trifluoperazine	>100	NI†	18.6	>100
Chlorpromazine	NI	NI	232	>500
Papaverine	4.72	22.4	15.4	155
Methylisobutylxanthine	11.8	9.94	10.8	10.8
Ro 20-1724	>250	63.5	>250	95.0
Ro 7-2956	270	124	>1000	96.8
SQ 20,009	2.13	1.85	16.4	9.8
Quinidine	1120	806	1010	778

* The I_{50} value was that concentration of inhibitor required to produce 50% inhibition in the rate of hydrolysis of each cyclic nucleotide at 1 μM. Values were determined in the presence of either 1 mM EGTA or 250 μM calcium. Control velocity was determined under corresponding conditions in the absence of inhibitor. The I_{50} value was estimated using linear regression analysis of double-reciprocal transformations of velocity versus inhibitor concentration where the coefficient of correlation exceeded 0.90. A minimum of five inhibitor concentrations in duplicate was used in each determination.

† Negligible inhibition.

differential and selective action of these agents may be attributed to the association of one form of phosphodiesterase with calmodulin [6, 8, 12]. Thus, calmodulin-dependent forms of phosphodiesterase should be susceptible to phenothiazine inhibition and calcium would be expected to modulate the response of the enzyme to different inhibitors.

Although calmodulin is often associated with phosphodiesterases which are more specific for cyclic GMP than for cyclic AMP [4], a high affinity, cyclic GMP-specific phosphodiesterase unresponsive to calmodulin has been reported [29]. In this study, the cyclic GMP phosphodiesterase of cardiac nuclear fraction appears to be predominantly associated with the calcium-dependent regulatory protein based on the inhibition of this activity by phenothiazine and its reversal by exogenous calmodulin and the pronounced modulation of inhibition of this activity by calcium. Furthermore, the inhibition of the particulate cyclic GMP phosphodiesterase by EGTA alone was approximately 53% which was 3-fold greater than the inhibition of cyclic AMP phosphodiesterase activity. This differential behavior was not due to a selective loss of calmodulin during preparation of the membranes since addition of purified brain calmodulin (1–10 μg/ml) to the membrane fraction had no effect on either phosphodiesterase activity measured at 1 μM substrate and 250 μM calcium. Preliminary studies indicate, however, that the major cyclic AMP phosphodiesterase activity can acquire calcium sensitivity upon release from the nuclear fraction by sonication. Solubilization of phosphodiesterase of the nuclear fraction by sonification resulted in the loss of differential inhibition of cyclic AMP and cyclic GMP phosphodiesterase by TFP and Ro 7-2956 in the presence of calcium. This suggests that, within the intact membrane, association of cyclic AMP phosphodiesterase with calmodulin may be impeded.

Depending upon the site of interaction with cyclic GMP phosphodiesterase, the inhibitory potency of specific drugs may be enhanced, suppressed, or unaltered by the presence or absence of calcium. Calcium enhanced the inhibitory potency of phenothiazines, suppressed inhibition by imidazolidinones, and had little or no effect on inhibition of cyclic GMP hydrolytic activity by quinidine, 1-methyl-3-isobutylxanthine and compound SQ 20,009. Studies [9, 10, 15, 30] have shown the imidazolidinones to be 20- to 100-fold more potent as inhibitors of cyclic AMP than of cyclic GMP phosphodiesterase. Similar results were obtained with membranous phosphodiesterases of this study; however, this difference in potency was not observed when EGTA, was present, suggesting that the calmodulin-deficient cyclic GMP phosphodiesterase is more sensitive to inhibition by the imidazolidinones. The presence of EGTA decreased the I_{50} value for cyclic GMP hydrolytic activity and increased the maximum percentage of inhibition by both Ro 20-1724 and Ro 7-2956 (Fig. 3). The most potent inhibitor of cyclic AMP phosphodiesterase activity, SQ 20,009, was not affected by calcium addition. SQ 20,009 was 5- to 8-fold more effective as an inhibitor of cyclic AMP than of cyclic GMP phosphodiesterase, which is analogous to results obtained with the soluble enzyme from lung tissue [9–11]. Based on these studies, select pharmacological inhibitors should prove useful as probes in characterizing the membrane-bound forms of the phosphodiesterases and in identifying potential interrelationships between particulate and cytosolic forms.

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