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Chronotropic and Cyclic Adenosine Monophosphate Response of Fetal Rat Heart in Organ Culture to Isoproterenol, Quinidine, and a Dysrhythmogenic Agent

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Abstract □ The fetal rat heart in organ culture was used to investigate rate changes by various cardioactive agents. Concomitantly, the effect of these pharmacologically induced rate changes on steady-state cyclic adenosine monophosphate levels was determined. Isoproterenol increased the fetal rat heart rate and cyclic adenosine monophosphate in a concentration-related fashion. Quinidine produced a concentration-related decrease in heart rate and no change in cyclic adenosine monophosphate level. The dysrhythmogenic agent produced concentration-related negative chronotropism in the fetal rat heart preparation and significant elevations in cyclic adenosine monophosphate at concentrations without chronotropic action. No correlation between chronotropic effect of a drug and cyclic adenosine monophosphate levels was observed.

Keyphrases □ Isoproterenol—effect on fetal rat heart rate related to levels of cyclic adenosine monophosphate, organ culture □ Quinidine—effect on fetal rat heart rate related to levels of cyclic adenosine monophosphate, organ culture □ Dysrhythmogenic agent—effect on fetal rat heart rate related to levels of cyclic adenosine monophosphate, organ culture □ Adenosine monophosphate, cyclic—levels in fetal rat heart in culture, related to rate changes caused by isoproterenol, quinidine, and dysrhythmogenic agent □ Cardioactive agents—isoproterenol, quinidine, and dysrhythmogenic agent, effect on fetal rat heart rate related to levels of cyclic adenosine monophosphate, organ culture □ Chronotropic effects—isoproterenol, quinidine, and dysrhythmogenic agent, fetal rat heart in culture, related to levels of cyclic adenosine monophosphate

Although the role of cyclic adenosine monophosphate (I) in cardiac inotropism has been investigated (1–3), there have been few attempts to study its role in cardiac chronotropism. The pacemaker region of the heart has been considered too small to be of use in determining adenyl cyclase activity, phosphodiesterase activity, or I levels (4). One study (5) demonstrated that chronotropic and inotropic cardiac responses to catecholamines were very similar with respect to dose dependence and sensitivity to β-blocking agents. This result might be considered as indirect evidence of a common mechanism for cardiac rate and force of contraction. Much evidence linking chronotropism and I comes from the study of cultured, beating heart cells isolated from the neonatal rat. The pulsation rate of these cells is accelerated by epinephrine, a known stimulator of adenyl cyclase (6). Dibutyryl I also imposes positive chronotropism in the same preparation (7).

Entire hearts from fetal mice can be cultured in such a manner as to beat consistently for a period of time (8). The cultured fetal mouse heart has been used as a pharmacological tool. Positive chronotropism was demonstrated using this preparation with liothyronine and levarterenol, and negative chronotropism was found with acetylcholine (9). The fetal mouse heart is capable of responding to these agents as early as the 12th day of gestation (10). The presence of the β-receptor was demonstrated in this same preparation (11).

In this study, the entire fetal rat heart was cultured and used to investigate the relationship between chronotropic effects and steady-state I levels in the myocardium. The fetal rat heart was chosen because it was larger than the mouse heart; this larger size facilitated tissue assay.

Three agents were chosen for pharmacological intervention. Isoproterenol was used for its positive chronotropic and β-adrenergic actions. Quinidine was employed for its negative chronotropic effect. Ethyl 3-ethoxycarbonyl-4-hydroxy-2*H*-1,2-benzothiazine-2-acetate 1,1-dioxide[1] (II), an experimental compound that produces ventricular fibrillation (12), was used to investigate the role of I in cardiac dysrhythmogenesis.

EXPERIMENTAL

Organ Culture—On their 19th day of gestation, pregnant Sprague–Dawley rats were sacrificed by cervical separation. The fetuses were quickly removed by Caesarean section.

The fetuses were decapitated, the chests were cut open, and the fetal hearts were removed as quickly and aseptically as possible. The hearts were trimmed of excessive connective tissue, rinsed in sterile saline, and

[1] McN-2165, McNeil Laboratories.

Table I—Formula of Medium Used to Culture Fetal Rat Hearts

Ingredient	Amount
Sodium chloride	116.40 mmoles
Potassium chloride	5.40 mmoles
Magnesium sulfate heptahydrate	0.80 mmole
Glucose	5.60 mmoles
Monobasic sodium phosphate monohydrate	1.00 mmole
Calcium chloride dihydrate	1.84 mmoles
Sodium bicarbonate	26.20 mmoles
Phenol red	20 mg
Human serum (not normally part of Earle's salts)	33 ml
Water	*q.s.* to 1000 ml
Penicillin G[a]	25 mg/200 ml
Streptomycin[a]	25 mg/200 ml
Hydrocortisone[a]	0.02 mg/200 ml
Insulin U-100 regular[a]	0.24 ml/200 ml

[a] Added just prior to use; the pH was adjusted to 7.40.

positioned carefully on stainless steel grids in 16 × 55-mm disposable petri dishes[2] as described by Wildenthal (13).

The culture medium was prepared from Earle's salts with various additives necessary to optimize survival of the organ culture (8) (Table I). Before being placed in the sterile petri dishes, the culture medium was sterilized by filtration through a 0.45-μm filter[3]. After removal, hearts were kept in their petri dishes in a laminar flow hood until transferred to an incubation chamber.

The organ cultures were placed in a humidified incubation chamber. The chamber was then filled with 95% O_2–5% CO_2, sealed, and incubated at 37° for 48 hr to allow the heart rhythm to stabilize.

After 48 hr of incubation, the culture medium was exchanged for freshly prepared medium. The hearts were equilibrated for at least 2 hr in the fresh medium, and each culture was then transferred to a constant-temperature chamber set at 37° (Fig. 1). The chamber was filled with 95% O_2–5% CO_2 and sealed. After 5 min for temperature equilibration, the hearts were observed with the aid of a dissecting microscope. The number of ventricular contractions was recorded for 1 min. The atrial rate and any anomalies of rhythm also were recorded. Hearts performing at a ventricular rate between 30 and 80 beats/min were retained for study, and the remainder was discarded.

After the predrug rate was recorded, the culture medium was exchanged for fresh medium containing a given drug concentration. The heart preparation was then returned to the incubation chamber for 90 min. (All drugs used in this study produced recognizable pharmacological effects within 45 min, and the effect was consistent for more than 120 min.) After this second incubation period, the petri dish was returned to the 37° incubation chamber and gassed with 95% O_2–5% CO_2. After equilibration for 5 min, the ventricular contractions were again counted for 1 min. Immediately after the second count, the heart was frozen in liquid nitrogen, weighed, and homogenized in 5 ml of ice-cold 6% trichloroacetic acid.

The homogenates were centrifuged at 2500×*g* in a clinical centrifuge for 20 min. The supernates were transferred to 50-ml screw-capped glass tubes and extracted four times with 20 ml of water-saturated ether. The samples were then placed in boiling water, the ether was boiled off, and the samples were stored at −20° until a I assay could be performed (14). The precipitated protein was saved and used to determine the total amount of protein in each heart.

Changes in heart rate were calculated as the ratio of the rate before incubation with the drug (predrug rate) to the rate after 90 min of drug incubation (postdrug rate). This value, R, would be greater than one if the heart rate increased after drug exposure and less than one if the heart rate decreased. Four to eight fetal hearts were generally used for each drug concentration.

Control hearts were randomly selected from various litters, and their heart rates were recorded in the same manner as the drug-treated hearts. Controls were incubated for 2 hr in fresh medium, counted for 1 min, incubated for 90 min, and again counted for 1 min to obtain an R value for each heart. The R values obtained for each concentration of a given drug were statistically compared with the control values and with each other.

Assay for I—Assay for I was performed by the saturation assay method of Brown *et al.* (15). The assay relies upon competition between tritium-labeled I and unlabeled I for binding sites in a protein fraction isolated from beef adrenals.

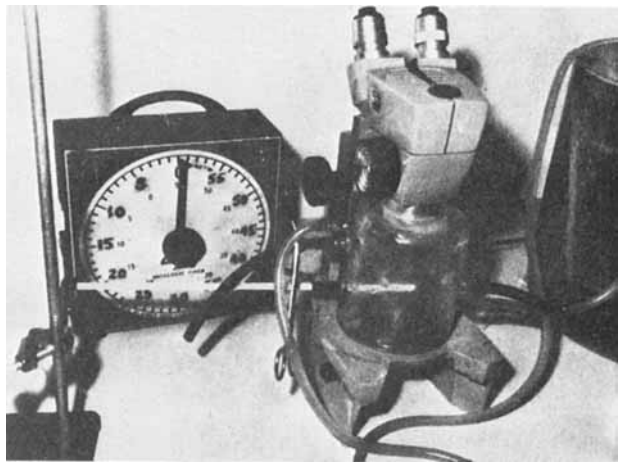

Figure 1—*Constant-temperature observation apparatus. Dark hoses are the gas inlet and outlet, and light hoses are the water jacket inlet and outlet. The thermometer lies across the face of the clock. The water bath is on the extreme right. A Spencer A/O binocular dissecting microscope is inserted through the top of the chamber.*

Assay tubes (50 × 10 mm) were prepared by the addition of 0.1 ml of tritiated I[4] (100,000 cpm/ml), 0.1 ml of sample or standard, and 0.3 ml of the appropriate dilution of binding protein. A standard curve was constructed using known concentrations of unlabeled I from 0.25 to 8 pmoles/tube. All standards and samples were determined in duplicate. Tubes were incubated at 4° for 12–16 hr in a shaker at low speed.

At the end of the incubation period, 0.5 ml of saturated ammonium sulfate was added to each tube to precipitate the binding protein (16). The tubes were immediately centrifuged at 60,000×*g* for 15 min in an ultracentrifuge. A 0.5-ml portion of supernate was transferred to a scintillation vial containing 10 ml of scintillation fluid[5]. All samples were counted to 50,000 counts in a liquid scintillation counter[6]. Since counting efficiency was essentially constant, ratios of counts per minute were used throughout. The ratio of counts per minute in each sample to the counts per minute in samples containing no unlabeled I was calculated and called the "fraction unbound."

A standard curve of the fraction unbound *versus* log picomoles per tube was constructed. Sample values were obtained by extrapolation from the standard curve.

The protein content of the fetal heart was determined by the method of Lowry *et al.* (17). Levels of I were expressed as picomoles per milligram of protein.

Statistical Procedures—Means and standard errors were calculated for each treatment group. The pooled Student t test was used to compare drug-treated values with their respective controls. Analysis of variance and Duncan's new multiple range test were employed to compare each value in an experiment with the other values in that experiment (18).

RESULTS AND DISCUSSION

Levels of I in the fetal hearts not treated with any drug were 25.88 ± 2.50 pmoles/mg of protein or 1.69 ± 0.31 nmoles/g of fresh weight. These values are about the same as reported values of 1.42 nmoles/g of fresh weight obtained (19) with the protein kinase assay in the adult rat ventricle. However, experimental values (20) with the bioluminescent assay were as high as 2.7 nmoles/g of fresh weight.

Quinidine sulfate decreased the heart rate significantly from the control value ($p < 0.05$) at concentrations of 1×10^{-4} M and greater (Table II). The response to the 5×10^{-6} M concentration was not significantly different from those of the 1×10^{-5} M concentration. At a concentration of 1×10^{-4} M, two hearts out of five stopped beating. The mean heart rate at this highest concentration was depressed to approximately 40% of the predrug level when the two hearts that stopped beating were included, and this value was significantly different from all other concen-

[2] Falcon 1007.
[3] Millipore type HA.
[4] New England Nuclear, 22.1 Ci/mM.
[5] PCS, Amersham/Searle.
[6] Packard Tri-Carb.

Table II—Effect of Quinidine Sulfate on Fetal Rat Heart Rate and I Level[a]

Concentration, M	n	R^b	R Mean Comparisons[c]	I, pmoles/mg of Protein	I Mean Comparisons[c]
Control	6	1.005 ± 0.044	α	25.88 ± 2.50	α, β
5×10^{-6}	5	0.939 ± 0.104	α, β	16.85 ± 4.09	β
1×10^{-6}	8	0.818 ± 0.053	β, γ	19.51 ± 4.26	β
5×10^{-5}	9	0.666 ± 0.067	γ	30.68 ± 3.52	α
1×10^{-4}	5[d]	0.405 ± 0.168	—	14.22 ± 1.80	β

[a] Values of R and I represent the mean ± SE. Sample size = n. [b] R = postdrug rate/predrug rate. [c] Treatment means that contain a common Greek letter were not significantly different from each other at the 5% level of significance. [d] Two hearts stopped beating after a concentration of 1×10^{-4} M quinidine sulfate.

trations. Levels of I were not significantly different from controls at any of these concentrations.

Although quinidine can prevent arrhythmias without altering heart rate, it produces negative chronotropism at less than toxic doses (21). At concentrations of 1–5 × 10^{-6} M, quinidine decreased the pulsation rate of cultured cells from the neonatal rat ventricle (22). In humans, blood levels greater than 2.5 × 10^{-5} M generally produced negative chronotropism (23). This result correlates well with the negative chronotropic response to quinidine observed in this study. A concentration of 5 × 10^{-5} M quinidine sulfate, equivalent to 1 × 10^{-5} M quinidine, was the lowest concentration of quinidine that produced significant negative chronotropism.

With use of analysis of variance, no I levels in the drug-treated group were significantly different from the control value. However, at the 1 × 10^{-4} M concentration, two hearts stopped beating at some time during the 90-min incubation. These two hearts had I levels of 19.30 and 17.61 pmoles/mg of protein as compared with 12.90, 10.95, and 10.33 pmoles/mg of protein for the three hearts still beating. Since it is not known when in this 90-min period the hearts stopped beating, the possibility that these two values were elevated after the hearts stopped beating must be considered.

Namm and Mayer (24) demonstrated that changes in I levels in dog and rat hearts were detectable only when the tissue could be frozen rapidly. The biopsy method could not detect changes in I levels because the tissue was not frozen quickly enough. Schmidt *et al.* (25) found that the I levels in brain tissue increased severalfold when rat brains were allowed to stand after decapitation before denaturation. From these studies, it seems reasonable that I levels could fluctuate considerably when the tissue is dying. However, Sutherland *et al.* (1) demonstrated that cardiac tissue retains its I responses to hormones and drugs even after it is no longer beating.

When the 1 × 10^{-4} M concentration of quinidine sulfate was compared with the control value for I by means of the Student t test, a significant difference was revealed ($p < 0.01$). The possibility that a high concentration of quinidine may lower I levels cannot be ignored. However, this concentration is lethal to about half of the hearts and probably this decrease in I levels is of no importance in the clinical usage of this drug.

Isoproterenol hydrochloride increased the heart rate significantly ($p < 0.05$) from the control value at concentrations of 5 × 10^{-6} and 1 × 10^{-5} M (Table III). At the highest concentration, the heart rate was approximately 1.7 times the predrug rate. The heart rate was gradually increased over the concentration range of 5 × 10^{-8}–1 × 10^{-5} M, but only the highest two concentrations caused significantly different rates from the control value at the 95% level of confidence. The rate at 5 × 10^{-8} M was significantly different from the rate at 1 × 10^{-5} M only, and rates at 1 × 10^{-7} and 5 × 10^{-7} M were not significantly different from any other rates at the 95% level of confidence.

Table III—Effect of Isoproterenol Hydrochloride on Fetal Rat Heart Rate and I Level[a]

Concentration, M	n	R^b	R Mean Comparisons[c]	I, pmoles/mg of Protein	I Mean Comparisons[c]
Control	6	1.005 ± 0.044	α	25.88 ± 2.50	α
5×10^{-8}	5	1.258 ± 0.126	α, β	36.93 ± 4.53	α
1×10^{-7}	4	1.306 ± 0.157	α, β, γ	36.94 ± 5.42	α
5×10^{-7}	6	1.342 ± 0.069	α, β, γ	34.28 ± 5.00	α
5×10^{-6}	6	1.698 ± 0.255	β, γ	41.65 ± 6.09	α
1×10^{-5}	8	1.741 ± 0.149	γ	66.35 ± 9.42	—

[a] Values of R and I represent the mean ± SE. Sample size = n. [b] R = postdrug rate/predrug rate. [c] Treatment means that contain a common Greek letter were not significantly different from each other at the 5% level of significance.

Table IV—Effect of II on Fetal Rat Heart Rate and I Level[a]

Concentration, M	n	R^b	R Mean Comparisons[c]	I, pmoles/mg of Protein	I Mean Comparisons[c]
Control	6	1.005 ± 0.044	α	25.88 ± 2.50	—
1×10^{-6}	8	0.850 ± 0.030	α, β	67.68 ± 9.12	α
5×10^{-6}	7	0.711 ± 0.083	β	74.80 ± 16.24	α
5×10^{-5}	8	0.539 ± 0.068	γ	54.86 ± 3.80	α
1×10^{-4}	7	0.452 ± 0.064	γ	119.51 ± 42.46	α

[a] Values of R and I represent the mean ± SE. Sample size = n. [b] R = postdrug rate/predrug rate. [c] Treatment means that contain a common Greek letter were not significantly different from each other at the 5% level of significance.

Although the I levels gradually increased with increasing concentrations of isoproterenol, only values at 1 × 10^{-5} M were significantly different from values at any other concentration ($p < 0.05$). At this highest concentration, the I level was 66.35 ± 9.42 pmoles/mg of protein, as compared to 25.88 ± 2.50 pmoles/mg of protein for the control value.

Isoproterenol had cardioaccelerator properties in laboratory animals and humans (26). The observed 74% increase in rate (Table III) is close to the 60% increase in heart rate found by Wollenberger (6) in response to β-adrenergic stimulation by epinephrine in isolated rat pacemaker cells. In the organ-cultured mouse heart, isoproterenol promoted an overall increase in rate (11). The same preparation showed concentration-related positive chronotropism in response to 10^{-8}–10^{-6} M epinephrine and levarterenol. These responses can be blocked by propranolol, which implicates β-adrenergic receptors. Concentrations of 10^{-7} and 10^{-6} M isoproterenol also increased heart rate 20 and 50%, respectively, in the cultured fetal mouse heart (27). These values compare favorably with the data in Table III, which show approximately a 34% increase in rate at 5 × 10^{-7} M and a 70% increase at 5 × 10^{-6} M isoproterenol.

Isoproterenol, 10^{-8} M, previously produced approximately a 100% increase in the I level of the electrically driven, isolated perfused adult rat heart (28). As perfusion without drug continued, the level returned to the control value within 4 min. In the same study, 10^{-7} M isoproterenol produced the same degree of increase in I levels. The same effects were shown with epinephrine in isolated rat and rabbit adult perfused hearts (1, 29). Continuous infusion of epinephrine produced a rapid rise in I followed by a decrease to a level approximately three times higher than the control value in adult perfused rat hearts (30).

Table III shows a similar tripling effect in the present system when the fetal rat hearts were incubated for 90 min in the presence of a sufficiently high concentration of isoproterenol. The apparent lack of effect of lower concentrations of isoproterenol seen in this study when compared with the results of Øye and Langslet (28) was related possibly to the observation time after the onset of treatment. These investigators gave isoproterenol as a single dose, and the I levels rapidly returned to the control level. The initial rise caused by a low concentration of isoproterenol in this study perhaps could have disappeared before the 90-min observation time. The I level for 5 × 10^{-6} M isoproterenol was not significantly different from the control value when analysis of variance was employed but was significantly different from the control value at the 5% level of significance when the two means were compared using the Student t test.

The rise in I levels following isoproterenol administration indicates that the β-adrenergic receptor subunit on the adenyl cyclase is present at Day 19. Clark *et al.* (31) found this to be true in the fetal rat heart as early as Day 16, although the glucagon receptor was not present until 28 days postpartum.

Compound II decreased the heart rate significantly from the control value at concentrations of 5 × 10^{-6} M and higher (Table IV). At the highest concentration of 1 × 10^{-4} M, the heart rate was depressed to approximately 45% of the predrug level. The heart rate at 5 × 10^{-6} M was not significantly different from the rate at 1 × 10^{-6} M but was significantly different from the control value and from the rates at higher concentrations. Rates at concentrations of 5 × 10^{-5} and 1 × 10^{-4} M were significantly different from rates at all other concentrations but were not significantly different from each other. At no time during the observation of the effects of II on the heart rate was any evidence of arrhythmias, other than bradycardia, detected. Ventricular tachycardia, fibrillation, and ectopic foci were never present when the fetal rat hearts were treated with II.

Levels of I were significantly elevated above the control level at all concentrations of II. There was no significant difference in the I levels between the drug-treated groups.

Intravenous administration of II to anesthetized adult dogs consis-

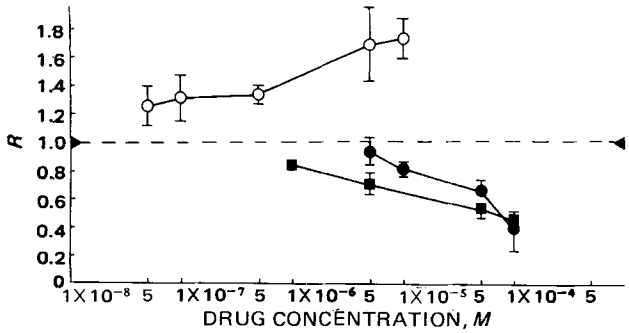

Figure 2—*Effects of quinidine sulfate (●), isoproterenol hydrochloride (○), and II (■) on fetal rat heart rate. The broken line represents the mean control value, and the base of the triangle is equal to the standard error for the control value. Vertical bars represent ± 1* SE.

tently produced a prolongation of the QRS interval (slowing the spread of action potential) and ventricular ectopic beats. These events often terminated in ventricular tachycardia and fibrillation (12). The same effect was shown in the isolated adult rat heart[7]. The fetal rat heart, however, showed only a negative chronotropic response to II. Even at the highest concentration, the fetal rat hearts showed no evidence of tachycardia, ectopic foci, or fibrillation. Cardiac standstill was not observed for any heart treated with II. The variation in the *R* values obtained at various concentrations of II was considerably less than with any other drug treatment.

In contrast, the variation in the fetal heart I levels at a given concentration of II was generally greater than for either quinidine or isoproterenol. Compound II elevated I levels above that of the control at all concentrations. However, the variation in the data was such that no statistically significant difference could be detected between I levels at any drug concentration.

As shown in Figs. 2 and 3, comparison of the effects of isoproterenol (increased rate and increased I concentration), quinidine (decreased rate and no change in I level), and II (decreased rate and increased I level) shows no correlation between changes in the heart rate and the level of I in the entire heart after a 90-min incubation. When the heart rate is altered pharmacologically, certain possibilities exist in which I could still be the mediator of chronotropism. A rapid flux of I could alter some other cellular component, *e.g.*, membrane phosphorylation, and this condition

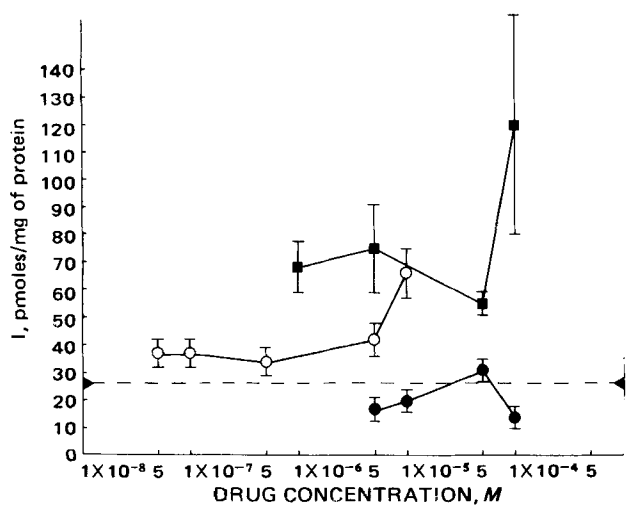

Figure 3—*Effects of isoproterenol hydrochloride (○), quinidine sulfate (●), and II (■) on I levels in the fetal rat heart. The broken line represents the mean control value, and the base of the triangle is equal to the standard error for the control value. Vertical bars represent ± 1* SE.

[7] Unpublished data.

could determine the rate that would still be in effect when the I level had returned to the control value. A separate cellular pool of I responsible for altering the heart rate could be changed without a similar change in the net cellular I level. Epstein *et al.* (2) postulated such separate cellular pools for the regulation of myocardial contractility and glycogenolysis. If changes in I levels were necessary only in pacemaker cells to alter the heart rate, this change could also be masked by the total I concentration of the heart.

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