BRIEF COMMUNICATIONS

Rat Cardiac Muscle Single Cell Automaticity Responses to α- and β-Adrenergic Agonists and Antagonists

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SUMMARY. Isolated cardiac muscle cells from neonatal rat ventricular myocardium have both α- and β-adrenergic, positive chronotropic responses, with sensitivity to applied adrenergic agents more than 100 times greater than the intact neonatal heart. These highly sensitive isolated single cells and small groups of cells also reveal partial agonist activity of the α-adrenergic antagonist, phentolamine, and the β-adrenergic antagonist, propranolol. The most likely explanation for the high sensitivity is the lack of a desensitization process that could involve both a desensitization substance and changes in receptors. These experiments suggest that all adrenergic antagonists could possibly show partial agonist activity. (Circ Res 51: 532-537, 1982)

We have previously shown the high sensitivity of isolated single cardiac muscle cells in culture to norepinephrine and acetylcholine (Hermsmeyer and Robinson, 1977; Marvin et al., 1979). The inherent cell sensitivity, with diffusion barriers and innervation removed, appears to be 100 times greater than in isolated hearts from the same animals. One explanation of higher sensitivity is that the concentrations of transiently released norepinephrine to which the heart responds are greatly limited by the factors of tissue organization.

The high sensitivity in our cell cultures may also reflect developmental changes of adrenergic responses in these cells from neonatal animals. Sensitivity of myocardial cells to adrenergic stimulation is known to be variable as a function of the degree of innervation, i.e., denervation supersensitivity (Fleming et al., 1973) and ontogeny (Pappano, 1975, 1977; Rosen et al., 1977). Myocardial cells not yet adrenergically innervated show rapid and profound desensitization on first exposure to norepinephrine (Hermsmeyer and Robinson, 1977).

Another consideration is the existence of α-receptor-mediated positive inotropic and chronotropic effects in cardiac muscle reported in several mammals (reviewed by Benfey, 1980). It appears likely that cells from the neonatal rats used for cultures express both α- and β-adrenergic receptor function, which could be genetically encoded but normally repressed during later stages of normal development.

In these experiments, we studied both α- and β-adrenergic chronotropic effects in isolated single cells or small groups of cultured cardiac muscle cells, using combinations of adrenergic agonists and antagonists to functionally define cell expression of membrane receptor stimulation in these highly sensitive cells. Just as in the earlier experiments on isolated cardiac muscle cells (Hermsmeyer and Robinson, 1977), some of the results were unexpected.

Methods

Cell Cultures

Only primary cell cultures were used for each of the 38 batches of cell cultures. Cardiac muscle cells were prepared as follows each week and used for studies after 3-7 days in culture. Using aseptic technique, ventricles of 15-20 decapitated neonatal (0- to 3-day-old) rats from the Kyoto-Wistar normotensive (WKY) rat colony at the University of Iowa were excised and placed in CV3M, which consists of 85% MEM-Earle's salts, 15% horse serum, and also contains 4 mM L-glutamine, 20 μg/ml gentamicin, 20 mM Hepes buffer at pH 7.3, and 16 mM HCO3. After being rinsed in CV3M, they were minced with fine dissecting scissors into fragments that were approximately 1 mm in diameter. Ventricular fragments then were exposed to eight 15-minute incubations in 15 ml of 1 mg/ml trypsin in KG solution, consisting of (mM): 133 K-glutamate, 20 Hepes, 16 NaHCO3, 0.5 NaH2PO4, 15.6 dextrose, and 16 NaHCO3. The supernatant from the first incubation was discarded, and all subsequent supernatants were collected and placed in 25 ml of CV3M. These were then centrifuged for 15 minutes at 200 g and washed twice by suspension in CV3M and sedimentation at 200 g for 5 minutes. The cell pellets thus obtained were resuspended in CV3M and plated onto polylysine-coated glass coverslips. Further details of our cell culture procedures are explained elsewhere (Marvin et al., 1979).

Quantitation of Responses

Measurements of spontaneous contraction frequency of isolated single cultured cells or isolated small groups (2-5 cells) were made during 15-second observations on a Leitz
Diervert enhanced contrast microscope. Cultured cells were attached to 9 × 22 mm polylysin-coated coverslips which were studied in designed laminar flow chambers containing less than 300 μl volume; these chambers are described in greater detail elsewhere (Hermsmeyer and Robinson, 1977). For all experiments, cells were suffused with isotonic solution for mammals (ISM), consisting of (in mm): 143 Na+, 4.7 K+, 1.8 Ca++, 0.8 Mg++, 16 HCO3-, 0.4 SO42-, and 17 Hepes. All solutions were saturated with 95% O2-5% CO2 and had a pH of 7.3-7.4 at 37°C as they flowed at approximately 2 ml/min through the laminar flow chamber. After visual recording of spontaneous contraction frequency of a cell, drugs were introduced by either 10- or 20-μl Eppendorf pipettes at the chamber inlet as a front, which swept over the cells without turbulence. Spontaneous contraction frequency was then observed over the next 15-second period, the duration of exposure to the drug. Each of the laminar flow chambers used in these experiments had been previously studied by addition of dye (tryphan blue, methylene blue, phenol red, sodium fluoresceinate, etc.) at the inlet and found to give uniform movement of the dye across the area of measurement as a front with step transition between no dye and full concentration within 100 msec, as determined by photometry. In addition, the vehicle for each drug was tested for chronotropic response due to application artifact. With practice and careful placement, <5% change in spontaneous beating rate (the control variation) with vehicle alone could be achieved. For antagonist vs. agonist experiments, the antagonists were always introduced in the suffusion solution for at least 10 minutes before and during agonist exposure.

Isolated Heart Studies

Entire hearts were isolated from neonatal (1 to 4-day-old) rats from the WKY colony for studies of sensitivity of the entire organ. The entire heart containing the sinus node was pinned in a silicone rubber perfusion chamber similar in principle to the cell culture chamber, and changes in the spontaneous contraction frequency on addition of drugs were recorded by intracellular atrial action potentials. Drugs were added at the chamber inlet and reached the heart as a front (at full concentration) when applied by Eppendorf pipet, as in the cultured cell experiments.

Drugs used in these experiments included: isoproterenol HCl (Sigma), phenylephrine HCl (Winthrop), d, l-dichloroisoproterenol HCl (Sigma), phenoxylbenzamine HCl (Smith, Kline, and French), phenolamine HCl (Ciba), d, l-propranolol HCl (Sigma Chemical Co.), d-propranolol HCl (99.6%, Ayrst), l-propranolol HCl (99.3%, Ayrst), and l-norepinephrine bitartrate (Sigma).

Calculations and Statistics

For sensitivity calculations, data were expressed as log concentrations of the ED50 determined by logit transformation curve fit for each batch of prepared cells or whole heart, each of which was averaged to give an ED50 for all cultured cells or isolated hearts, according to log-normal distribution theory (Fleming et al., 1972). Statistical comparisons were made by group f-test comparison of cell cultures and isolated hearts, with the 0.01 confidence level accepted as significant.

Results

High Sensitivity of Isolated Cells to β-Adrenergic Receptor Stimulation

When the spontaneously contracting ventricular myocardial cells were exposed to a pulse application of isoproterenol, the contraction frequency increased according to the isoproterenol concentration, as shown in Figure 1. Isoproterenol application was in 10- to 20-μl pulses applied in the concentration plotted on the axis, with stimulation artifacts due to the mechanical process of applying the drug carefully tested for each group of cells. The cells were exposed to the applied drug for approximately 15 seconds and the spontaneous contraction frequency was quantitated for 15 seconds in all cases. When water was added instead of a drug, there was no change in spontaneous frequency on the average, with normal variation in spontaneous frequency of less than 20% over a 1-hour period (this long period used only to illustrate the long-term stability of spontaneous frequency). For each response to an applied drug concentration, a cell or cell group was used only one time, as the responses to repeat applications were always strongly diminished in a repeatable pattern. The decrease in second response is interpreted as desensitization, which would account for the lesser response of the cells at concentrations beyond the peak of the dose-response curve. At these higher concentrations, the increase to maximal frequencies lasted for much less than 15 seconds. The maximum average increase in spontaneous contraction frequency was greater than 2-fold the basal contraction frequency, with maximum frequencies of about 300/min observed in individual cases. Control contraction frequencies were selected to be approximately 75/min, which we have found to be the most constant.

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Isoproterenol increased spontaneous contraction frequency over a range of concentrations from 10 pM to 1 μM. Each cell or cell group was exposed to only one concentration to avoid desensitization, which is also evident in the decrease in frequency above 1 nm. After 10 minutes of exposure to 1 μM dichloroisoproterenol (DCI), contraction frequency increases were limited to 45% of those without DCI. For the curve without DCI, control contraction frequency was 64 ± 1, and after DCI, 75 ± 1 (mean ± se). Each point represents the mean ± se of eight measurements. *Indicates point at −24% as contraction frequency increase lasted only seconds, followed by transient quiescent period.
TABLE 1
Comparison of Sensitivity to Adrenergic Stimulation in Cultured Cells and Freshly Isolated Hearts

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Isolated cells</th>
<th>Whole heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol</td>
<td>2</td>
<td>250</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>50</td>
<td>750</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>6,000</td>
<td></td>
</tr>
</tbody>
</table>

$E_{D50}$ values are from pulse applications of 10 µl to cultured cells or 100 µl to isolated hearts to give equivalent spontaneous contraction frequency increases.

contributing to the consistency of the recorded data. Spontaneous contraction frequency of randomly selected cells in all culture dishes averaged 85 ± 12 (±SD) per minute. The $E_{D50}$ for pulse applied isoproterenol was 2 nM, a value > 100X lower than found in freshly isolated neonatal rat hearts (Table 1). Norepinephrine caused stimulation similar to isoproterenol in both single cells and isolated hearts (not shown).

High Sensitivity of Isolated Cells to α-Adrenergic Receptor Stimulation

Sensitivity to the α-adrenergic agonist, phenylephrine, was also found in these isolated cardiac muscle cells. Increasing concentrations of phenylephrine caused higher spontaneous contraction frequencies up to the peak of the dose-response curve, with an $E_{D50}$ of 50 nM (Fig. 2). The sensitivity to phenylephrine in single myocardial cells was also much greater than in isolated hearts (Table 1). The maximum effect observed with phenylephrine was a 1.5X increase, although individual cells showed increases as large as 2X over the control contraction frequency. As with isoproterenol, the response to repeat concentrations of phenylephrine was greatly diminished. At concentrations above the peak of the curve, the initial increase in contraction frequency faded after several seconds, resulting in lower frequencies at high concentrations.

α-Adrenergic Antagonists

The α-adrenergic receptor stimulation could be blocked by the noncompetitive antagonist, phenoxybenzamine (1 µM). Figure 2 shows the shift to the right of the phenylephrine dose-response curve in the presence of phenoxybenzamine. In contrast, when phentolamine was introduced to the cells there was a dose-related increase in spontaneous contraction frequency (Fig. 3), indicating that phentolamine acted as a partial agonist. The phentolamine dose-response curve indicated a much lower sensitivity ($E_{D50}$ = 300 nM) than stimulation by isoproterenol or phenylephrine. Figure 3 also shows that the stimulation by phenolamine could also be blocked by 10-minutes perfusion with 10 µM phenoxybenzamine. Although phentolamine could have been used at low concentrations as an α-antagonist, partial agonist activity prevented such use of phenolamine for the rest of the experiments. No partial agonist activity of phentolamine up to 1 mM was detected in isolated hearts.

β-Adrenergic Antagonists

Figure 1 also shows β-adrenergic blockade as a partial block of the isoproterenol dose-response curve by 1 μM dichloroisoproterenol. There was an average

![Phenylephrine increased spontaneous contraction frequency over a range of molar concentrations 100X higher than isoproterenol (cf. Fig. 1). Each cell was exposed to only one concentration. After 10 minutes of exposure to 10 µM phenoxylbenzamine (Pbz), contraction frequency increased to a limit of only 40% of that without Pbz. For the upper curve, control contraction frequency was 80 ± 3, and for Pbz, was 77 ± 2. Each point represents the mean ± se of eight measurements.](image1)

![Phentolamine acted as a partial agonist to increase spontaneous contraction frequency at molar concentrations three orders of magnitude greater than isoproterenol or norepinephrine. Each cell was exposed to only one concentration, and each point is the mean ± se of three measurements. Control contraction frequency was 68 ± 2 without and 71 ± 1 with 10 µM Pbz. After 10 minutes of exposure to Pbz, the phenolamine simulation of increased contraction frequency was completely blocked.](image2)
of 6% increase of baseline spontaneous contraction frequency in 1 μM dichloroisoproterenol alone. Higher concentrations of dichloroisoproterenol caused increased contraction frequency, with a peak average increase of 55% at 100 μM and a peak at 30 μM. The choice of dichloroisoproterenol as the β-adrenergic blocking agent in Figure 1 was based on the relatively smaller effect of 1 μM dichloroisoproterenol compared to 1 μM d,l-propranolol, and the wider range of concentrations of propranolol which increased frequency (Fig. 4). In isolated (noncultured) hearts, there was a 30% increase in spontaneous contraction frequency caused by 100 μM dichloroisoproterenol, but no partial agonist activity of up to 1 μM propranolol was detected.

Antagonism of Stimulation by the 2-Propranolol Stereoisomers

Both d- and l-propranolol caused increased isolated cell spontaneous contraction frequency when applied separately. Figure 5 shows the dose-response curve to l-propranolol alone, with an ED₅₀ approximately one-half that for racemic propranolol. Figure 5 also shows the partial (40%) blockade of the l-propranolol spontaneous contraction frequency increase by 1 μM dichloroisoproterenol, and complete blockade by the combination of 1 μM dichloroisoproterenol and 10 μM phenoxybenzamine. For these experiments using propranolol as an agonist, the dichloroisoproterenol and/or phenoxybenzamine were added as described for antagonists in the methods, i.e., 10 minutes previous to the pulse of propranolol. Figure 6 shows the contraction frequency increase to d-propranolol alone. There was a smaller (25% peak average) dose-dependent increase in contraction frequency. The d-propranolol agonist effect was not antagonized by dichloroisoproterenol, but was completely blocked by phenoxybenzamine.

Discussion

These experiments provide further evidence that single rat ventricular myocardial cells are highly sensitive to stimulation by adrenergic agonists, and therefore, are a confirmation of earlier reports of high sensitivity of cardiac and vascular muscle cells (Hermsmeyer and Robinson, 1977; Marvin et al., 1979). With such highly sensitive cells, it is possible to study postsynaptic adrenergic mechanisms in great detail. With the drug application method we have devised, all factors, including access of the drug to a large fraction of the total cell surface area within 0.1 sec, are optimized for highest sensitivity to agonists. In particular, the process of desensitization, which we earlier demonstrated is the strongest factor limiting catecholamine sensitivity in experiments on cultured cardiac muscle cells (Hermsmeyer and Robinson, 1977), was limited. Another possibility is that ventricular muscle cell pacemaker mechanisms are more sensitive than sinoatrial node pacemaker mechanisms.
However, this possibility seems unlikely based on the even higher sensitivity found in isolated rat sinoatrial nodal cells (Marvin and Hermsmeyer, unpublished observations).

Our experiments have shown that both $\alpha$- and $\beta$-adrenergic stimulation produce positive chronotropic effects, as in other young animal ventricles studied (reviewed by Benfey, 1980). It appears possible that $\alpha$-adrenergic receptor function is expressed at early ages, but then increases with age, as has been recently suggested by Cheng et al. (1980). Receptor binding studies have suggested that the number of $\alpha$-adrenoceptors in cardiac muscle of the mouse peaks in early neonate (7 to 21 days old), but the decrease in sensitivity during ontogeny may reflect changes in regulatory proteins for adrenergic sensitivity, i.e., the proposed densensitization protein (Terasaki et al., 1978). Whereas cell culture studies have shown that both muscle cells and fibroblasts have $\beta$-receptors (Lau et al., 1980), which would also complicate any whole heart homogenate binding study, increased contraction frequency demonstrates both $\alpha$ and $\beta$

stimulation in myocardial cells (Karsten et al., 1977; present study) and allows detection of partial agonist activity (present study).

With the nondesensitized cells in our cultures, the partial agonist activity of adrenergic antagonists was prominent and possible to quantitate. The fairly high intrinsic agonist activity of phentolamine was not surprising in view of earlier reports on the arterial circulation (Weiner, 1980). Given the presence of $\alpha$-adrenergic receptors in cardiac muscle, such an action of phentolamine (that is not shared by phenoxymethylamine) might have been predicted. On the other hand, the partial agonist activity of propranolol was unexpected in these experiments (Ahlquist, 1979). The partial agonist activity of dichloroisoproterenol is known (Ahlquist, 1979) and was also found here (Fig. 4). However, the previously undetected intrinsic sympathomimetic activity of propranolol was even more of a complication (to its use as a pure antagonist) in highly sensitive isolated cardiac muscle cells.

The evidence that propranolol is a partial adrenergic agonist appears unequivocal in the single cell experiments. With the $d$- and $l$-stereoisomers of propranolol, we could separate the dose-response curve of racemic propranolol into the $l$-propranolol component, which was not purely $\beta$-receptor, but was blocked by the combination of $\alpha$- and $\beta$-antagonists, and the $d$-propranolol component, which was apparently purely $\alpha$, being blocked completely by phenoxymethylamine. These novel effects of propranolol might be involved in the poorly understood wide variation of sensitivity and withdrawal symptoms to propranolol observed in patients (Meier et al., 1980).

Although we have not tested other agents in the growing series of $\beta$-adrenergic antagonists, it appears likely, especially in view of the deliberate design of the partial agonist activity into certain antagonists, e.g., alprenolol (Ablad et al., 1980) and pindolol (Weil, 1980), that the characterization of adrenergic antagonists might increasingly take the form of selectivity ratio, rather than absolute statements of complete freedom from agonist activity. These experiments have demonstrated the need to explore further the $\alpha$- and $\beta$-stimulation of cardiac muscle automatically with combinations of agonists and antagonists and—considering that partial agonist activity may be involved—especially where antagonists increase rather than decrease heart rate.

### References


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