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ß-adrenergic receptor function in cultured AT-1 cardiomyocytes

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Summary: AT-1 cells are highly differentiated, contracting cardiomyocytes derived from atrial tumours in transgenic mice. The aim of this study was to characterize ß-adrenergic receptor function and associated intracellular calcium regulation in AT-1 cells. Equilibrium binding experiments with [^{3}H]-CGP-12177 showed a K_d=0.30±0.08nM and a B_{max}=2.25±0.47 fmol/10^5cells. Competition binding experiments with CGP-20712A showed presence of predominantly $ß_1$-adrenoreceptors. S-(-)propranolol, atenolol and R-(+)propranolol showed a competitive inhibition of binding with successively lower affinity. Isoproterenol, 2 µM, for 48 hours down-regulated the number ($p<0.05$) of ß-adrenergic receptors/cell by about 50%; 10 µM for one hour increased the cAMP concentration ($p<0.05$) by about 100%. Cytosolic [Ca^{2+}] was measured flourimetrically in spontaneously and synchronously beating AT-1 cells. The resting cytosolic concentration was 94±10 nM. The observed sinusoidal Ca^{2+} oscillation frequency increased after addition of 10 µM isoproterenol ($p<0.02$). This effect was antagonized by 10 µM alprenolol ($p<0.01$). In conclusion, AT-1 cells have functional ß-adrenoreceptor signalling pathways and constitute an important tool in cardiac biology.

Heart cells in culture are a valuable experimental tool in cardiac biology. However, cardiomyocytes do not divide, change phenotype when plated and dedifferentiate rapidly in prolonged culture [1,2,3]. These characteristics have proven to be a major obstacle for comparative studies addressing the function of cardiomyocytes.

A breakthrough was achieved when the AT-1 cells were developed [4]. These cells are derived from atrial tumours in mice carrying an ANF promotor-SV40 large T-antigen transgene. The AT-1 cells are unique since they represent a population of spontaneously beating cardiomyocytes that maintain an advanced state of differentiation and yet retain the ability to divide. In primary culture the cells grow to a syncytium which morphologically, histologically and electrophysiologically display a highly differentiated cardiac phenotype [5,6,7].

ß-adrenergic receptors play a major role in cardiac biology. Altered responses to adrenergic stimuli have been implicated in several clinically important cardiac diseases as for example; heart failure [8,9,10], cardiomyopathies [11] and myocardial ischemia [12,13,14]. Several studies characterising ß-adrenergic receptor function have been performed on cultured [15,16]

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and isolated [17,18] cardiomyocytes. The major obstacle when performing studies on cultured cardiomyocytes is dedifferentiation. Single cell suspensions from collagenase treated heart tissue contain endothelial cells, smooth muscle cells, fibroblasts, macrophages and blood cells as well as cardiomyocytes. The AT-1 cells are the first cardiomyocytes, which retain the characteristics of the original cell in *pure* primary culture and it is of great importance to study function and characteristics of ß-adrenoreceptors in this unique celltype.

The regulation of intracellular Ca^{2+} concentration is essential in excitation-contraction coupling in cardiac muscle. Spontaneous oscillations in cytosolic Ca^{2+} are known to exist in cultured cardiac muscle cells [19]. Consequently the aim of this study was to characterize ß-adrenergic function and intracellular Ca^{2+} regulation in spontaneously beating AT-1 cells.

Methods

Cell culture: AT-1 cells were kindly provided by Dr LJ Field (Indiana University, Indianapolis, IN, USA). Primary cultures were prepared as earlier described [4,5,6,7]. Shortly, subcutaneously growing AT-1 tumours were excised from a syngenic host and minced into small pieces. The tumour was then washed with 2 x 50 ml sterile phosphate buffered saline. A single cell suspension was obtained from sequential digestion with 0.1% CLS I collagenase (Worthington Biochem. Corp. NJ, USA) in 37° C for 70 minutes. The cells were centrifugated, washed, pooled and plated at a density of 0.1 x 10^6 cells/cm^2. Cells were maintained in Dulbeccos modified Eagle´s medium (Sigma, St Louis, MO, USA) supplemented with penicillin (100 U/ml) (Sigma, St Louis, MO, USA), streptomycin (100 µg/ml) (Sigma, St Louis, MO, USA), 10% fetal bovine serum and 2% L-glutamine (Sigma, St Louis, MO, USA). Cells were grown on Falcon Primaria plates. DDT-MF-2 smooth muscle cells (CRL-1701, ATCC, Rockville, MD, USA) were cultured in Dulbeccos modified Eagle´s medium supplemented with penicillin (100 u/ml), streptomycin (100 µg/ml), 5% fetal bovine serum and 2% L-glutamin. DDT-MF-2 cells were cultured for 2 days before experiments.

Measurements of ß-adrenergic receptors: Binding experiments were performed in Ringer solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM glucose and 20 mM HEPES, pH 7.4) on AT-1 cells cultured for at least 6 days. Equilibrium binding constants for [^{3}H]-CGP-12177 (Du Pont-NEN, Boston, MA, USA) were obtained following incubation with a range of concentrations of the radioligand±1000x excess of S-(-)propranolol (Sigma, St Louis, MO, USA) for 100 min at +4°C. In desensitization experiments, isoproterenol (Sigma, St Louis, MO, USA) was added directly to the culture medium and the cells were then cultured for 48 h in 37° C and 5% CO_2. Routinely, ß-adrenergic receptor levels were quantitated following binding of 2 nM [^{3}H]-CGP-12177±1000x excess of S-(-)propranolol for 100 min at +4°C. In binding competition experiments, S-(-)propranolol (unspecific $ß_1$- and $ß_2$-antagonist), R-(+)propranolol (unspecific $ß_1$- and $ß_2$-antagonist with low affinity) (Sigma, St Louis, MO, USA), atenolol ($ß_1$-specific antagonist and a very weak $ß_2$-antagonist) (Sigma, St Louis, MO, USA) or CGP-20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2 imidazolyl)-phenoxy]-2-propanol methanesulfonate) (highly $ß_1$-specific antagonist) (CIBA-Geigy, Basel, Switzerland) were added to the Ringer solution containing 2 nM [^{3}H]-CGP-12177. Bound radioactivity was measured (LKB, Rackbeta 1214, Bromma, Sweden) following extraction with 1 M NaOH and 1 M HCl.

Calcium measurements: Fura-2 was used to measure cytosolic free Ca^{2+}. AT-1 cells cultured for 6 days were washed with Ringer solution. The cells were incubated for 1 h in the same solution with 2 µM Fura-2 AM (Sigma, St Louis, MO, USA) and thereafter washed twice. Measurements were performed at room temperature in 1 ml Ringer solution. Fura-2 fluorescence was measured in a CAF-100 fluorimeter (Jasco, Tokyo, Japan) at 340/380 nm and 505 nm excitation- and emission wavelengths, respectively. Recordings were done in ratio mode. Maximum flourescence was determined by addition of 0.1% Triton X-100 and minimum

fluorescence was determined by addition of 5 mM EGTA [21]. Intracellular Ca^{2+} was determined with the equation $[Ca^{2+}] = K_d x(F-F_{min}/F_{max}-F)$, where F denotes flourescence. An estimated K_d of 224 nM [20] was used for calculation of $[Ca^{2+}]$.

Measurement of cAMP: Cultured AT-1 cells were washed three times with Ringer solution. The cells were then exposed to isoproterenol for 60 min at 37°C in Ringer solution with 0.3 mM IBMX (isobutylmethylxanthine, Sigma, St Louis, MO, USA). To stop the experiment, the cells were exposed to 10% percloric acid for 30 min at room temperature. To precipitate excess percloric acid, 2 M KCl with 0.1 M HEPES was added and the samples were put on ice for 30 min. Following centrifugation at 3000g for 5 min, the supernatants were saved and the cAMP content measured with a radioimmunoassay (Du Pont-NEN, Boston, MA, USA).

Calculations: Determination of K_d and B_{max} were evaluated using the non-linear Hill equation [22]; $LR=(B_{max}xL^n)/(K_d^n+L^n)$. Calculation of K_i values were done with the Cheng-Prusoff equation [22]; $K_i=IC_{50}/(1+L/K_d)$. Competition binding experiments (determination of IC_{50}) were evaluated by a non-linear four parameter logistic (4pl)-model [22]; $LR=((B_{max}-B_{min})/(1+(I/IC_{50})^F))+B_{min}$. LR denotes bound radioligand; B_{max} and B_{min}, maximum and minimum binding, respectively; L is free radioligand and K_d the equilibrium dissociation constant; I, added concentration of binding inhibitor; IC_{50}, concentration of binding inhibitor at half maximal binding of radioligand; F, slope factor.

Statistics: Values are given as means with standard deviation. Paired t-tests were used for calculating the effects of isoproterenol on ß-adrenergic receptor density, cAMP levels and Ca^{2+} oscillation frequency.

Ethics: The AT-1 cell culture and mouse inoculation of tumour were approved by the animal scientific ethical commitee of the Karolinska Institute.

Results

[^{3}H]-CGP-12177 binding to intact AT-1 cardiomyocytes had a K_d of 0.30±0.08 nM and B_{max} of 2.25±0.47 fmol/10^5 cells (figure 1A). CGP-20712A, a ß1-specific ligand, almost totally inhibited binding of [^{3}H]-CGP-12177. S-(-)propranolol totally inhibited [^{3}H]-CGP-12177 binding (figure 1B). Thus, the total amount of ß2 receptors were less than 10%. CGP-20712A bound to a single class of binding sites with a K_i of 1.18 nM.

DDT-MMF-2 is a leiomyosarcoma cell-line expressing almost exclusively ß2 receptors. S-(-)propranolol binds with approximately the same affinity in AT-1 (IC_{50}=0.69nM) and DDT-MF-1 (IC_{50}=1.0 nM), whereas different affinities were observed for atenolol (IC_{50}=0.68 nM (AT-1) and 4.1µM (DDT-MF-2)) and R-(+)propranolol (IC_{50}=3.1 µM (AT-1) and 26.9 µM (DDT-MF-2)) (figure 1C and 1D).

Isoproterenol, 1 µM, for 48 hours in the culture medium down-regulated the ß-adrenergic receptor density by approximately 50 % ($p<0.05$) from 2.25±0.29 to 1.15±0.05 (expressed as fmol/10^5 cells). Isoproterenol, 10 µM, for 1 hour up-regulated total cAMP concentration by approximately 100% ($p<0.05$) from 89.7±8.9 to 204.0±14.1 nM (in extracts).

Fura-2 measurements showed a resting cytosolic Ca^{2+} concentration of 94±10 nM. The cells responded with an increase in cytosolic Ca^{2+} when exposed to 20 mM KCl (figure 2A) and a decrease when exposed to 20 mM EGTA (figure 2B). We observed sinusoidal oscillations in cytosolic Ca^{2+} concentrations (n=10) (figure 2B). EGTA also abolished the spontaneous oscillations (figure 2B). Addition of 10 µM isoproterenol increased the Ca^{2+}

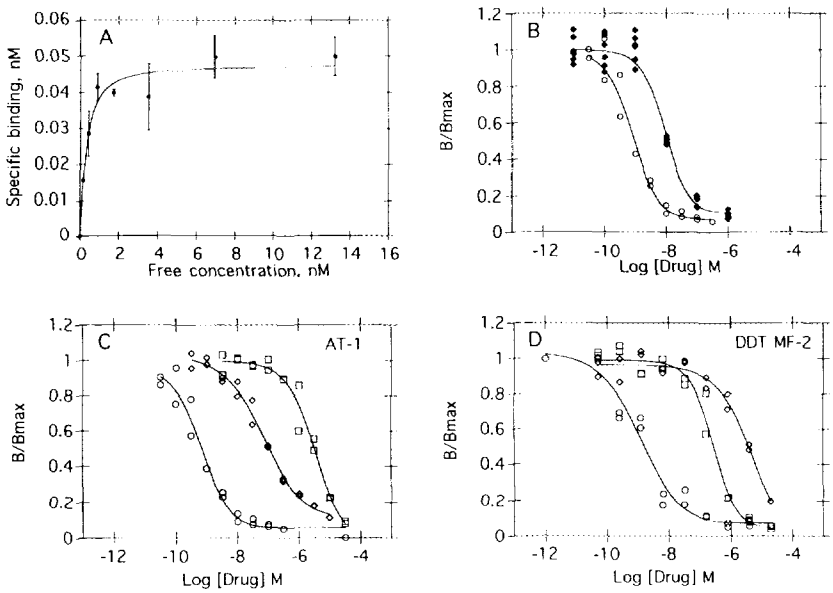

Figure 1. **A**, Equilibrium binding experiment with [^{3}H]-CGP-12177 in AT-1 cells done in triplicate. Error bars show standard deviation. **B**, Equilibrium binding experiment with [^{3}H]-CGP-12177 done in hexaplicate and addition of increasing concentrations of the cold ß$_1$-specific antagonist CGP-20712A (-♦-) compared to the non-specific ß-receptor antagonist S-(-)propranolol (-o-). The binding of [^{3}H]-CGP-12177 is almost totally inhibited with CGP-20712A. The amount ß$_2$ receptors is less than 10%. The Y-axis denotes bound radioligand/maximal binding. **C and D**, Competition binding experiment with [^{3}H]-CGP-12177 done in triplicate with addition of increasing concentrations S-(-)propranolol (-o-), atenolol (-◊-) and R-(+)propranolol (-❑-). In AT-1 cells, with predominantly ß$_1$-adrenergic receptors, (C), the affinities for the three cold ligands was different from that found in the DDT MF-2 cells, which express predominantly ß$_2$-adrenergic receptors (D).

oscillation frequency from 2.24±2.84 to 5.7±5.5 ($p<0.02$)($n=5$)(figure 2C). Subsequent addition of 10 µM alprenolol reduced the intracellular Ca^{2+} oscillating frequency from 5.7±5.5 min^{-1} to 2.7±5 min^{-1} ($p<0.01$) (figure 2C).

Discussion

The present study shows that the AT-1 cells express functional ß-adrenergic receptors. The receptors are predominantly of the ß$_1$ type. The total number of receptors and the K_d are in the same range as previously reported by other groups using [^{3}H]-CGP-12177 [16,17,18,23] with other types of cardiomyocytes, ie. isolated or cultured adult and neonatal rat. Other studies using [^{125}I]-iodocyanopindolol (ICYP) and isolated or cultured rat cardiomyocytes have shown different B_{max} [15,24]. This is probably due to differences in lipid solubility and the very high non-specific binding of [^{125}I]-ICYP. Another important factor is the incubation temperature, which in different studies varies from +4°C to +37°C as compared to the present study, where we used +4°C.

The AT-1 cell line expresses almost exclusively ß$_1$-adrenoreceptors. In previous studies with purified adult ventricular rat cardiomyocytes, a similar relationship between ß$_1$ and ß$_2$

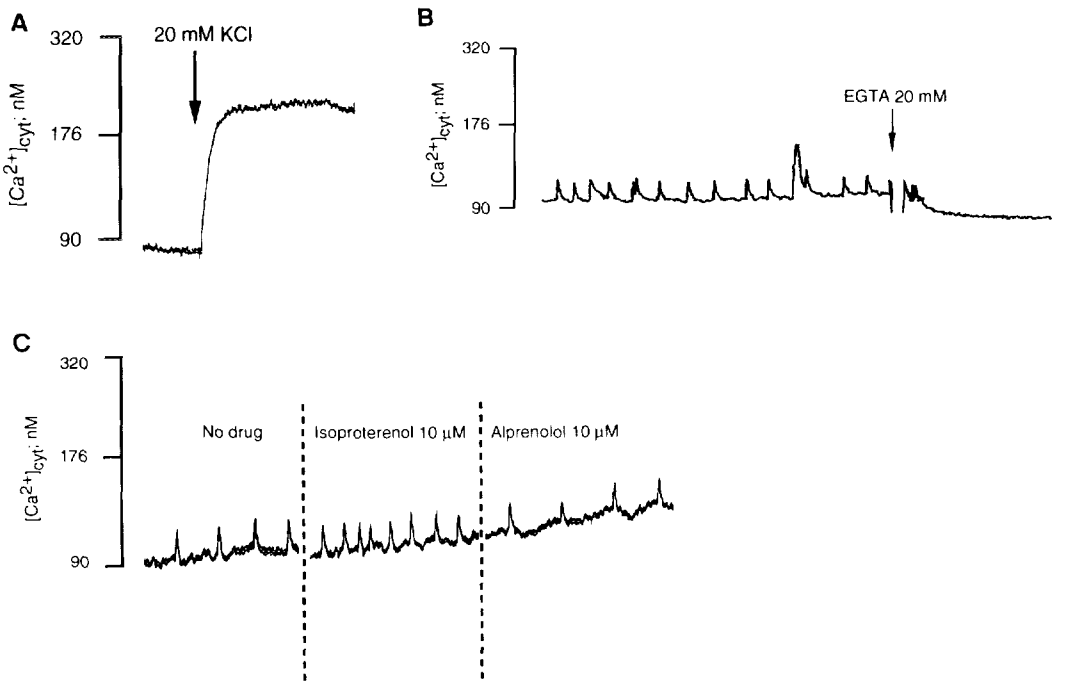

Figure 2. **A**, Addition of 20 mM KCl increased cytosolic $[Ca^{2+}]$. The emission ratio between 340 and 380 nm excitation wavelengths is shown. **B**, Spontaneously beating AT-1 cells with cytosolic $[Ca^{2+}]$ oscillations. 20 mM EGTA abolished the oscillations and decreased the cytosolic Ca^{2+} concentration. **C**, Increase in frequency of Ca^{2+} oscillations by 10 µM isoproterenol and antagonism by 10 µM alprenolol.

receptors was reported [15]. Other studies, on rat and human heart, showed a higher amount of β_2 receptors, but a major difference is that these were performed on homogenized hearts, including all the different celltypes within the ventricle [25,26].

The AT-1 cells bind S-(-)propranolol, atenolol and R-(+)propranolol with affinities which imply ß-adrenoreceptors with β_1 binding characteristics. A different pattern is seen in the DDT MF-2 cells, where the β_1-specific drug atenolol has a significantly higher IC_{50}. This is expected since DDT MF-2 cells mainly express β_2 receptors. The difference in binding of atenolol between the two cell lines also validates the assay.

Isoproterenol down-regulated ß-adrenergic receptor density by approximately 50%. Several earlier studies have reported similar effects of isoproterenol on ß-adrenergic receptor density in rat cardiomyocytes [16,18,27]. The magnitude of the downregulation in these studies were in the same range as for the AT-1 cells in the present study.

Regulation of cAMP concentration by isoproterenol is essential when proving the presence of functional ß-adrenergic receptors. The AT-1 cells responded as previously reported for adult canine and guinea pig cardiomyocytes [28,29]. The total cAMP level increased 100 % in the AT-1 cells whereas the increase was approximately 300-400% in the earlier studies [28,29]. These studies were mostly performed on whole hearts or freshly isolated cardiomyocytes, which may account for the difference.

The resting cytosolic Ca^{2+} concentration in the AT-1 cells was in the same range as previously reported for guinea pig and rat cardiomyocytes using Fura-2 [30,31]. Oscillating Ca^{2+} transients have previously been reported by other investigators in isolated cells and primary cultures of rat, guinea pig and ferret cardiomyocytes [19,31,32]. The spontaneously beating AT-1 cells do not seem to differ from other types of cardiomyocytes in this respect. The finding that EGTA abolishes the oscillations indicates that the these are, in part, caused by influx of Ca^{2+} through the cell membrane. This is, however, the first study to report that these oscillating transients can be modulated in *frequency* by ß-adrenergic stimulation and blockade.

In conclusion, the AT-1 cardiomyocytes express functional ß-adrenergic receptors which modulate cytosolic Ca^{2+} transients. The AT-1 cells constitute an accurate and important model for the study of ß-adrenoreceptor function in cardiac research.

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