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DIFFERENTIAL EFFECTS OF PHOSPHODIESTERASE INHIBITORS ON ACCUMULATION OF CYCLIC AMP IN ISOLATED VENTRICULAR CARDIOMYOCYTES

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Abstract—The intracellular actions of phosphodiesterase (PDE) inhibitors on the accumulation of cyclic nucleotides were studied in isolated ventricular cardiomyocytes from adult Sprague–Dawley rats. Elevated levels of cyclic AMP, due to the effects of selective PDE inhibitors, were detected only when the levels of cyclic nucleotide were enhanced with forskolin (10 μM). The time course for the elevation of cyclic AMP levels was similar for all the PDE inhibitors tested, following the pattern of an initial rise in the first 2–4 min, proceeded by a steady state at 67 ± 6% of the maximum stimulation. HN-10200 (2-[3-methoxy-5-methylsulfinyl-2-thienyl]-1*H*-imidazo-[4,5-*c*]-pyridine hydrochloride), a new imidazopyridine derivative, had a similar concentration-dependent profile to the structurally related compound, sulmazole (AR-L 115 BS, 2-[2-methoxy-4-methylsulfinyl)phenyl]-1*H*-imidazo-[4,5-*b*]-pyridine). Both the non-selective inhibitor, 3-isobutyl-1-methylxanthine (IBMX), and the selective PDE IV inhibitor, Ro 20-1724 (4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone), potentiated the forskolin-stimulated levels of cyclic AMP with a much greater efficacy than sulmazole or HN-10200. The concentrations of forskolin required by IBMX, sulmazole and HN-10200 (10^{-3} M) to increase levels of cyclic AMP by 4 pmol/mg protein were 3.2×10^{-6} M, 1.32×10^{-5} M and 1.46×10^{-5} M, respectively. Enoximone failed to cause an increase in the levels of cyclic AMP, even when stimulated with maximal concentrations of forskolin. Furthermore, in the presence of forskolin, enoximone attenuated the response of Ro 20-1724 and IBMX in a concentration-dependent manner. Enoximone, similarly to HN-10200, sulmazole, Ro 20-1724 and IBMX did not produce any significant effect on levels of cyclic GMP under elevated conditions in the presence of sodium nitroprusside. The combined action of Ro 20-1724, with either HN-10200, sulmazole, or IBMX (10^{-4} M), on intracellular levels of cyclic AMP, was not greater than the response to Ro 20-1724 alone. These data demonstrate the differential actions of PDE III and PDE IV inhibitors in rat ventricular cardiomyocytes. It is suggested that enoximone has a high selectivity for the PDE III isoenzyme so that hydrolysis of cyclic AMP by the PDE IV isoenzyme is not inhibited, in accordance with the lack of increase in cyclic AMP by enoximone in rat cardiomyocytes. HN-10200 and sulmazole, producing small increases in intracellular levels of cyclic AMP, are less selective PDE III inhibitors than enoximone. In contrast, the non-selective PDE inhibitor, IBMX, and the PDE IV inhibitor, Ro 20-1724, produced large increases in intracellular levels of cyclic AMP and it is likely, therefore, that Ro 20-1724 has effects on both the PDE III and PDE IV isoforms in rat cardiomyocytes.

Key words: phosphodiesterase inhibitors; cyclic nucleotides; ventricular cardiomyocytes; Ro 20-1724; sulmazole; enoximone

Intense research efforts have been directed over the past decade, to the development of cardiotonic agents with PDE†-inhibiting properties, for the effective management of congestive heart failure [1–3]. Although the physiological effects of these compounds have been well documented, their mechanisms of action are still relatively unclear [4]. Intracellular levels of cyclic AMP are regulated by the relative rate of formation of cyclic AMP versus the rates of its degradation and removal [5]. PDEs inactivate cyclic nucleotides by hydrolysing the 3′-ribose phosphate bond to produce the biologically inert 5′-monophosphate. Most tissues contain multiple isoenzymes of cyclic nucleotide PDE; five isoenzyme families have been defined and designated PDE I to PDE V in terms of substrate specificity (cyclic AMP or cyclic GMP), regulation characteristics and sensitivity to competitive inhibitors [6–8]. In recent years most attention has been focused on the PDE III and PDE IV isoforms which have a high affinity for cyclic AMP in cardiac tissues [9, 10]. Inhibition of these enzymes may provide alternative therapy in severe heart failure where conventional cardiotonic agents are not well tolerated.

Rat cardiac ventricle contains four PDE isoenzymes (PDE I, II, III, IV) which have been resolved definitively due to their chromatographic behaviour and kinetic properties [11]. These isoforms include PDE I which is stimulated by Ca^{2+}/calmodulin, and PDE II which is stimulated by low concentrations of cyclic GMP. PDE III exhibits similar K_m values for cyclic AMP and cyclic GMP

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†Abbreviations: PDE, phosphodiesterase; HN-10200, 2-[3-methoxy-5-methylsulfinyl-2-thienyl]-1*H*-imidazo-[4,5-*c*]-pyridine hydrochloride; IBMX, 3-isobutyl-1-methylxanthine; Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone; KRB, Krebs–Ringer bicarbonate.

(0.1–0.3 μM) but cyclic AMP is hydrolysed with a much higher V_{max} than cyclic GMP and consequently the latter acts as an inhibitor of this isoform. PDE IV which has a higher affinity for cyclic AMP than cyclic GMP (K_m values of 2 and 100 μM, respectively), and is not affected by cyclic GMP. In isolated ventricular cardiomyocytes, however, only three isoenzymes have been identified, that is, the PDE II, PDE III and PDE IV isoforms [12]. Bode *et al.* [12] suggested that the soluble Ca^{2+}/calmodulin-sensitive isoform was most likely present in non-cardiomyocytes such as vascular smooth muscle cells.

Little is known of the intracellular mechanisms by which selective inhibitors of PDE isoenzymes act in the isolated heart muscle cell. This study investigates the actions of a number of compounds with differing selectivities for PDE isoenzymes in isolated ventricular cardiomyocytes. Using this model, two criteria were achieved which are difficult, if not impossible, to accommodate in tissue or organ models. These are a homogeneous population of heart cells and a preparation which is uncomplicated by the interactions of neural, hormonal and mechanical influences in whole tissue. Use of such a model is particularly suited to biochemical studies involving determination of intracellular cyclic nucleotides where the physical and chemical environment can be specifically controlled. Compounds with selectivity for the PDE III isoenzyme including enoximone and sulmazole, and the novel compound, HN-10200, were compared with the selective PDE IV inhibitor, Ro 20-1724. By investigating the combined actions of the selective PDE III inhibitors and the selective PDE IV inhibitor, and comparing these effects with that of the non-selective PDE inhibitor, IBMX, it was possible to obtain information concerning the individual isoenzymes in modulating levels of cyclic AMP in isolated ventricular heart muscle cells.

MATERIALS AND METHODS

Cell preparation. Ventricular cardiomyocytes from adult male Sprague–Dawley rats (200–250 g) were prepared by a modification of a method described previously [13]. Hearts were perfused retrogradely through the ascending aorta, at 37° for 25 min, with an oxygenated (95% O_2/5% CO_2) KRB solution containing (mM) 110 NaCl, 2.6 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 11 glucose (pH 7.4) [14], supplemented after 5 min with 0.04% (w/v) collagenase (Serva, Heidelberg, West Germany), and 25 μM $CaCl_2$. Following enzymatic digestion, the hearts were cut at the atrioventricular junction, sliced vertically towards the apex to flatten the tissue, and chopped into cubes of 0.7 mm^3 using a mechanical tissue chopper (McIlwain Chopper, Mickle Laboratory Engineering, Surrey, U.K.). The minced tissue was placed in a teflon beaker containing perfusate to which 1% (w/v) BSA had been added and the mixture was gassed gently with 95% O_2/5% CO_2 at 37° for approximately 5 min. Release of the myocytes was enhanced by triturating the tissue suspension gently using a 10 mL serological pipette at a rate of twice per minute. The disaggregated cell suspension was filtered through a nylon mesh gauze of pore size, 200 μm, and centrifuged at 25 g for 60 sec. The sediment was washed twice in KRB buffers containing 250 and 500 μM $CaCl_2$, respectively, and the resultant cell suspensions were centrifuged at 25 g after each wash. Finally, the cells were layered onto a solution of 4% (w/v) BSA containing 1 mM $CaCl_2$ and then left to settle by gravity at 37°. After 5 min the supernatant was aspirated and the resulting cell material suspended at a density of 1–2 mg protein/mL in a modified Tyrode's solution, containing 125 mM NaCl, 1.2 mM KH_2PO_4, 2.6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES at pH 7.4.

Modulation of adenylate cyclase. Cells were incubated for 1 hr at 37°, in an atmosphere of 95% O_2/5% CO_2 prior to their use. Effects on the accumulation of cyclic AMP were studied using a method previously described [15]. All experiments were performed in the presence of adenosine deaminase (5 units/mL), which was used to degrade endogeneous adenosine. The temporal effects of a number of PDE inhibitors on the accumulation of cyclic AMP within the cell were examined over a period of 30 min, both in the presence and absence of forskolin. In the subsequent experiments, cells were incubated at 37° for 15 min with the various PDE inhibitors. For studies in which the effects of PDE inhibitors were compared, forskolin (10 μM) was used to potentiate the levels of cyclic AMP; forskolin was added to the dishes 5 min after the PDE inhibitor. Experiments were terminated by centrifugation of the suspensions for 1 min at 12,000 g through a 1:1 mixture of silicon oils (AR 20/AR200), obtained from Wacker (Munich, West Germany), into 6% (w/v) perchloric acid. After neutralization with a Tris–HCl (0.05 M) buffer (pH 7.4) in which K_2CO_3 (0.5 M) had been dissolved, potassium perchlorate was removed by centrifugation for 2 min at 12,000 g. Concentrations of cyclic AMP were determined using a protein binding assay incorporating [^{3}H]cyclic AMP [16] (Amersham International, Amersham, U.K.). The assay was based on the competition between unlabelled material and a fixed quantity of radiolabelled cyclic AMP for binding to a protein which had a high specificity and affinity for cyclic AMP. Separation of the protein-bound cyclic AMP from the unbound nucleotide was performed by adsorption of the free nucleotide unto dextran-coated charcoal followed by centrifugation for 2 min at 12,000 g. The supernatant was removed for determination of radioactivity in the presence of 6 mL liquid scintillant (Optiphase 'Hisafe', LKB Products, Leicestershire, U.K.) using a Wallac 1410 liquid scintillation counter (Pharmacia, Finland).

Modulation of guanylate cyclase. Cyclic GMP was released from ventricular cardiomyocytes using a procedure similar to that described for cyclic AMP. The effects of the PDE inhibitors were investigated in the presence of sodium nitroprusside (10 μM) which was used to potentiate the levels of cyclic GMP. Cyclic GMP was determined using a radioimmunoassay procedure based on competition between unlabelled material and ^{125}I-labelled cyclic GMP for a number of binding sites on a cyclic GMP-specific antibody. The complex of antibody bound

to cyclic GMP was then reacted with a second antibody in solution bound to magnetizable polymer particles (Amersham International). Separation of the antibody-bound cyclic GMP from the unbound nucleotide was carried out by centrifugation of the mixture at 12,000 *g* for 4 min. The supernatant was aspirated and discarded, and the radioactivity of the pellet determined using a gamma-emission detector (Nuclear Enterprises, NE 1600). The amount of unlabelled cyclic GMP in the sample was determined by interpolation of the calibration curve for the relationship between %B/Bo and concentration of cyclic GMP where %B/Bo = (average counts per minute in standard or sample solutions × 100)/ counts per minute in control solution.

Protein determination. Protein concentrations were determined by a modified Lowry procedure [17, 18] using BSA as a standard over a range of concentrations, 0–100 mg/mL. Samples of the cell suspension obtained from each experiment were solubilized with an equal volume of sodium hydroxide (1 N).

Materials. Drugs were obtained from the following sources: HN-10200 was a gift from Dr Rosenhow, Hafslund Nycomed, Linz, Austria; sulmazole (AR-L 57 Cl) from Karl Thomae, Biberach/Riss, West Germany; Ro 20-1724 from Roche Products (Herts, U.K.); enoximone from Merrell Dow Pharmaceuticals Ltd (Middlesex, U.K.); and IBMX, forskolin and adenosine deaminase from the Sigma Chemical Co. (Poole, U.K.). Stock solutions of IBMX, sulmazole and Ro 20-1724 (0.2 M) were prepared by dissolving the compounds in DMSO and further dilutions made using a modified Tyrode's buffer (pH 7.4). Stock solutions of all compounds were prepared, enough for each series of five to six experiments, and frozen for a maximum of 1 week. Care was taken to prevent precipitation of compounds by adding solutions in DMSO to a large volume (1:1000) of modified Tyrode's solution and immediately agitating the mixture using a Vortex mixer (Leicestershire, U.K.). HN-10200 was soluble directly in modified Tyrode's buffer. Twice distilled water which had been de-ionized through a Millipore-Q system (Millipore, Harrow, U.K.) was used in all experiments.

Data analysis. Concentrations of cyclic AMP and cyclic GMP were expressed as picamoles per milligram of protein. Data are presented as mean values ±standard error of three to eight heart cell preparations. Levels of cyclic AMP were calculated using the Multicalc immunoassay data management program (Pharmacia) by which a calibration curve is fitted and the concentrations of unlabelled cyclic AMP in the sample solutions are subsequently calculated. A quadratic equation was fitted to the concentration–response data (SPSS-PC) and the potencies of the PDE inhibitors were determined by calculating either, the concentration of forskolin required to increase the level of cyclic AMP by 4 pmol/mg protein or, the concentration of PDE inhibitor required to increase the level of cyclic AMP by 2 pmol/mg protein. IC_{50} values were calculated using a Dose–Effect Analysis programme (Elsevier Biosoft, Cambridge, U.K.). Statistical analysis of the data was performed using multivariate analysis with repeated measures and multiple comparison tests (Student–Neuman–Keuls).

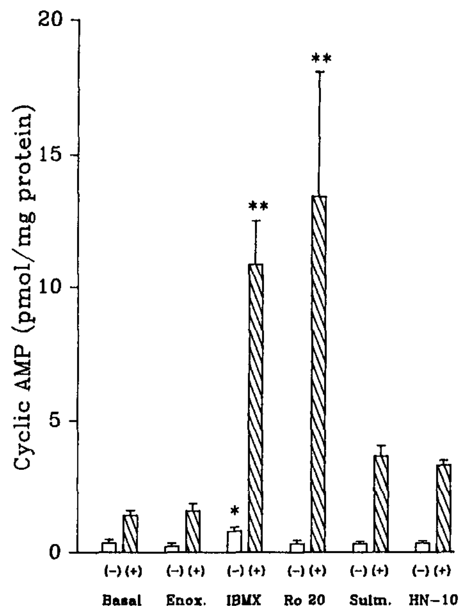

Fig. 1. Effect of PDE inhibitors (3×10^{-4} M) on the accumulation of cyclic AMP in rat ventricular cardiomyocytes which were stimulated with forskolin (10 μM). Data are presented as the mean ± SE for six heart cell preparations. * Denotes in the absence of forskolin, a significant difference ($P < 0.05$) of treatment with respect to basal conditions. ** Denotes, in the presence of forskolin, a significant difference ($P < 0.05$) of treatment with respect to the response to forskolin alone (10 μM). Enox. = enoximone, Ro 20 = Ro 20-1724, Sulm. = sulmazole, HN-10 = HN-10200.

RESULTS

Effects of PDE inhibitors in the presence and absence of forskolin

In the absence of forskolin, no appreciable changes in the levels of cyclic AMP were produced by HN-10200, sulmazole or Ro 20-1724 at a concentration of 3×10^{-4} M (Fig. 1). A concentration of 0.36 ± 0.28 (mean ± a significant difference) pmol cyclic AMP/mg protein was recorded under basal conditions, in the absence of both forskolin and PDE inhibitors, whereas in the presence of HN-10200, sulmazole or Ro 20-1724 alone, the mean cellular content of cyclic AMP was in the range of 0.22–0.35 pmol/mg protein. The non-selective PDE inhibitor, IBMX (3×10^{-4} M), however, produced a small, but significant increase ($P < 0.05$) in the level of cyclic AMP to 0.82 ± 0.09 (mean ± SE) pmol/mg protein in the absence of forskolin.

Forskolin (10 μM) alone produced a relatively small increase in the accumulation of cyclic AMP (4-fold), in contrast to the greater responses observed in the presence of the PDE inhibitors (7–40-fold) (Fig. 1). In the presence of forskolin, the non-selective PDE inhibitor, IBMX (3×10^{-4} M), produced a larger response (10.85 ± 1.65 [mean

Table 1. Effects of forskolin (10^{-5} M) and isoprenaline (10^{-5} M) on accumulation of cyclic AMP in the presence of IBMX, sulmazole and HN-10200 (10^{-3} M)

	Concentration of cyclic AMP (pmol/mg protein)			
	Forskolin		Isoprenaline	
Drug	Basal	Stimulated	Basal	Stimulated
IBMX	0.874 ± 0.133	10.86 ± 1.79*	1.534 ± 0.121	4.727 ± 0.204†
Sulmazole	0.231 ± 0.119	2.862 ± 0.755*	0.456 ± 0.101	1.000 ± 0.121
HN-10200	0.370 ± 0.320	3.840 ± 0.86*	0.393 ± 0.110	0.736 ± 0.180

Data are presented as mean values ± SE of three heart cell preparations.

* Denotes significant difference ($P < 0.05$) by comparison to basal values in the absence of forskolin.

† Denotes significant difference ($P < 0.05$) by comparison to basal values in the absence of isoprenaline.

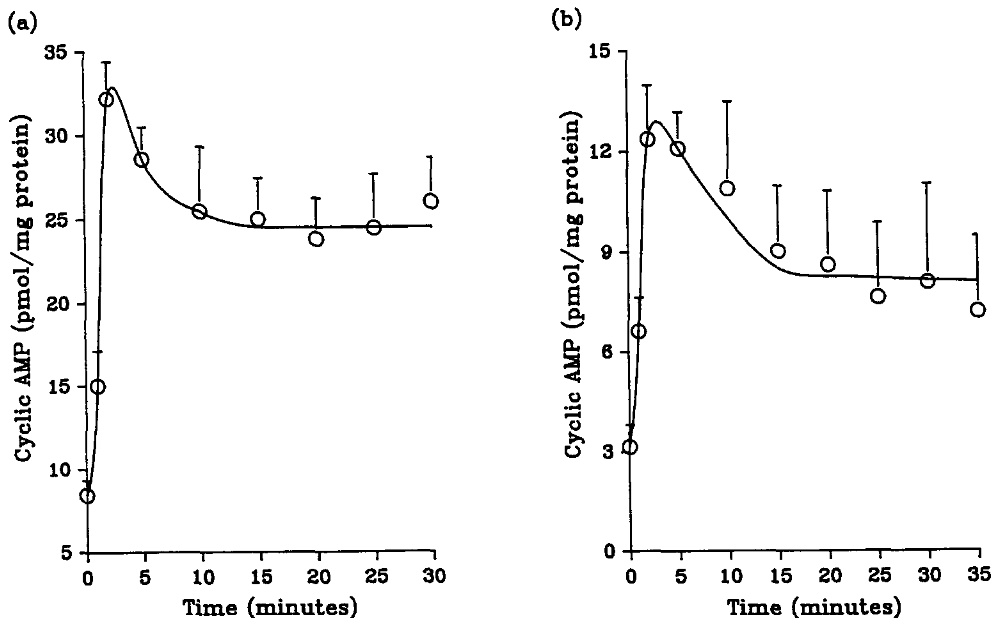

Fig. 2. The temporal relationship for the increase in cyclic AMP after the addition of (a) IBMX (10^{-3} M) in the presence of forskolin (10^{-4} M), (b) sulmazole (10^{-3} M) in the presence of forskolin (10^{-4} M). Data are presented as the means ± SE of three heart cell preparations.

±SE] pmol cyclic AMP/mg protein) than both the selective PDE III inhibitor, sulmazole, and the structurally related compound, HN-10200, (3.31 ± 0.19 and 3.65 ± 0.41 [mean ± SE] pmol cyclic AMP/mg protein, respectively), but at equimolar concentrations had comparable efficacy to that of the PDE IV inhibitor, Ro 20-1724 (13.42 ± 4.70 [mean ± standard error] pmol/mg protein). Sulmazole and HN-10200 increased levels of cyclic AMP to a similar degree, while enoximone, at an equimolar concentration, did not have any effect on the stimulated levels of cyclic AMP. The concentrations of cyclic AMP under control conditions with forskolin, and with the addition of enoximone, were 1.42 ± 0.19 and 1.58 ± 0.28 (mean ±SE) pmol/mg protein, respectively (Fig. 1).

Effects of PDE inhibitors in the presence of isoprenaline

In the presence of isoprenaline (10 μM), the PDE inhibitors increased concentrations of cyclic AMP to a much lesser extent than in the presence of forskolin, at an equimolar concentration (Table 1). IBMX, sulmazole, and HN-10200 (3×10^{-4} M), produced 3.1-, 2.2- and 1.9-fold increases in cyclic AMP over basal levels, respectively, in the presence of isoprenaline, by comparison to the 13.4-, 13.4- and 10.4-fold increases, respectively, recorded in the presence of forskolin (Table 1).

The temporal relationship of the effects of PDE inhibitors in the presence and absence of forskolin

Similar time-dependent profiles of the accumu-

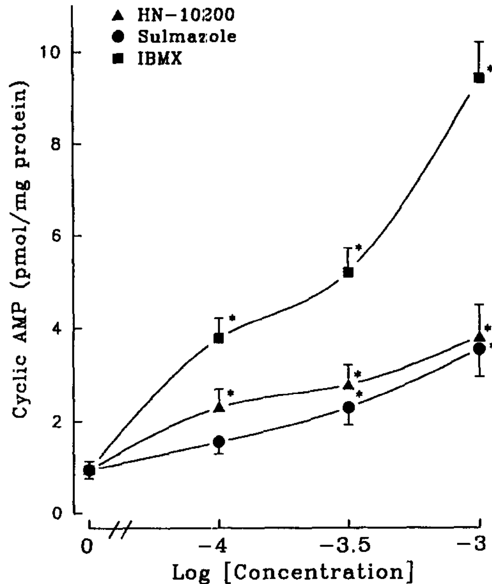

Fig. 3. Concentration-dependent effects of IBMX, HN-10200, and sulmazole on the accumulation of cyclic AMP in ventricular cardiomyocytes in the presence of forskolin (10 μM). Data are presented as the means ± SE of six heart cell preparations. * Denotes a significant difference (P < 0.05) with respect to the appropriate basal value.

lation of cyclic AMP were obtained in the presence of all PDE inhibitors, either without the addition of forskolin, or stimulated with forskolin. Figure 2 represents the time-course followed by IBMX and sulmazole (10^{-3} M) in the presence of forskolin (10^{-4} M); however, a similar maximum peak stimulation occurred between 1 and 5 mins for all the compounds investigated. Independent of their concentration or degree of stimulation by forskolin, a maximum accumulation of cyclic AMP was followed after 15 min by a steady state plateau, which occurred at 67 ± 6% (mean ±SE) of the peak concentration.

Differences were noted between PDE inhibitors with regard to the levels of cyclic AMP reached at the steady state plateau by comparison with the basal levels of cyclic AMP. In the presence of forskolin (10^{-4} M), the steady state levels of cyclic AMP obtained with IBMX and sulmazole (10^{-3} M) were 195 and 160%, respectively (Fig. 2a and b), greater than basal values at 0 min. In contrast the steady state plateau of HN-10200, at a concentration of 10^{-4} M and in the presence of lower stimulation by forskolin (10^{-5} M), was only 10% greater than the basal value. However, Ro 20-1724 (5×10^{-4} M) alone, produced a steady state accumulation of cyclic AMP which was 160% greater than basal levels, whereas IBMX (10^{-4} M) alone produced a steady state increase of 49%. Forskolin (10^{-5} M), in the absence of PDE inhibitor, produced a maximum response after 5 min followed by a steady state over 25 min.

Concentration-dependent effects of PDE inhibitors

In the presence of forskolin (10 μM), high concentrations (10^{-4}–10^{-3} M) of sulmazole, HN-10200, and IBMX increased the accumulation of cyclic AMP in a concentration-dependent manner (Fig. 3). The concentrations of PDE inhibitor which increased the accumulation of cyclic AMP by 2 pmol/mg protein were 0.50×10^{-4}, 1.93×10^{-4} and 2.98×10^{-4} M, respectively, such that potencies were of the order: IBMX > HN-10200 > sulmazole. Efficacies were of a similar order, in which IBMX, HN-10200 and sulmazole (10^{-3} M) increased levels of cyclic AMP by 10.0-, 4.0- and 3.8-fold, respectively, over the basal level observed in the absence of PDE inhibitors. Investigation of the effects of higher concentrations of all of the PDE inhibitors and forskolin was prevented by limited solubility of the compounds. Forskolin and enoximone were maximally soluble each at a concentration of 10^{-4} M, and IBMX, sulmazole, HN-10200 and Ro 20-1724, each at a concentration of 10^{-3} M.

In determining the effects of PDE inhibitors under conditions which altered the cellular levels of cyclic AMP variously, the concentration-dependent effects of the individual PDE inhibitors were demonstrated over a range of concentrations of forskolin (10^{-7}–10^{-4} M). Forskolin alone produced an increase in levels of cyclic AMP at concentrations greater than 10^{-6} M and likewise, the potentiating effects of sulmazole, HN-10200 and IBMX were found only under such conditions of high stimulation. Figure 4b shows that significant elevation (P < 0.05) of cyclic AMP levels by sulmazole was evident only at a maximal (10^{-3} M) concentration of the PDE inhibitor and at high levels of forskolin (>10^{-6} M). An analogous concentration–response relationship was obtained for the novel compound, HN-10200 (Fig. 4a). Both compounds demonstrated comparable efficacies, which were 9.22 ± 1.03 and 10.35 ± 1.63 (mean ±SE) pmol cyclic AMP/mg protein at maximal concentrations of the PDE inhibitors (10^{-3} M) (HN-10200 and sulmazole, respectively). Similarly, IBMX produced a significant shift in the concentration–response curve of forskolin only at a concentration of 10^{-3} M (Fig. 4c). IBMX had a greater efficacy (18.93 ± 4.45 [mean ± SE] pmol/mg protein) at the maximal concentration than either sulmazole or HN-10200.

Table 2 shows the potencies of the various compounds expressed as the concentrations of forskolin which increased cyclic AMP by 4 pmol/mg protein in the presence of each PDE inhibitor. Sulmazole and HN-10200 were equally potent, and at maximal concentrations (10^{-3} M) required approximately 3-fold less concentration of forskolin to increase the accumulation of cyclic AMP to the same level as under basal conditions. IBMX (10^{-3} M), on the other hand, required a 13-fold less concentration of forskolin to increase the level of cyclic AMP accumulation to the same degree, and 3.5-fold less stimulation when a lower concentration of IBMX was used (10^{-4} M). Comparable data obtained for sulmazole and HN-10200 (10^{-4} M) were 1.34- and 1.63-fold changes, respectively. In contrast,

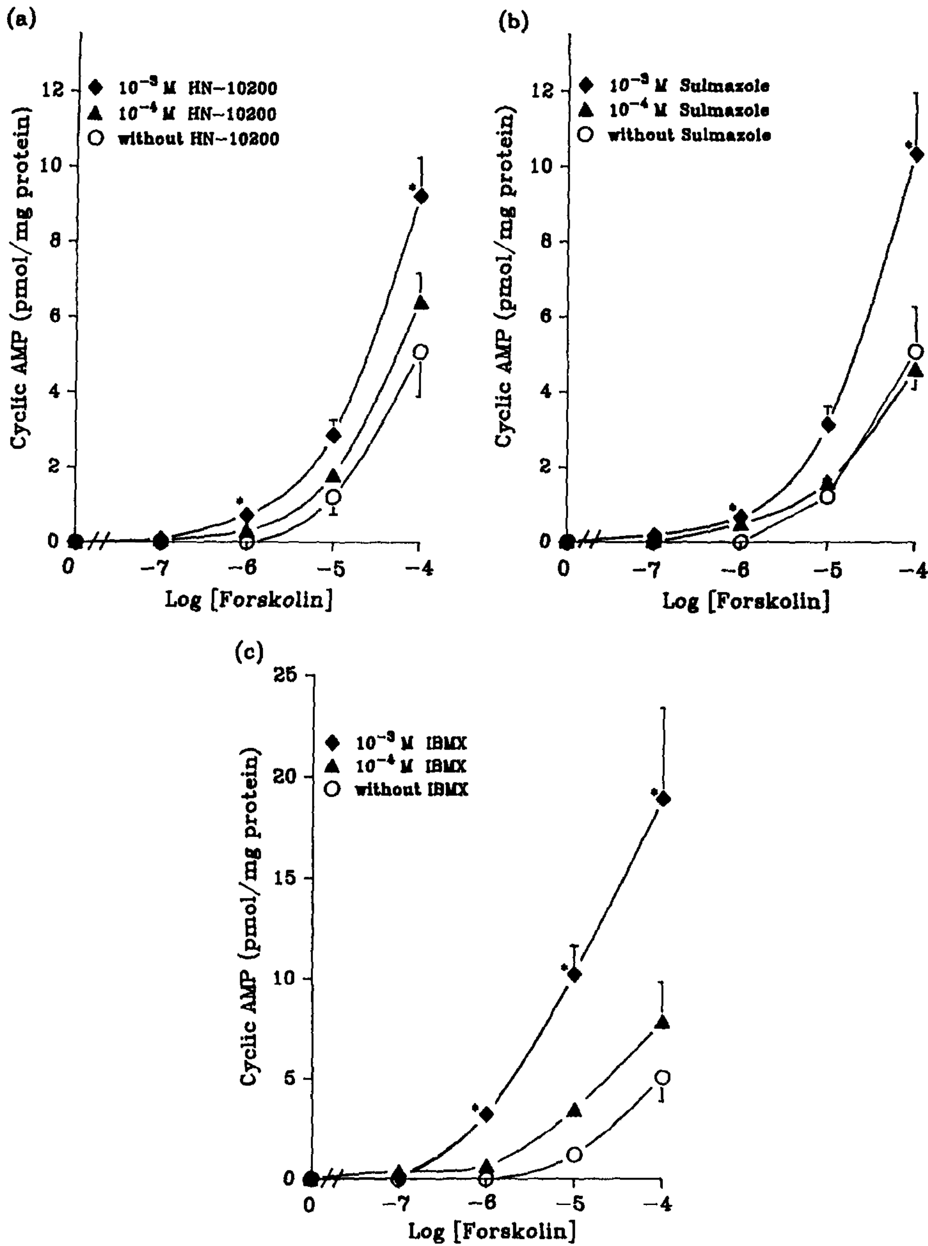

Fig. 4. Effects of (a) HN-10200, (b) sulmazole and (c) IBMX on the accumulation of cyclic AMP in rat ventricular cardiomyocytes, stimulated with increasing concentrations of forskolin. Data are presented as the means ± standard error of six heart cell preparations. Values are presented as the increase in levels of cyclic AMP produced by forskolin. Absolute values in the absence of forskolin are 0.36 ± 0.28 (mean ± SE) pmol/mg protein. * Denotes a significant difference ($P < 0.05$) with respect to control values in the absence of PDE inhibitor.

however, enoximone did not produce an increase in levels of cyclic AMP, even at maximal concentrations.

Interaction of PDE IV inhibitor, Ro 20-1724, with other PDE inhibitors

Ro 20-1724 (10^{-4} M) stimulated the accumulation of cyclic AMP by 5.3-fold by comparison with the basal level, to a concentration of 13.96 ± 1.65 pmol cyclic AMP/mg protein. The addition of Ro 20-1724 to each of the PDE inhibitors (10^{-4} M) tested resulted in increased accumulation of cyclic AMP in each case ($P < 0.05$). However, the combined effects of Ro 20-1724, with the various PDE inhibitors, were smaller in each case than the total of the individual responses as follows: IBMX alone, 6.99 ± 1.21; IBMX in combination with Ro 20-1724, 12.52 ± 2.49; HN-10200 alone, 3.43 ± 0.48; HN-10200 in combination with Ro 20-1724,

Table 2. Potencies of PDE inhibitors to increase cellular accumulation of cyclic AMP in the presence of forskolin

Drug	Concentration (M)	[Forskolin] $\times 10^{-5}$ M
(Basal)	—	4.12 ± 0.47
Sulmazole	10^{-4}	3.08 ± 0.80
	10^{-3}	1.32 ± 0.34
HN-10200	10^{-4}	2.83 ± 0.51
	10^{-3}	1.46 ± 0.33
IBMX	10^{-4}	1.18 ± 0.25
	10^{-3}	0.32 ± 0.06

*Denotes the concentration of forskolin required to increase cyclic AMP levels by 4 pmol/mg protein. Data are given as mean values ± SE of six heart cell preparations.

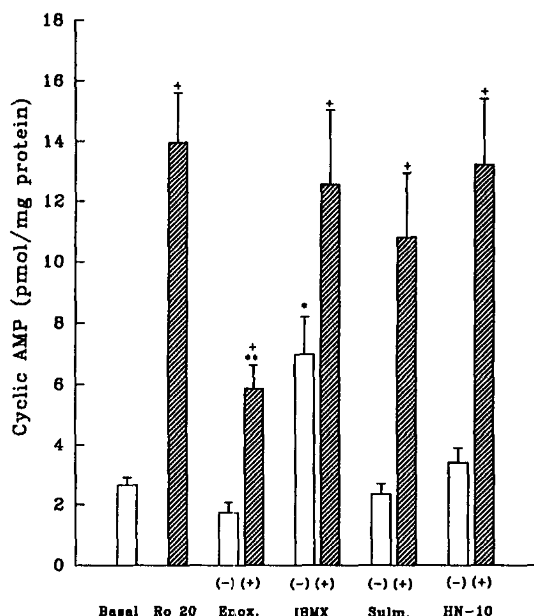

Fig. 5. Effects of enoximone, IBMX, sulmazole and HN-10200 on the accumulation of cyclic AMP in the presence of forskolin (10 μM), and in the absence (−) and presence (+) of Ro 20-1724 at a concentration of 10^{-4} M. Data are presented as the mean ± SE of six heart cell preparations. * Denotes, in the absence of Ro 20-1724, a significant difference ($P < 0.05$) with respect to the basal response. ** Denotes, in the presence of Ro 20-1724, a significant difference ($P < 0.05$) with respect to Ro 20-1724 alone. + denotes a significant difference ($P < 0.05$) of treatments with Ro 20-1724 compared to those in the absence of Ro 20-1724.

13.19 ± 2.19; sulmazole alone, 2.36 ± 0.34; and sulmazole in combination with Ro 20-1724, 10.79 ± 2.15 (mean ±SE) pmol/cyclic AMP/mg protein. In all cases, the cumulative effects of Ro 20-1724 with the other PDE inhibitors tested were no greater than that of Ro 20-1724 alone. No significant difference was noted between the accumulation of cyclic AMP in the presence of Ro 20-1724 alone and the combined effects of sulmazole, HN-10200, or IBMX with Ro 20-1724 (Fig. 5).

Ro 20-1724 did not increase the accumulation of cyclic AMP in the presence of enoximone to the same extent as with the other PDE inhibitors (enoximone, 1.742 ± 0.337; enoximone in combination with Ro 20-1724, 5.86 ± 0.77 [mean ± SE] pmol/mg protein); in other words, while Ro 20-1724 did elevate the response of enoximone, the combined effects of both PDE inhibitors were significantly less than that of Ro 20-1724 alone (Fig. 5). A similar pattern was noted using a higher concentration of the PDE inhibitors (3×10^{-4} M) (results not shown).

Interaction of enoximone with other PDE inhibitors

Enoximone attenuated the maximal response of all PDE inhibitors tested (Table 3). A concentration-dependent relationship was established for antagonism of the effects of both IBMX and Ro 20-1724 by enoximone (Fig. 6). IC_{50} values for the response to enoximone were of a similar order under basal conditions, in the presence of IBMX, or in the presence of Ro 20-1724 (1.01, 0.93 and 1.04 $\times 10^{-4}$ M, respectively). The possibility that the negative effects of enoximone might be due to the constituents of the medium in which the compound is available as a commercial preparation was investigated. A solution (vehicle) containing polyethylene glycol (41.3% w/v), alcohol (9.9% v/v) and sodium hydroxide (0.12% w/v) was found to have no effect on forskolin-stimulated accumulation of cyclic AMP, nor did this solution have any effect on the actions of the other PDE inhibitors tested, that is, Ro 20-1724 and IBMX. Furthermore, enoximone (10^{-4} M) did not produce any change in the pH of the buffer solution used.

Effect of PDE inhibitors on cyclic GMP

In the presence of sodium nitroprusside (10^{-5} M), enoximone (3×10^{-4} M) increased the accumulation of cyclic GMP by 68%. The effects of the other PDE inhibitors (sulmazole, HN-10200, IBMX and Ro 20-1724) on accumulation of cyclic GMP was not significantly different from that of enoximone. However, enoximone apparently decreased the accumulation of cyclic GMP in the presence of the PDE inhibitors, HN-10200, sulmazole, IBMX, or Ro 20-1724 (10 μM), but these effects were not significant statistically.

DISCUSSION

Cyclic AMP is produced in the cell as a result of catalysis of adenosine triphosphate by the enzyme, adenylate cyclase. Activation of adenylate cyclase occurs either by a direct stimulation of the enzyme or indirectly by means of the activation of membrane receptors coupled to stimulatory G proteins, such as is the case for β-adrenoceptors. Inhibition of the PDE isoenzymes in the cytosol prevents the breakdown of the cyclic nucleotides. Basal levels of cyclic AMP were low in isolated ventricular cardiomyocytes similar to isolated tissues [4, 19] Hypothetically, the effects of PDE inhibitors on levels of cyclic nucleotides should be related in part to the rates of synthesis of the cyclic nucleotides;

Table 3. The effects of PDE inhibitors on the accumulation of cyclic AMP in the presence of forskolin (10^{-5} M), and in the absence (−) and presence (+) of enoximone (3×10^{-4} M)

		Concentration of cyclic AMP (pmol/mg protein)		
	Concentration	Enoximone		
Drug	(M)	(−)	(+)	%Decrease
Sulmazole	(10^{-4})	2.015 ± 0.33	1.652 ± 0.22	18.0
HN-10200	(10^{-4})	1.801 ± 0.46	1.429 ± 0.25	21.0
Ro 20-1724	(10^{-4})	8.573 ± 0.73	4.850 ± 0.55*	45.0
IBMX	(10^{-4})	5.615 ± 0.87	2.428 ± 0.17*	55.0
IBMX	(10^{-5})	1.982 ± 0.11	1.428 ± 0.04	28.0

Data are given as mean values ± SE of six heart cell preparations.
* Denotes a significant difference ($P < 0.05$) by comparison to control value in the absence of enoximone.

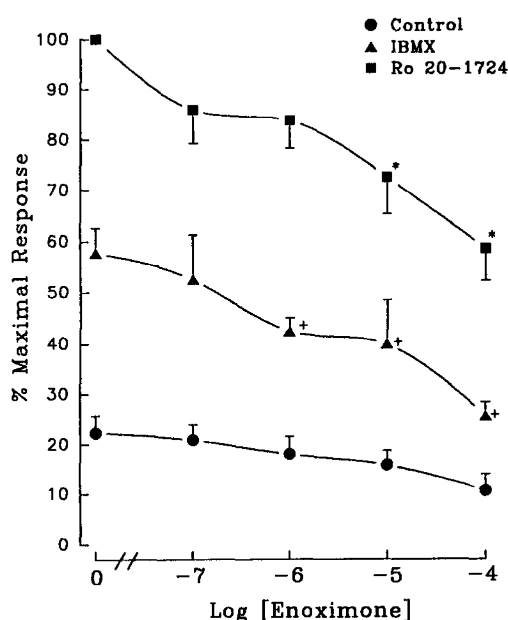

Fig. 6. Inhibitory effect of enoximone on the accumulation of cyclic AMP in the presence of forskolin (10 μM), and in the presence of either IBMX, or Ro 20-1724 (10^{-4} M). Data are presented as the mean ± SE of six heart cell preparations. * Denotes a significant difference ($P < 0.05$) between Ro 20-1724 alone and in the presence of increasing concentrations of enoximone. + Denotes a significant difference ($P < 0.05$) between IBMX alone and in the presence of increasing concentrations of enoximone.

that is, PDE inhibitors should have greater effects on the intracellular content of cyclic AMP when adenylate cyclase is stimulated. The results from this study support this postulate. Forskolin, which acts on the catalytic subunit of adenylate cyclase to activate the enzyme [20–23], was used to magnify the effect of the PDE inhibitors investigated. When used alone, none of the selective PDE inhibitors elevated the content of cyclic AMP under basal conditions, but they all potentiated forskolin-induced accumulation of cyclic AMP.

Forskolin was more effective than the β-adrenoceptor agonist, isoprenaline, at producing an accumulation of cyclic AMP in ventricular cardiomyocytes, in the presence of the PDE inhibitors. Isoprenaline produced an approximate 2-fold increase in cyclic AMP, in contrast to the 12-fold increase observed using forskolin, in the presence of either sulmazole, HN-10200 or IBMX. Hence, forskolin proved to be more useful than isoprenaline for investigating the actions of PDE inhibitors on levels of cyclic AMP in isolated cardiomyocytes. The differences in effects of both stimulators of adenylate cyclase may be explained by the fact that forskolin directly stimulates all the adenylate cyclase present within the cell and, consequently, many adenylate cyclase units that are not coupled to β-adrenoceptors will be activated [21].

Investigation of the time dependency of the response to the various PDE inhibitors on intracellular concentrations of cyclic AMP showed profiles of similar shape in which an initial peak occurred before establishment of the steady state level. Independent of the degree of stimulation by forskolin, the various PDE inhibitors all elevated levels of cyclic AMP by a similar proportion compared to steady state levels within the first few minutes. Such an initial rise in levels of cyclic nucleotides may be explained by a primary rapid burst of free cyclic nucleotide formed in the cytosol following inhibition of the PDE isoenzymes. Then the level of cyclic AMP within the cytosol declines as a certain amount of the cyclic nucleotide transfers to other compartments [9]. These membrane-bound fractions are not included in the assay as the cells are lysed to release only the soluble component of the cyclic nucleotide. By comparing levels of cyclic AMP at the steady state in relation to the peak levels it is clear that the former is dependent both on the degree of stimulation by forskolin, and also on the efficacy of the compounds as inhibitors of PDE isoenzymes which have a high affinity for cyclic AMP.

The differential effects of the various PDE inhibitors have been clearly demonstrated in isolated ventricular cardiomyocytes. IBMX and Ro 20-1724 increased levels of cyclic AMP, in the presence of

forskolin, by 7.7- and 9.5-fold, respectively, compared to the smaller increases in the levels of cyclic AMP of 1.1-, 2.6- and 2.4-fold in the presence of enoximone, sulmazole and HN-10200, respectively. These differences raise the question of whether sulmazole, HN-10200 and enoximone are less effective PDE inhibitors, or whether the PDE inhibitors, Ro 20-1724 and IBMX, stimulate accumulation of cyclic AMP to a greater degree through additional mechanisms. Other mechanisms involving increases in levels of cyclic AMP, apart from inhibition of PDE isoenzymes, have been reported for a number of compounds. IBMX, sulmazole, enoximone, piroximone, amrinone and milrinone, have been shown to increase intracellular levels of cyclic AMP through mechanisms such as antagonism at adenosine A_1 receptors and inhibition of the function of inhibitory G proteins in rat adipocyte membranes [24–28]. It would be expected that if these mechanisms were involved in the present study using ventricular cardiomyocytes, the PDE inhibitors should have produced some degree of increase in the levels of cyclic AMP in the absence of forskolin. However, neither sulmazole, HN-10200, enoximone or Ro 20-1724, produced any significant stimulation of basal levels of cyclic nucleotides although a small increase was observed in the presence of the non-selective PDE inhibitor, IBMX. Furthermore, adenosine deaminase was present in incubation solutions in these studies making it unlikely that these compounds have an effect at the A_1 receptor.

The similar effect of HN-10200, on accumulation of cyclic AMP, to that of sulmazole suggests that HN-10200 may affect the PDE isoenzymes in a manner comparable to that of the structurally similar compound, sulmazole. The lesser effect of sulmazole and HN-10200 compared to IBMX, on accumulation of cyclic AMP, may be an indication of the greater selectivity of these compounds for the PDE III isoenzymes as suggested by other investigators [25], but it is thought to more likely be a consequence of a less potent action and possibly less selective inhibitory effect with regard to the PDE III and PDE IV isoenzymes [29].

Interestingly, enoximone did not augment the forskolin-stimulated effect on the accumulation of cyclic AMP in contrast to the positive action of all the other PDE inhibitors examined, including sulmazole which inhibits the PDE III isoenzyme. The inability of enoximone to increase levels of cyclic AMP in isolated ventricular cardiomyocytes is in marked contrast to those of other investigators [28, 30, 31] who indicated a significant elevation in levels of cyclic AMP, in accordance with evidence showing that this compound is a highly selective inhibitor of the PDE III isoform. A similar peculiarity was noted using sulmazole, which did not increase levels of cyclic AMP in guinea-pig ventricular slices [31]. In general, however, this compound has been shown to increase levels of cyclic AMP in accordance with sulmazole's inhibitory effect on the PDE III isoenzyme in cardiac muscle [24, 32–34]. Conflicting findings have also been reported for a few other compounds which have selectivity for PDE III isoenzymes. While earlier studies failed to detect any significant effect of amrinone on cardiac PDE and cyclic AMP levels in guinea-pig [35] rabbit [36] and rat heart muscle [37, 38], recent studies have demonstrated an increase in accumulation of cyclic AMP in agreement with inhibition of the PDE III isoenzyme [33, 39–42]. DPI 201–106, on the other hand, has been found to inhibit the PDE III isoenzyme, but with no effect on levels of cyclic AMP in guinea-pig, rabbit and rat heart muscle [43]. An explanation for the lack of effect of these compounds on accumulation of cyclic AMP has been discussed in terms of subcellular compartmentalization of cyclic AMP within myocardial cells. It has been suggested that the increases in levels of cyclic AMP are localized presumably near the membrane compartments of the cell and thus may contribute only modestly to a change of the average cytosolic concentration [44–46].

However, the lack of effect of enoximone on levels of cyclic AMP in isolated rat cardiomyocytes is most simply understood by considering a complete selectivity of the compound for the PDE III isoform with no action on the PDE IV isoform; therefore, although enoximone inhibited the PDE III isoenzyme, cyclic AMP could still be hydrolysed by the PDE IV isoenzyme. Hence, the lack of effect of enoximone in cardiomyocytes highlights the degree of selectivity of this compound, especially when compared to sulmazole which has been suggested to be selective for the PDE III isoenzyme [25, 34]. Other investigators [32] report selectivity of enoximone for the PDE III isoenzyme (IC_{50} value 1–8 μM) compared to the PDE IV isoform (IC_{50} value > 300 μM).

Such arguments however, do not explain the effect of enoximone in isolated rat ventricular cardiomyocytes under conditions of elevated levels of cyclic AMP. Further to demonstrating a lack of positive effect of enoximone, this compound had an antagonistic action on the forskolin-stimulated accumulation of cyclic AMP in ventricular cardiomyocytes. Also, enoximone attenuated the response of all PDE inhibitors studied. Moreover, under conditions which produced elevated levels of cyclic AMP in the cardiomyocyte, enoximone was found to have a more pronounced antagonistic effect on levels of cyclic AMP. This phenomenon is not due to the high pH of the compound in solution or the vehicle in which enoximone was dissolved, as solutions of identical composition were added to the cell without effect. Moreover, in a test of whether there was any combined effect of the vehicle with the PDE inhibiting compound, the vehicle solution was added to preparations of all of the other PDE inhibitors, and was found to have no effect. A further property which was investigated was that of the stability of the compound. Enoximone oxidizes on exposure to air, but if stored properly the compound is stable for up to a week (Merrell Dow Pharmaceuticals). Hence, the behaviour of enoximone was not attributed to the preparation of the compound.

In the heart, cyclic GMP can antagonize the effects of cyclic AMP through activation of the PDE II isoenzyme [47, 48]. Therefore, it was of particular interest to investigate the negative effect of

enoximone on accumulation of cyclic AMP in relation to the effects of this compound on levels of cyclic GMP. Since inhibition of PDE can result in increases in either cyclic AMP and/or cyclic GMP, it was important to clarify the role of the latter cyclic nucleotide in the inotropic response produced by the selective PDE inhibitors. The effects of enoximone on accumulation of cyclic GMP did not differ from those of the other PDE inhibitors which produced increases in levels of cyclic AMP at equimolar concentrations. Therefore, it was concluded that the negative response of enoximone on accumulation of cyclic AMP was not due to increased levels of cyclic GMP within the cells. This is in agreement with reports from other investigators who have shown that a number of selective PDE III inhibitors increase the accumulation of cyclic AMP without any effect on levels of cyclic GMP in cardiac tissue [32, 49, 50].

The attenuated response of enoximone on levels of elevated cyclic AMP are less easily explained. It is possible that enoximone may also act through a completely different mechanism at high concentrations; however, it is also conceivable that under stimulated conditions of the cyclic nucleotide, elevated concentrations of enoximone may in fact reverse the PDE-inhibiting effect and cause a small activation of the PDE III isoenzyme. Such was found to be the case in developing selective PDE II inhibitors; that is, while the compounds were found to inhibit the catalytic site of the enzyme, they also appeared to bind with a high affinity to the allosteric site of the enzyme, causing activation of the isoenzyme [51, 52].

Isoforms of PDE other than those with high affinity for cyclic AMP were noted to be relatively unimportant in regulating cyclic AMP levels in rat ventricular cardiomyocytes since the combined effect of the PDE III inhibitor, sulmazole, and the PDE IV inhibitor, Ro 20-1724, was no greater than that of IBMX, a compound that inhibits all four PDE isoforms. This agrees with the findings of Torphy and Undem [53] who noted that the combined effect of the PDE III inhibitor, SK&F 94120 and Ro 20-1724 on isoprenaline-stimulated accumulation of cyclic AMP, was equal to that of the non-selective inhibitor, IBMX, in intact canine trachealis. Furthermore, a lack of effect of zaprinast, a PDE I inhibitor, on the accumulation of cyclic AMP was reported [54]. In addition, sulmazole, enoximone and RMI 82,249 failed to significantly inhibit the hydrolysis of cyclic AMP by PDE I and PDE II in cardiac muscle [31, 55].

Little information is available concerning the contribution of the individual PDE isoforms in rat cardiac muscle in relation to other species. Furthermore, most of this information has been accumulated from studies using PDE inhibitors. While the rat cardiomyocyte contains PDE II, III and IV, their role in the hydrolysis of cyclic nucleotides differ from those of other species. It has been suggested by Verrijk *et al.* [34] that in the rat and hamster, selective PDE III inhibitors seem to be much less effective than in other animal species, both *in vivo* and *in vitro* [36, 56, 57]. Cellular localization also explains differences in activity of the PDE inhibitors among different species. PDE III and PDE IV isoenzymes are present in the cytoplasm in isolated rat ventricular cardiomyocytes whereas in dog cardiac muscle, the PDE III isoenzymes are in a membrane-bound form and are associated with a more effective inotropic response [9].

It has also been reported that PDEs isolated from rat heart contain a higher proportion of the PDE IV isoform in relation to the PDE III isoform than the respective isoenzymes from dog heart [58]. This would explain why Ro 20-1724 produced a greater effect on the accumulation of cyclic AMP than the selective PDE III inhibitors in rat cardiomyocytes; in other words, following inhibition of the PDE IV isoenzyme, a smaller proportion of PDE III isoenzyme was available to hydrolyse the accumulated levels of cyclic AMP.

In isolated cardiomyocytes, the PDE IV inhibitor, Ro 20-1724 had a more pronounced effect on levels of cyclic AMP than any of the other PDE inhibitors examined in this study, including the non-selective PDE inhibitor, IBMX. Furthermore, the effects of Ro 20-1724 on the accumulation of cyclic AMP were non-additive with those of the selective PDE inhibitors. Moreover, since the combined effects of Ro 20-1724 were not greater than the effects produced by Ro 20-1724 alone, it would appear that in addition to inhibiting PDE IV isoenzymes, Ro 20-1724 may also have an effect on the PDE III isoenzymes in isolated cardiomyocytes. Such an effect was demonstrated recently by Schudt *et al.* [44] who reported that the inhibitory potencies of Ro 20-1724 for PDE III and PDE IV isoenzymes were very similar in rat cardiac ventricle, in which IC_{50} values were 1.02 and 1.7 μM, respectively. In addition, rolipram which potently inhibits PDE IV and significantly increases cyclic AMP levels in guinea-pig ventricular slices, was found also to weakly inhibit PDE III [8, 59]. In contrast, Ro 20-1724 selectively inhibited the PDE IV isoform with K_i values of 62 and 3.1 μM, respectively, for the PDE III and IV isoenzymes in guinea-pig and human myocardium [9].

In conclusion, differences in the effects of selective and non-selective PDE inhibitors on the accumulation of cyclic AMP were demonstrated in the presence of forskolin, in isolated rat ventricular cardiomyocytes. Sulmazole and HN-10200 produced a more moderate effect than that of the non-selective PDE inhibitor, IBMX. On the other hand, Ro 20-1724 produced an effect similar to that of IBMX, suggesting that Ro 20-1724 inhibits both the PDE III and PDE IV isoenzymes, and also indicates a more prominent role of the PDE IV isoenzyme in the rat ventricular cardiomyocyte. It is clear that isoenzymes of PDE III and IV have an important role to play in the hydrolysis of cyclic AMP in isolated ventricular cardiomyocytes. It is likely that the high selectivity of enoximone for the PDE III isoform resulted in no increase in the levels of cyclic AMP as a result of its hydrolysis by the PDE IV isoform.

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REFERENCES

1. Colucci WS, Wright RF and Braunwald E, New positive inotropic agents in the treatment of congestive heart failure. *N Engl J Med* **314**: 290–299, 1986.
2. Schmitz W, van der Leyden H, Meyer W, Neumann J and Scholz H, Phosphodiesterase inhibition and positive inotropic effects. *J Cardiovasc Pharmacol* **14**: S11–S14, 1989.
3. van der Leyen H, Phosphodiesterase inhibition by new cardiotonic agents: mechanism of action and possible clinical relevance in the therapy of congestive heart failure. *Klin Wochenshr* **67**: 605–615, 1989.
4. Nicholson CD, Challiss RAJ and Shahid M, Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol Sci* **12**: 19–27, 1991.
5. Weishaar RE, Kobylarz-Singer DC, Quade MM and Kaplan HR, Role of cyclic AMP in regulating cardiac muscle contractility: novel pharmacological approaches to modulating cyclic AMP degradation by phosphodiesterase. *Drug Dev Res* **12**: 119–129, 1988.
6. Beavo JA, Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv Second Messenger Phosphoprot Res* **22**: 1–38, 1988.
7. Thompson WJ, Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. *Pharmacol Ther* **51**: 13–33, 1991.
8. Beavo JA and Reifsnyder DH, Primary sequence of cyclic nucleotide phosphodiesterase isoenzymes and the design of selective inhibitors. *Trends Pharmacol Sci* **11**: 130–155, 1990.
9. Reeves ML, Leigh BK and England PJ, The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem J* **241**: 535–541, 1987.
10. Weishaar RE, Kobylarz-Singer DC and Kaplan HR, Subclasses of cyclic AMP phosphodiesterase in cardiac muscle. *J Mol Cell Cardiol* **19**: 1025–1036, 1987.
11. Tenor TH, Bartel S and Krause EG, Cyclic nucleotide phosphodiesterase activity in the rat myocardium: evidence of four different PDE subtypes. *Biomed Biochim Acta* **10**: S749–S753, 1987.
12. Bode DC, Kanter JR and Brunton LL, Cellular distribution of phosphodiesterase isoforms in rat cardiac tissue. *Circ Res* **68**: 1070–1079, 1991.
13. Piper HM, Probst I and Schwartz P, Culturing of calcium stable adult cardiac myocytes. *J Mol Cell Cardiol* **14**: 397–412, 1982.
14. Powell T and Twist VW, A rapid technique for the isolation and purification of adult cardiac muscle cells having respiratory control and a tolerance to calcium. *Biochem Biophys Res Commun* **72**: 327–333, 1976.
15. Millar BC, Piper HM and McDermott BJ, The antiadrenergic effect of neuropeptide Y on the ventricular cardiomyocyte. *Naunyn Schmiedeberg's Arch Pharmacol* **338**: 426–429, 1988.
16. Tovey KC, Oldham KG and Whelen JA, A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. *Clin Chim Acta* **56**: (3): 221–234, 1974.
17. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
18. Ohishi ST and Barr JK, A simplified method of quantitating proteins using the biuret and phenol reagents. *Anal Biochem* **86**: 193–197, 1978.
19. Endoh M, Yanagisawa T and Taira N, Dissociation of cyclic AMP and contractile responses to isoprenaline: effects of dihydropyridine derivative, nicardipine (YC-93), on canine ventricular muscle. *Eur J Pharmacol* **67**: 225–233, 1980.
20. Lindner E and Metzger H, The action of forskolin on muscle cells is modified by hormones, calcium ions and calcium antagonists. *Arzneim Forsch* **33**: 1436–1441, 1983.
21. Rodger IW and Shahid M, Forskolin, cyclic nucleotides and positive inotropism in isolated papillary muscles of the rabbit. *Br J Pharmacol* **81**: 151–159, 1984.
22. Bristow MR, Ginsburg R, Strosberg A, Montgomery W and Minobe W, Pharmacology and inotropic potential of forskolin in the human heart. *J Clin Invest* **74**: 212–223, 1984.
23. Evans DB, Modulation of cyclic AMP: mechanism for positive inotropic action. *J Cardiovasc Pharmacol* **8** (Suppl 9): S22–S29, 1986.
24. Parsons WJ, Ramkumar V and Stiles GL, Isobutylmethylxanthine stimulates adenylate cyclase by blocking the inhibitory regulatory protein, G_i. *Mol Pharmacol* **34**: 37–41, 1988.
25. Parsons WJ, Ramkumar V and Stiles GL, The new cardiotonic agent sulmazole is an A_1 adenosine receptor antagonist and functionally blocks the inhibitory regulator G_i. *Mol Pharmacol* **33**: 441–448, 1988.
26. Ramkumar V and Stiles GL, The new positive inotrope sulmazole inhibits the function of guanine nucleotide regulatory proteins by affecting GTP turnover. *Mol Pharmacol* **34**: 761–768, 1988.
27. Dorigo P, Gaion RM, Belluco P, Mosti L, Borea PA and Maragno I, An analysis of the mechanism of the inotropic action of some milrinone analogues in guinea-pig isolated atria. *Br J Pharmacol* **104**: 867–872, 1991.
28. Endoh M and Blinks JR, Inhibition of isoproterenol-induced increase in peak calcium transients by theophylline in the isolated canine ventricular muscle. *J Muscle Res Cell Motility* **8**: 468, 1987.
29. Kelso EJ, McDermott BJ and Silke B, Cardiotonic actions of selective phosphodiesterase inhibitors in rat isolated ventricular cardiomyocytes. *Br J Pharmacol* **110**: 1387–1394, 1993.
30. Hsieh CP, Kariya T, Dage RC and Ruberg SJ, Effects of enoximone and isobutylmethylxanthine on contractile tension and cyclic nucleotide levels in isolated blood-perfused dog papillary muscle. *J Cardiovasc Pharmacol* **9**: 230–236, 1987.
31. Ahn HS, Eardley D, Watkins R and Prioli N, Effects of several newer cardiotonic drugs on cardiac cyclic AMP metabolism. *Biochem Pharmacol* **35**: 1113–1121, 1986.
32. Endoh M, Yanagisawa T, Morita T and Taira N, Differential effects of sulmazole (AR-L 115 BS) on contractile force and cyclic AMP levels in canine ventricular muscle: comparison with MDL 17,043. *J Pharmacol Exp Ther* **234**: 267–273, 1985.
33. Scholtz H and Meyer W, Phosphodiesterase-inhibiting properties of newer inotropic agents. *Circulation* **73**: III-99–III-108, 1986.
34. Verrijk R, Vleeming W, van Rooij HH, Wemer J and Porsius AJ. Positive inotropic effect of milrinone, sulmazole and AR-L100 on isolated normal and infarcted hearts of the rat. *Arch Int Pharmacodyn* **297**: 7–17, 1989.
35. Alousi AA, Farah AE, Lesher GY and Opalka CJ, Cardiotonic activity of amrinone—Win 40680 [5-Amino-3,4′-bipyridin-6(1*H*)-one]. *Circ Res* **45**: 666–677, 1979.
36. Schwartz A, Grupp I, Grupp G, Johnson C, Berner P, Wallick E, Imai K and Alousi A, Amrinone: a new inotropic agent, studies on organelle systems. *Circulation* **60**: 16, 1979.

37. Azari J and Huxtable RJ, Differential effects of amrinone on contractility and taurine influx in rat and guinea-pig hearts. *Eur J Pharmacol* **67**: 347–353, 1980.
38. Millard RW, Dube G, Grupp G, Grupp I, Alousi AA and Schwartz A, Direct vasodilator and positive inotropic actions of amrinone. *J Mol Cell Cardiol* **12**: 647–652, 1980.
39. Earl CQ, Linden J and Weglicke WB, Inhibition of cyclic AMP-dependent protein-kinase activity by the cardiotonic drugs amrinone and milrinone. *Life Sci* **39**: 1901–1908, 1986.
40. Alousi AA and Johnson DC, Pharmacology of the bipyridines: amrinone and milrinone. *Circulation* **73**: III-10–III-24, 1986.
41. Braunwald E, Newer positive inotropic agents; introduction. *Circulation* **73**: 1–3, 1986.
42. Sys SU, Goenen MJ, Chalant CH and Brutseart DL, Inotropic effects of amrinone and milrinone on contraction and relaxation of isolated cardiac muscle. *Circulation* **73**: 25–35, 1986.
43. Scholtysik G, Salzmann R, Berthold R, Herzig JW, Quast U and Markstein R, DPI 201–106, a novel cardioactive agent. Combination of cyclic AMP-independent positive inotropic, negative chronotropic, action potential prolonging and coronary dilatory properties. *Naunyn Schmiedeberg's Arch Pharmacol* **329**: 316–325, 1985.
44. Schudt C, Winder S, Muller B and Ukena D, Zardaverine as a selective inhibitor of phosphodiesterase isozymes. *Biochem Pharmacol* **42**: 153–162, 1991.
45. Barsony J and Marx SJ, Immunocytology on microwave-fixed cells reveals rapid and agonist-specific changes in subcellular accumulation patterns for cyclic AMP or cyclic GMP. *Proc Natl Acad Sci* **87**: 1188–1192, 1990.
46. Murray KJ, England PJ, Hallam TJ, Maguire J, Moores K, Reeves M, Simpson AWM and Rink TJ, The effects of siguazodan, a selective phosphodiesterase inhibitor, on human platelet function. *Br J Pharmacol* **99**: 612–616, 1990.
47. Hartzell HC and Fischmeister R, Opposite effects of cyclic-GMP and cyclic-AMP on Ca^{2+} current in single heart cells. *Nature* **323**: 273–275, 1986.
48. Komas N, Lugnier C, Le Bec A, Serradeil-Le Gal C, Barthelemy G and Stoclet JC, Differential sensitivity to cardiotonic drugs of cyclic AMP phosphodiesterases isolated from canine ventricular and sinoatrial-enriched tissues. *J Cardiovasc Pharmacol* **14**: 213–220, 1989.
49. Kauffmann RF, Schenck KW, Utterback BG, Crowe VG and Cohen ML, *In vitro* vascular relaxation by new intropic agents: relationship to phosphodiesterase inhibition and cyclic nucleotides. *J Pharmacol Exp Ther* **242**: 864–872, 1987.
50. Iwatsuki K, Horiuch A, Ren LM and Chiba S, Effects of the cyclic nucleotide phosphodiesterase inhibitors, rolipram, 3-isobutyl-1-methylxanthine, amrinone and zaprinast, on pancreatic exocrine secretion in dogs. *Eur J Pharmacol* **209**: 63–68, 1991.
51. Erneux C, Miot F, Boeynaems J and Dumont JE, Paradoxical stimulation by 1methyl-3-isobutylxanthine of rat liver cyclic AMP phosphodiesterase activity. *FEBS lett* **142**: 251–254, 1982.
52. Yamamoto T, Yamamoto S, Osborne JC and Manganiello VC, Complex effects of inhibitors on cyclic GMP-stimulated cyclic nucleotide phosphodiesterase. *J Biol Chem* **258**: 14173–14177, 1983.
53. Torphy TJ and Undem BJ, Phosphodiesterase inhibitors: new opportunities for treatment of asthma. *Thorax* **46**: 512–523, 1991.
54. Torphy TJ and Cieslinski L, Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol Pharmacol* **37**: 206–214, 1990.
55. Kariya T, Wille LJ and Dage RC, Biochemical studies on the mechanism of cardiotonic activity of MDL 17,043. *J Cardiovasc Pharmacol* **4**: 509–514, 1982.
56. Kobylarz DC, Steffen RP, Weishaar RE and Evans DB, Relationship between inhibition of ventricular peak III phosphodiesterase and the *in vivo* cardiotonic effect of CI-914 in several species. *Pharmacologist* **27**: 603, 1985.
57. Drexler H, Faude F, Hoing S and Just H, Blood flow distribution within skeletal-muscle during exercise in the presence of chronic heart failure; effect of milrinone. *Circulation* **76**: 1344–1352, 1987.
58. Pang DC, Cyclic AMP and cyclic GMP phosphodiesterases: target for drug development. *Drug Dev Res* **12**: 85–92, 1988.
59. Gristwood RW and Owen DAA, Effects of rolipram on guinea-pig ventricles *in vitro*: evidence of an unexpected synergism with SK&F 94120. *Br J Pharmacol* **87**: 91P, 1986.