



Regulation of A₁ Adenosine Receptors by Amiodarone and Electrical Stimulation in Rat Myocardial Cells *In Vitro*

Dalia El-Ani,* Kenneth A. Jacobson† and Asher Shainberg*‡

*THE OTTO MEYERHOFF DRUG RECEPTOR CENTER, DEPARTMENT OF LIFE SCIENCES, BAR-ILAN UNIVERSITY, RAMAT-GAN 52900, ISRAEL; AND †MOLECULAR RECOGNITION SECTION, LABORATORY OF BIOORGANIC CHEMISTRY, NIH, NIDDK, BETHESDA, MD 20892, U.S.A.

ABSTRACT. The effects of conditions that either increase or decrease heart rate on the pharmacological properties of adenosine receptors in cultured rat myocytes were examined. Levels of A₁ adenosine receptors, following prolonged treatment with electrical stimulation (ES) or the antiarrhythmic drug amiodarone, were determined using radioligand binding with the specific A₁ receptor antagonist [³H]1,3-dipropyl-8-cyclopentylxanthine (CPX). The effects of lowering temperature were also explored. Exposure to amiodarone for 4 days reduced the density of A₁ receptors by 19% (from 24.7 ± 0.4 to 20.09 ± 0.3 fmol/dish) and inhibited the rate of contraction by 60% (from 188 ± 16 to 76 ± 30 beats/min), without changing the receptor affinity, protein content, creatine kinase (CK) activity or cell number. Electrical stimulation at 25°C elevated the density of A₁ adenosine receptors by 185% (from 4.1 ± 0.4 to 11.69 ± 2.1 fmol/dish). Four days of reduced temperature (from 37°C to either 30 or 25°C) lowered the density of A₁ adenosine receptors by 69 or 86%, respectively (from 24.1 ± 1.2 to 7.4 ± 0.4 or 3.4 ± 0.3 fmol/dish), with no significant change in the receptor affinity, activity of CK, or lactate dehydrogenase (LDH), protein content or cell number. The observed up- and down-regulation of A₁ adenosine receptors in primary myocyte cultures in response to conditions that exogenously alter the rate of contraction, is indicative of the role of adenosine receptors in adaptation of heart cells to stress. *BIOCHEM PHARMACOL* 54;5:583–587, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. adenosine receptor; heart rate; amiodarone; electrical stimulation; temperature; cardiocytes

Adenosine and amiodarone are antiarrhythmic agents that slow conduction, suppress premature heart beats, are used for the emergency treatment of supraventricular tachycardia [1–3], and are used for long-term treatment of atrioventricular tachycardia [4]. Both drugs decrease heart rate as a result of direct effects on the sinoatrial (SA) node [5, 6].

The A₁ adenosine receptor is upregulated in hyperthyroidism [7], an effect accompanied by stimulation of myocardial contractility and a biochemical transition of slow-twitch (“red”) muscle to the fast-twitch (“white”) muscle type [8]. Treatment with amiodarone, which bears a structural similarity to thyroxine, decreased the serum level of T₃ [9] and caused similar symptoms of hypothyroidism [10]. Amiodarone decreased sensitivity of the heart to catecholamines via a noncompetitive inhibition of catecholamine receptors and decreased the number of β-adrenoceptors in the myocardium [10, 11].

Electrical stimulation (ES) inhibited the synthesis of

nicotinic acetylcholine receptors [12] and increased the density of L-type Ca²⁺ channels [13] in cultured muscle cells. ES also reduced the levels of muscarinic acetylcholine receptors [14] in myocardial cells *in vitro*.

In the present study we have examined the effects of conditions that either increase or decrease heart rate, i.e. ES at low temperature or amiodarone, respectively. As in the previous study utilizing adrenergic and cholinergic stimulation [7], the change in contraction rate in rat heart cell cultures was associated with changes in the density of A₁ adenosine receptors.

MATERIALS AND METHODS

Preparation of Heart Cell Cultures

Rat hearts (1–2 days old) were removed under sterile conditions and washed three times in phosphate-buffered saline (PBS) to remove excess blood cells. The hearts were minced to small fragments and then gently agitated at 25°C for a few cycles of 10 min each in a solution of the proteolytic enzyme mixture RDB (Biological Institute, Ness-Ziona, Israel, diluted 1:50 in PBS), prepared from a fig tree extract, as described previously [7, 8, 10]. Following agitation the suspension was centrifuged at 500 × g for 5 min, the supernatant phase was discarded, and the cells

‡ Corresponding author: Prof. Asher Shainberg, Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel. TEL: 972-3-5318265; FAX: 972-3-5351824; email: shaina@ashur.cc.biu.ac.il.

Abbreviations: CK, creatine kinase; CPX, 8-cyclopentyl-1,3-dipropylxanthine; ES, electric stimulation; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; and SA, sinoatrial.

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were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Biological Industries, Kibbutz Beit Haemek, Israel) containing high glucose (5 mg/mL) and supplemented with 10% heat-inactivated horse serum (Biolab, Jerusalem, Israel) and 2% chick embryo extract. The suspension of cells was diluted to 1.0×10^6 cell/mL with the same medium, and aliquots of 1.5 mL were placed in 35 mm collagen-gelatin-coated plastic culture dishes. Cultures were incubated in a humidified atmosphere of 5% CO₂, 95% air at 37°C. The incubated cells were washed well 24 hr after plating to remove unattached cells. Confluent monolayers, which exhibit spontaneous contractions, developed in culture within 2 days. The growth medium was replaced every 3–4 days.

Materials

Amiodarone hydrochloride (Sigma, St. Louis, MO) was dissolved in H₂O at 60°C to 1 mM, and applied to the cell cultures to final concentration of 10 µM. CPX was purchased from RBI (Natick, MA). [³H]CPX was obtained from Dupont NEN (Boston, MA).

Radioligand binding studies were performed as previously described [7, 15]. Intact cells were incubated at room temperature (23–25°C) for 60 min, with various concentrations of [³H]CPX, 1 µM diprydamole, 2 U/mL adenosine deaminase in PBS, pH 7.4. Incubation was stopped by quickly rinsing the cells 5× with cold (4–8°C) PBS. The cells were solubilized with 0.2 mL of 0.5 N NaOH, and the solution was neutralized upon addition of 0.1 mL 2 N Tris-HCl, pH 3.7. After the addition of 3 mL of scintillation fluid, radioactivity was determined using a β counter. Nonspecific binding of [³H]CPX was defined in the presence of theophylline (5 mM). Specific binding of [³H]CPX was calculated as the total radioactivity bound minus the nonspecific binding (less than 20%).

Measurement of Contractility

Changes in the contractile state of individual cells in the monolayer cardiocytes were performed by using an optical video system. The cells were viewed with 20× objective and the image monitored by a television camera connected to a video motion detector that followed cell movements.

Protein content

Protein determination was performed following washing of the cells with PBS (2×) according to the method of Bradford [16], using bovine serum albumin as a standard.

Creatine Kinase (CK) and Lactate Dehydrogenase (LDH) Activity

The cardiomyocytes were washed with cold PBS (2×), and the cells were homogenized in the same buffer. CK and LDH activities were determined using CK and LDH-L kits

(Sigma, St. Louis, MO) and the product of the enzyme was measured spectrometrically at 23–25°C at a wavelength of 340 nm.

Effect of Electrical Stimulation (ES)

Cardiomyocytes grown for 4 days in culture in a 35 mm dish at 37°C were transferred to a temperature of 25°C for 3 days prior to application of ES, to reduce the rate of spontaneous contractions. ES of heart cells was carried out by placing in the culture medium a pair of platinum electrodes fastened to a cover glass to keep them separated by a distance of 25 mm [12]. Pulses of 20 V of a duration of 1 msec each were applied at a frequency of 4 Hz. After ES, the density of A₁ adenosine receptors was determined using radioligand saturation binding.

Statistics

The data are presented as mean ± SE. Statistical significance was estimated using the Student's *t*-test, with a *P* value of less than 0.05 considered significant. Binding data were represented graphically using Scatchard plots, and the parameters of *K_d* and *B_{max}* were calculated using the LIGAND program.

RESULTS

Effect of Amiodarone on [³H]CPX Binding

Myocardial cells (5 days old) were treated with 10 µM amiodarone at 37°C for 4 days. Scatchard analysis of [³H]CPX binding indicates that the maximum binding sites were 24.7 ± 0.4 fmol/dish in untreated cells and 20.09 ± 0.30 fmol/dish in amiodarone treated cells (Fig. 1). The *K_d* for [³H]CPX binding was 0.22 ± 0.1 nM and 0.28 ± 0.09 nM for the control group and amiodarone treated group, respectively. Thus, a 19% decrease in the density (*B_{max}*) of [³H]CPX binding was observed following 4 days of amiodarone treatment in comparison to control, while the affinity for the radioligand was nearly unchanged. The onset of this change in receptor density was delayed, because no significant change was observed in the level of binding to A₁ adenosine receptors during the initial 1–24 hr of treatment (data not shown).

The dose dependence of the depressant effect of amiodarone at 37°C on heart rate is shown in Fig. 2. Amiodarone (0.1–30 µM) inhibited (15–80%) heart rate following 24, 48, and 72 hr. There were no significant differences in protein content, CK activity and cell number, between control and amiodarone-treated cells (Table 1).

Effect of Electrical Stimulation (ES) and Temperature on [³H]CPX Binding

To decrease the spontaneous contractions the cardiac cells were transferred from 37 to 25°C, as described in Materials and Methods, prior to electrical stimulation. Scatchard

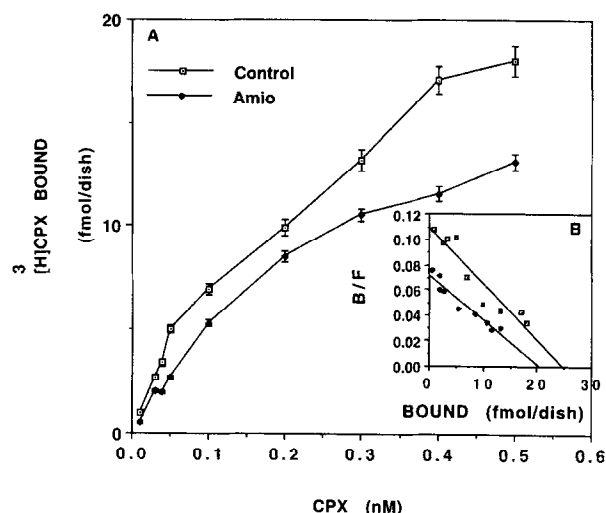


FIG. 1. Effect of amiodarone on [³H]CPX binding to intact myocyte cultures. Five-day-old cardiocytes were treated with 10 μ M amiodarone at 37°C for 4 days, and specific binding of [³H]CPX was determined. (A) Saturation of binding to A₁ receptors in control and treated cultures. (B) Scatchard transformation of data from Fig. 1A. $K_d = 0.22 \pm 0.1$ and 0.28 ± 0.09 nM and $B_{max} = 24.7 \pm 0.4$ and 20.09 ± 0.30 fmol/dish were calculated for the control and amiodarone treated cells, respectively. Each point is the mean (\pm SE) of triplicate determinations of a representative experiment out of three experiments that gave similar results.

analysis showed an increase of A₁ adenosine receptors from 4.14 ± 0.4 to 11.69 ± 2.1 fmol/dish following 36 hr of electrical stimulation, without a significant change in the affinity of [³H]CPX (Fig. 3). There were no significant differences in protein content, cell number, CK, and LDH activity between control and ES treated cells (Table 2). Four-day-old cultures that had been transferred from 37 to

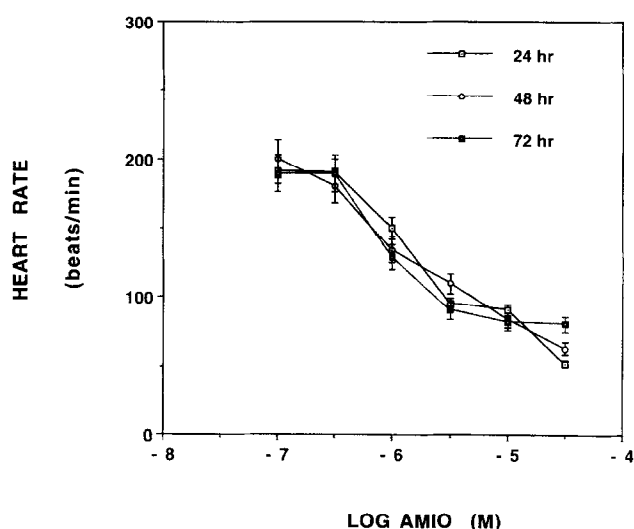


FIG. 2. Dose response of amiodarone on heart rate in myocyte cultures. Five-day-old cardiocytes were treated at 37°C with different concentrations of amiodarone. Contraction rate was determined at the indicated times. Each point represents the mean (\pm SE) of triplicate determinations of a representative experiment out of three experiments that gave similar results.

TABLE 1. Summary of the effects of amiodarone on rat cardiac cells in primary culture

	Control	Amiodarone	P
CK (U/dish)	0.40 ± 0.01	0.42 ± 0.1	NS
Protein (mg/dish)	0.89 ± 0.10	0.99 ± 0.08	NS
Cell number ($\times 10^4$)	121 ± 20	133 ± 25	NS
Rate of Contractions (beats/min)	179 ± 20	72 ± 28	<0.05
Adenosine receptors (fmol/dish)	24.7 ± 0.4	20.09 ± 0.30	<0.05

Five-day-old cardiocytes were treated with 10 μ M amiodarone at 37°C for 4 days. The indicated data were measured on separate dishes according to Materials and Methods. The results are expressed as means \pm SE of triplicate determinations of a representative experiment out of three experiments that gave similar results.

NS, not significant.

25°C for 4 days, displayed contractility reduced from 96 ± 8 to 36 ± 6 beats/min. Lowering the temperature also reduced the density of adenosine receptors from 24.1 ± 1.2 fmol/dish at 37°C to 3.4 ± 0.3 and 7.4 ± 0.4 fmol/dish at 25 and 30°C, respectively (Fig. 4). Following this treatment, cell number, protein content, CK, and LDH activity remained essentially unchanged (Table 2).

DISCUSSION

The *in vitro* model of the neonatal cultured rat heart has enabled us to examine the direct effects of ES, amiodarone, and temperature changes on myocytes, particularly with respect to regulation of A₁ adenosine receptors.

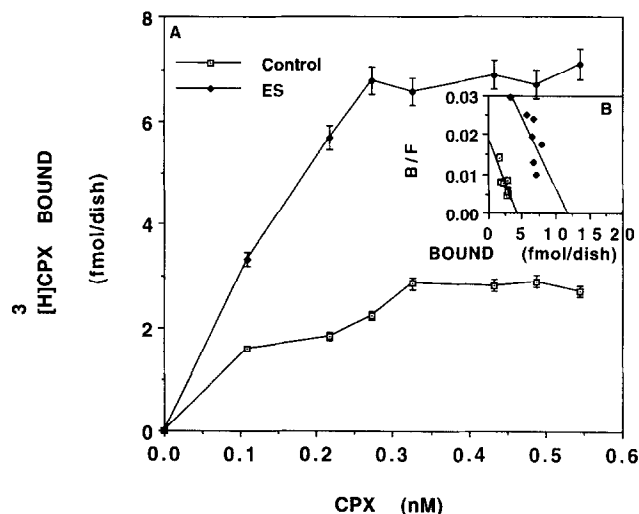


FIG. 3. The effect of ES on [³H]CPX binding to intact myocyte cultures. Four-day-old cardiocytes were transferred from 37 to 25°C for 3 days. The cells were then electrically stimulated for 36 hr at 4 Hz, and specific [³H]CPX binding was determined. (A) Saturation of binding to A₁ receptors in control and treated cultures. (B) Scatchard transformation of data from Fig. 3A. $K_d = 0.22$ and 0.28 nM and $B_{max} = 4.1 \pm 0.4$ and 11.69 ± 2.1 fmol/dish were calculated for the control and electrically stimulated cells, respectively. Each point is the mean (\pm SE) of triplicate determinations of a representative experiment out of three experiments that gave similar results.

TABLE 2. Summary of the effects of ES on rat cardiac cells in primary culture

	Control	ES	P
CK (U/dish)	0.50 ± 0.09	0.45 ± 0.15	NS
LDH (U/dish)	0.69 ± 0.13	0.60 ± 0.1	NS
Protein (mg/dish)	0.98 ± 0.09	1.1 ± 0.12	NS
Adenosine receptors (fmol/dish)	4.14 ± 0.4	11.69 ± 2.1	<0.05

Four-day-old cardiocytes were transferred from 37 to 25°C for 3 days and then were electrically stimulated (4 Hz) as described in Materials and Methods. The results are expressed as means ± SE of triplicate determinations of a representative experiment out of three experiments that gave similar results.

NS, not significant.

Amiodarone acts as a class III antiarrhythmic drug [17, 18], and has been shown to inhibit ATP-sensitive potassium channels [19], to interact with myocardial Na⁺ K⁺-ATPase in guinea pig microsomal preparations [20] and to protect the ischemic rat heart by inhibiting oxidative phosphorylation [21]. Intramuscular or intravenous amiodarone treatment has been used in controlling tachyarrhythmia in children [22] and young adults [23], and results in sustained bradycardia clinically. The mechanism of action of amiodarone in reducing heart rate is unclear. One possibility is antagonism of catecholamines acting at β -adrenergic receptors [10, 11]. Other possibilities are uncoupling of electrical activity and contraction (unpublished), or inhibition of Ca²⁺ uptake to the SR [24].

The present results confirm that amiodarone depresses the rate of contraction of cardiac myocytes in primary culture. Furthermore, amiodarone specifically attenuated the density of radioligand binding to A₁ adenosine receptors by 19% without changing the K_d value. The findings that amiodarone decreased contractility supports the idea that amiodarone-treated cardiocytes decrease their sensitiv-

ity to A₁ adenosine agonist. Indeed, increase in the EC₅₀ of the A₁ adenosine receptor agonist R-PIA in depressing spontaneous contractility in cardiac cell cultures following chronic amiodarone treatment was observed (data not shown). This finding may have clinical implications for further research to consider the effect of modulating adenosine receptors in antiarrhythmic therapy [25], perhaps using a combination of amiodarone and an adenosine receptor ligand [1, 26].

Amiodarone was first developed for use as an antiangina agent (for review, see [17], whereas adenosine injections have been reported to be accompanied by angina pain [27]. We raise the possibility here that amiodarone, by reducing the level of adenosine receptors, may reduce the susceptibility to adenosine-induced angina pain.

Electrical stimulation has been shown to cause membrane depolarization, biochemical, and physiological changes in the cells [28, 29]. In our myocyte cultures, CK, and LDH activity, protein content, and cell number remained nearly unchanged following ES. However, 36 hr of electrical stimulation, caused 185% elevation in A₁ adenosine receptors with no change in the affinity. This increase is probably a result of increased receptor synthesis because the protein synthesis inhibitor cycloheximide (5 μ g/mL), prevented elevation of the level of adenosine receptors elicited by ES (data not shown). This increase also indicates that the A₁ adenosine receptors become a compensation under such stress conditions of excessive contractions, as chronic treatment with amiodarone caused downregulation of β -adrenergic receptors [11]. The elevation in A₁ adenosine receptors probably increases cardiac sensitivity to adenosine and indicates by which mechanism the heart cells may adapt to electrical stimulation stress. Further support for this mechanism of adaptation to stress was demonstrated by the effect of thyroid hormones and catecholamines that accelerated heart rate and also upregulated the A₁ adenosine receptor [7, 15].

The important role of the A₁ adenosine receptor and not A₂ receptors in relation to heart contractility was reported for electrically stimulated cardiocytes that were acutely treated with R-PIA [30, 31]. In those cells R-PIA decreased contractility, while CPX, a selective A₁ adenosine antagonist, antagonized the effect of R-PIA on contractile response. In the present study, the decrease in the level of A₁ adenosine receptors following amiodarone and low temperature treatment and the elevation of A₁ receptors following ES, suggests that the level of A₁ adenosine receptors is regulated by heart contractility and environmental conditions as a mechanism to restore normal heart rate.

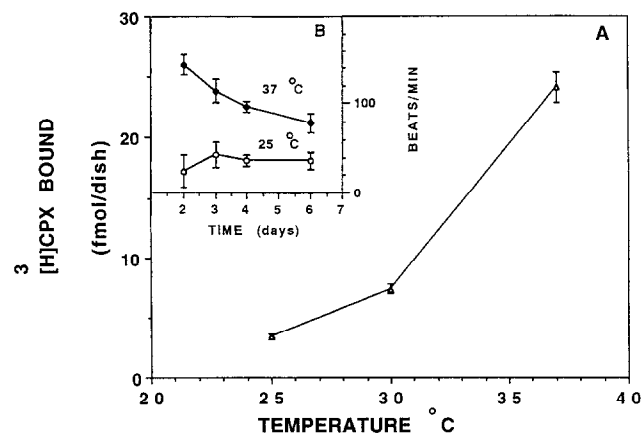


FIG. 4. The effect of temperature on [3H]CPX binding and heart rate. Four-day-old cardiocytes were transferred from 37°C to either 25°C or 30°C. Specific [3H]CPX binding to intact cells as a function of temperature following 4 days of treatment (A) and heart rate at the indicated times (B) were measured. Each point is the mean (±SE) of triplicate determinations of a representative experiment out of three experiments that gave similar results.

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References

- Manz M and Luderitz B, Supraventricular tachycardia and pre-excitation syndromes: Pharmacological therapy. *Eur Heart J* **14**: 91–98, 1993.
- Honerjager P and Schmidt G, Pharmacology of modern anti-arrhythmia drugs in therapy of supraventricular tachycardia. *Z Gesmte Inn Med* **48**: 425–429, 1993.
- Kowey PR, Marinchak RA, Rials SJ, Rubin AM and Smith L, Electrophysiologic testing in patients who respond acutely to intravenous amiodarone for incessant ventricular tachyarrhythmias. *Am Heart J* **125**: 1628–1632, 1993.
- Bartlett TQ and Friedman PL, Current management of the Wolff-Parkinson-White syndrome. *J Cardiovasc Surg* **8**: 503–515, 1993.
- Murthy VS and Hwang TF, Antiarrhythmic drugs and the modulation of autonomic control of heart rate in rabbits. *Fed Proc* **45**: 2186–2190, 1986.
- Belardinelli L, Shryock JC, Song Y, Wang D and Srinivas M, Ionic basis of the electrophysiological actions of adenosine on cardiomyocytes. *FASEB J* **9**: 359–365, 1995.
- El-Ani D, Jacobson KA and Shainberg A, Characterization of adenosine receptors in intact cultured heart cells. *Biochem Pharmacol* **48**: 727–735, 1994.
- Brik H and Shainberg A, Thyroxine induces transition of red towards white muscle in cultured heart cells. *Basic Res Cardiol* **85**: 237–246, 1990.
- Venkatesh N, Padbury JF and Singh BN, Effects of amiodarone and desethylamiodarone on rabbit myocardial β -adrenoceptors and serum thyroid hormones-absence of relationship to serum and myocardial drug concentrations. *J Cardiovasc Pharmacol* **8**: 989–997, 1986.
- Disatnik MH and Shainberg A, Regulation of β -adrenoceptors by thyroid hormone and amiodarone in rat myocardial cells in culture. *Biochem Pharmacol* **41**: 1039–1044, 1991.
- Bjornerheim R, Froyssaker T and Hansson V, Effects of chronic amiodarone treatment on human myocardial beta adrenoceptor density and adenylate cyclase response. *Cardiovasc Res* **25**: 503–509, 1991.
- Shainberg A and Burstein M, Decrease of acetylcholine receptor synthesis in muscle cultures by electrical stimulations. *Nature* **264**: 368–369, 1976.
- Freud-Silverberg M and Shainberg A, Electric stimulation regulates the level of Ca-channels in chick muscle culture. *Neurosci Lett* **151**: 104–106, 1993.
- Waisberg M and Shainberg A, Characterization of muscarinic cholinergic receptors in intact myocardial cells in vitro. *Biochem Pharmacol* **43**: 2327–2334, 1992.
- El-Ani D, Jacobson KA and Shainberg A, Regulation of adenosine receptors in cultured heart cells. In: *Molecular and Subcellular Cardiology: Effects on Structure and Function* (Eds. Sideman S and Beyar R), pp. 205–215. Plenum Press, New York, 1995.
- Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**: 248–254, 1976.
- Rotmensch HH and Belhassen B, Amiodarone in the management of cardiac arrhythmias: Current concepts. *Cardiovasc Pharmacol* **72**: 321–358, 1988.
- Brentano FC, Rate-dependence of class III actions in the heart. *Clin Pharmacol* **7**: 51–59, 1993.
- Haworth RA, Goknur AB and Berkoff HA, Inhibition of ATP-sensitive potassium channels of adult rat heart cells by antiarrhythmic drugs. *Circ Res* **65**: 1157–1160, 1989.
- Dzimiri N and Almotrefi AA, Amiodarone: Biochemical evidence for its interaction with myocardial $\text{Na}^+ \text{K}^+$ -ATPase in guinea pig microsomal preparations. *Biochem Pharmacol* **41**: 470–472, 1991.
- Nokin P, Jungbluth L and Mouton J, Protective effects of amiodarone pretreatment on mitochondrial function and high energy phosphates in ischemic rat heart. *J Mol Cell Cardiol* **19**: 603–614, 1987.
- Perry JC, Intravenous amiodarone for life-threatening tachyarrhythmia in children and young adults. *J Am Coll Cardiol* **22**: 8–95, 1993.
- Wynnewood PA, Electrophysiologic testing in patients who respond to intravenous amiodarone for incessant ventricular tachyarrhythmias. *Am Heart J* **125**: 1628–1632, 1993.
- Weinstein L, Brik H, Rotmensch HH and Shainberg A, Characterization of sarcoplasmic reticulum in skinned heart muscle cultures. *J Cell Physiol* **148**: 124–132, 1991.
- Pelleg A and Belardinelli L, Cardiac electrophysiology of adenosine: Basic and clinical aspects. *Cardiol Res* **27**: 54–61, 1993.
- Grauer K, Cavallaro D and Gums J, New developments in cardiopulmonary resuscitation. *Am Fam Physician* **43**: 832–844, 1991.
- Sylvén C, Beermann B, Jonzon B and Brandt R, Angina pectoris-like pain provoked by intravenous adenosine in healthy volunteers. *Br Med J* **293**: 227–230, 1986.
- Luther PW, Peng HB and Lin JJC, Changes in cell shape and actin distribution induced by constant electric fields. *Nature* **303**: 61–64, 1983.
- Robinson HR, The response of cells to electrical fields: A review. *J Cell Biol* **101**: 2023–2027, 1985.
- Behnke N, Muller W, Neumann J, Schmitz W, Scholz H and Stein B, Differential antagonism by 1,3-dipropylxanthine-8-cyclopentylxanthine and 9-chloro-2,2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo(1,5-C) quinazolin-5-imine of the effects of adenosine derivatives in the presence of isoprenaline on contractile response and cAMP content in cardiomyocytes. Evidence for the coexistence of A_1 and A_2 adenosine receptors on cardiomyocytes. *J Pharmacol Exp Ther* **254**: 1017–1023, 1990.
- Stein B, Schmitz W, Scholz H and Seeland C, Pharmacological characterization of A_2 -adenosine receptors in guinea pig ventricular cardiomyocytes. *J Mol Cell Cardiol* **26**: 403–414, 1994.