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β-Adrenoceptor subtypes in young and old rat ventricular myocytes: a combined patch-clamp and binding study

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1 We used electrophysiological and binding techniques to assess the presence of β_1- and β_2-adrenoceptors (β_1AR and β_2AR) in rat cardiac myocytes and to determine their ratio during aging. Experiments were performed in left ventricular myocytes enzymatically dissociated from the heart of 3- (young) or 22-month-old (old) Wistar Kyoto rats.

2 In patch-clamp experiments, myocytes from old rats showed a prolonged action potential duration (at −20 mV: 41.7±3.6 vs 26.2±3.1 ms; at −60 mV: 154.4±17.7 vs 87.1±6.9 ms, $P<0.05$) and an augmented membrane capacitance (an index of cell size) (271.7±20.2 vs 164.3±14.6 pF, $P<0.05$) compared to young rats. β_2AR stimulation, achieved by superfusing myocytes with the selective β_2AR agonist, zinterol (10 μM) or with (-)-isoprenaline (1 μM) in the presence of the selective β_1 AR antagonist, CGP 20712A (0.1 μM), significantly increased L-type calcium current ($I_{Ca,L}$) in rat ventricular myocytes. The percentage increase was similar in both young and old rats, either with zinterol (26.9±3.6% and 24.2±2.8%, respectively) or isoprenaline plus CGP 20712A (30.4±3.7% and 22.4±4.1%, respectively). Isoprenaline alone (β_1AR and β_2AR stimulation) caused a much smaller increase in $I_{Ca,L}$ in old rats (58.4±12.1%) than in younger ones (95.3±8.1%) ($P=0.067$).

3 The number of βAR mg^{-1} protein, measured with saturation binding assays of the non selective βAR antagonist [^{3}H]-CGP 12177 was 1989.4±189.5 for 3- and of 1580.7±161.5 for 22-month-old rats. Competition for [^{3}H]-CGP 12177 binding by CGP 20712A gave biphasic curves which demonstrated two classes of binding sites. Densities (as percentages of total βAR density), and affinities for the two binding sites were: 80.4±2.2% (K_i=6.6±1.3 nM) β_1AR and 19.6±2.2% (K_i=6.9±2.2 μM) β_2AR in young rats and 66.1±1.2% (K_i=8.3±1.1 nM) β_1 AR and 33.9±1.2% (K_i=5.2±0.6 μM) β_2AR in old rats. The β_1AR/β_2AR ratio was significantly ($P<0.01$) reduced in old rats with respect to the younger ones.

4 By combining electrophysiological and binding measurements, we calculated β_1AR and β_2AR densities as number of receptors per μm^2 of cell surface. In old rats, β_1AR density was significantly decreased compared to young rats (8.4±2.0 vs 15.4±3.7 receptors μm^{-2}, $P<0.05$), while β_2AR density remained unchanged at both 3 and 22 months (3.8±0.7 and 4.2±1.1 receptors μm^{-2}, respectively).

5 Our results demonstrate that both β_1AR and β_2AR are functionally present in rat ventricular myocytes of young and old rats. The decreased responsiveness to βAR stimulation during aging appears to be associated with a selective reduction in the density of β_1AR.

Keywords: β-Adrenoceptor subtypes; aging; receptor binding; patch-clamp; isolated myocytes; calcium current; zinterol; CGP 20712A; ICI 118,551

Introduction

The electrophysiological and mechanical properties of the heart change significantly with age (Lakatta, 1987). In papillary muscles isolated from the heart of senescent rats (which is the animal generally used for studies on aging) the intracellular action potential, the myoplasmic Ca^{2+} transient and isometric contraction are of longer duration than in young adults (Lakatta, 1987). Individual myocytes isolated from the intact heart retain the morphological and functional properties of the multicellular preparations, i.e., myocytes isolated from the heart of old rats show a prolonged action potential due to selective modifications of ionic currents (Cerbai *et al.*, 1994a; Walker *et al.*, 1993).

Myocyte size increases with age, but except for a prolonged time course, the twitch contraction characteristics normalized for cell length do not change with age (Fraticelli, *et al.*, 1989; Sakai *et al.*, 1992); however, the contractile response of isolated myocytes to β-adrenergic stimulation is analogous to that observed in intact muscle studies, in that the inotropic response to β-adrenergic stimulation declines with age (Amerini *et al.* ,1985; Guarnieri, *et al.*, 1980; Lakatta, *et al.*, 1975; Sakai *et al.*, 1992). While several studies suggest that the density and affinity of cardiac β-adrenoceptors (βAR) is unchanged with age (Barbieri *et al.*, 1994; Guarnieri *et al.*, 1980; Lakatta & Yin, 1982; Lakatta, 1987), no studies have been performed to assess if the ratio β_1AR/β_2AR changes during aging, as it has been reported to do in other conditions such as heart failure (Harding *et al.*, 1994 for review; Bristow *et al.*, 1986; Brodde, *et al.*, 1989). One reason for the lack of such studies is certainly due to the fact that the presence of β_2AR in rat myocardium has been controversial for a long time (Wilson & Lincoln, 1984; Saito *et al.*, 1988; Buxton & Brunton, 1985; Mauz & Pelzer, 1990) and their presence in rat ventricular myocytes has been demonstrated only recently by means of functional studies (Xiao & Lakatta, 1993). A recent study suggests that the decreased β-adrenoceptor responsiveness which occurs in the human heart with aging is due to multiple mechanisms including β_1AR-down regulation and uncoupling of β_2AR (White *et al.*, 1994). A large body of data demonstrate that βAR subtypes are differentially regulated (Muntz *et al.*, 1994); they also show relevant differences in intracellular Ca^{2+} responses in cardiac myocytes (Xiao & Lakatta, 1993) and β_1AR stimulation is more arrhythmogenic than β_2AR stimulation (Cerbai *et al.*, 1990). Thus it would be of interest to assess if the relative proportion of β_1AR and β_2AR varies with age, since this may have relevant physiological implications. However, measurements of cardiac βAR subtypes with binding techni-

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ques is complicated by several factors especially in membrane preparations from heart homogenates since the heart is composed of several tissue types containing both subtypes of βAR (Muntz *et al.*, 1994). Even more, the senescent rat exhibits a moderate cardiac hypertrophy (Yin *et al.*,1982) and the myocytes isolated from these hearts are enlarged (Fraticelli *et al.*, 1989). Thus it would be difficult to distinguish a real down regulation of the receptors from their dilution on the plasma membrane without having a measurement of cell surface. In many models of cardiac hypertrophy βAR concentration has been reported to increase (Homcy *et al.*, 1991); but in rats with left ventricular hypertrophy due to pressure overload, βAR number, adenylyl cyclase activity and the inotropic response to isoprenaline were shown to be decreased (Chevalier *et al.*, 1989). In the same model of cardiac hypertrophy, others have reported either an increase (Limas, 1979) or no change (Cervoni *et al.*, 1981) in the number of βAR.

To obviate these confounding factors, in the present study both the electrophysiological and binding experiments were performed on isolated myocytes. In this way it was possible to measure only the β_1AR and β_2AR present in ventricular myocytes, having at the same time the biochemical and functional proof of their existence and information about their changes during ageing. Even more, since cell capacitance, which can be easily obtained during electrophysiological measurements, is an index of cell size, the number of receptors could be expressed not only per cell, but also per surface area.

Part of this work has been presented in abstract form (Cerbai *et al.*, 1994c; Guerra *et al.*, 1994).

Methods

Preparation of ventricular cardiomyocytes

The investigation conforms to the rules of the care and use of laboratory animals of the European community (86/609/CEE). Male Wistar Kyoto rats (WKY), 2 and 18 months of age, were obtained from Charles River (Italy). They were maintained in our animal facility until they were killed.

Single left ventricular myocytes were isolated from young (3 month old) or old rats (22-month-old) using a protocol based on previously described procedures (Barbieri *et al.*, 1994; Cerbai *et al.*, 1994a,b). Animals were injected with 500 iu heparin i.p. (Liquemin, Roche) and anaesthetized with ether, after which they were killed and the hearts rapidly excised and rinsed in cool low-calcium solution (LCS) containing (mM): NaCl 120, KCl 10, KH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 10, taurine 20, and pyruvate 5; the pH was adjusted to 7.20 with HEPES/NaOH. The heart was mounted in a Langendorff apparatus and perfused retrogradely with LCS maintained at 37°C and equilibrated with 100% O_2. The solution was then quickly changed to LCS plus 1 g l^{-1} collagenase, 0.03 g l^{-1} dispase and 1 g l^{-1} albumin. The time of perfusion with the enzymatic solution ranged from 5 min for 3-month-old rats to 20 min for 22-month-old rats, depending on the size of the heart. In fact perfusion was terminated when the heart became soft; the left ventricle including septum was then cut off, chopped into small pieces and gently stirred in LCS for 20 min. Cardiomyocytes that appeared in the supernatant were purified by gravity sedimentation, collected and stored in LCS at room temperature. The cell suspension was filtered through a nylon net (pore size: 300 x 300 μM). The yield of rod-shaped cells ranged from 75% at 2 months to 30% at 22 months. For the electrophysiological experiments, cells were kept in LCS supplemented with 1 mM $CaCl_2$ and used within 10 h of their isolation.

Radioligand binding assays

Saturation binding assays for β-adrenoceptors (βAR) were performed in triplicate with samples of 3×10^4 myocytes being used for each assay. Proteins were determined by the method of Lowry *et al.* (1951) at the end of the experiments; 3×10^4 myocytes from 3- and 22-month-old rats were used which amounted to 0.19 ± 0.02 mg and 0.26 ± 0.03 mg of proteins, respectively. This difference is in agreement with an increase in cell size during aging (Fraticelli *et al.*,1989). Myocytes, suspended in LCS, were incubated with increasing concentrations (0.03 – 5 nM) of the hydrophilic βAR antagonist [^{3}H]-CGP 12177 in a final volume of 250 μl. Inhibition experiments were performed by incubation in the same final volume 2 nM [^{3}H]-CGP 12177 in the presence of increasing concentrations (1 nM – 300 μM) of the selective β_1AR antagonist, CGP 20712A (Dooley *et al.*, 1986) or of the selective β_2AR antagonist, ICI 118,551 (Bilski *et al.*, 1980; O'Donnell & Wanstall, 1980).

Inhibition time for both saturation and inhibition experiments was 120 min at 37°C. The non-specific binding was determined in the presence of 10 μM (±)-propranolol and was about 20 – 30% in the range 0.03 – 2 nM and about 50% at 5 nM. The reaction was stopped by separation of the free from the bound radioligand by rapid vacuum filtration over Whatman GF/B glass fibre filters, using a Brandell cell harvester. Filters were washed three times with 5 ml of 50 mM Tris HCl (pH = 7.4) at 4°C; they were then placed in liquid scintillation vials, containing 5 ml of Instagel (Packard). Radioactivity retained on filters was determined in a Beckman Liquid Scintillation Spectrometer at 55% efficiency.

Electrophysiological measurements

The experimental set-up was similar to that described by Cerbai *et al.* (1994a,b). A drop of cells was placed in the experimental chamber (0.2 ml) and superfused by means of a peristaltic pump (Masterflex, model 7524/05, Cole-Parmer Instrument Company) at a flow rate of 1.8 ml min^{-1}; a three-line system controlled by electronic valves allowed solutions to be changed rapidly. The recording chamber was mounted on the stage of an inverted microscope (TMS, Nikon). The control solution was a modified Tyrode solution containing (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.2, HEPES 5, glucose 10; pH was adjusted to 7.35 with NaOH and the temperature was kept at 36 ± 0.5°C. The internal solution of the patch pipettes contained (mM): KCl 140, $MgCl_2$ 1, Na_2-ATP 5, ethyleneglycol-*bis*(β-aminoethyl ether)-*N,N,N',N'*-tetra acetic acid (EGTA) 5, HEPES 10, adjusted to pH 7.20 with KOH. To study the L-type Ca^{2+}-current ($I_{Ca,L}$), KCl was replaced by CsCl and KOH by CsOH in all solutions in order to block potassium currents interfering with $I_{Ca,L}$ measurement.

β_2AR stimulation was achieved by superfusion with the selective β_2AR agonist, zinterol (10 μM) or with 1 μM (-)-isoprenaline, a mixed β_1AR and β_2AR agonist, in the presence of the selective β_1AR antagonist, CGP 20712A (0.1 μM). β_2AR specificity was determined by antagonism with 10 nM ICI 118,551, a β_2AR antagonist. Stock solutions of (-)-isoprenaline (1 mM) and zinterol HCl (2 mM) containing 1 g l^{-1} ascorbic acid were prepared on the day of the experiment and diluted in Tyrode solution.

The whole-cell configuration of the patch-clamp technique was used. The electrical signal was recorded with a patch amplifier (Axopatch 1D, Axon Instrument Inc.), digitised (Labmaster TL-1 DMA, Scientific Solutions), and displayed on the monitor of a 386 personal computer and a digital oscilloscope (Nicolet 310, Nicolet Instrumentation Company). The cut-off frequency was 20 kHz. Current and voltage protocol generation, data acquisition, and analysis were performed with pClamp software (Vers 5.5.1, Axon Instrument Inc.). MicroCal Origin (MicroCal Software Inc.) was used for further analysis.

After gaining intracellular access, whole-cell membrane capacitance was measured by applying ± 10 mV voltage steps from a holding potential of −70 mV and calculated according to the equation:

$$C_m = \tau_c (I_0 - I_\infty)/\Delta V_m$$

where C_m is the membrane capacitance, τ_c is the time constant

of the membrane capacitance, I_0 is the initial current value, I_∞ is the amplitude of steady-state current and ΔV_m is the amplitude of voltage steps. To minimize the amplitude of the capacitative transient, thus facilitating the analysis of calcium current, whole cell membrane capacitance was compensated up to 100 pF (the maximal capacity compensation that could be achieved with our amplifier). Series resistance (R_s), calculated as $R_s = \Delta V_m / I_0$ (see above), ranged from 2 to 7 MΩ and was compensated in all cells by about 80%. Recording was started after 5 min dialysis of the cell. Action potentials were elicited at a rate of 0.2 Hz and sampled at 1 kHz. The following parameters were measured: action potential amplitude (AP), overshoot (OS), maximum diastolic potential (MDP), action potential duration at −20 mV (APD_{-20}) and −60 mV (APD_{-60}). L-type Ca^{2+}-current ($I_{Ca,L}$) was elicited by 160 ms depolarizing steps to 0 mV from a holding potential of −70 mV, preceded by a brief (15 ms) step to −40 mV to inactivate the Na^+-current. Steps were applied at low frequency (maximum rate: 0.2 Hz) and sampled at 5 kHz. $I_{Ca,L}$ amplitude was measured as the difference between steady state current, measured at the end of the depolarizing step at 0 mV, and peak inward current.

Materials

[^{3}H]-CGP 12177 ((-)-4-(3-*t*-butylamino-2-hydroxypropoxy-[5,7-^{3}H] benzimidazol-2-one) (specific activity: 43 Ci mmol^{-1}) was obtained from Amersham, Buckinghamshire, England; CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy) ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol methanesulphate was kindly provided by Ciba Geigy. ICI 118,551 (erythro-DL-1-(methyl-indan-4-yloxy)-3-isopropylaminobutan-2-ol) was a kind gift of Imperial Chemical Industries; zinterol (N-(5(2-((1,1-dimethyl-2-phenyl-ethyl)amino)-1-hydroxy-ethyl)-2-hydroxyphenyl)-monohydrochloride) was a gift from Bristol Meyers Squibb. Aquassure was obtained from NEN Research Products, (Boston, MA, U.S.A.); (-)-isoprenaline HCl, collagenase (Type I) and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) were purchased from Sigma Chemical Co.; dispase and albumin (fatty acid free fraction V) Boehringer, (Mannheim, FRG).

All other reagents were of analytical grade and obtained from commercial sources.

Data analysis

Saturation experiments were analysed by the LIGAND Computer programme (Munson & Rodbard, 1980). Receptor binding inhibition data were analysed by using the non linear least squares curve fitting programmes Dose-Reponse (Humrich & Richardson, 1983) and LIGAND for one or two binding sites. The two programmes gave essentially the same results. Final B_{max} and K_i values represent means ± s.e.mean of 8–9 separate experiments carried out in duplicate. For curve fittings, as shown in Figure 6, the binding data of all single experiments were used for the curve fitting procedure. The equation of Cheng & Prusoff (1973) was used for calculation of K_i values. Comparison between two groups was performed by Student's *t* test for grouped or paired data. Comparison of the number of receptors (either β_1AR or β_2AR) per μm^2 between the two groups of rats was performed by estimating the confidence limits (95%) of a ratio (independent observations) (Goldstein, 1964). A *P* value of less than 0.05 was considered significant.

Results

Figure 1 shows typical action potentials recorded from myocytes isolated from the heart of 3- and 22-month-old rats. It is apparent that the major difference is a significant prolongation of the action potential duration (see Table 1) the ionic basis of which has been recently clarified (Cerbai *et al.*, 1994a; Walker *et al.*, 1993). The mean action potential parameters are shown in Table 1 together with other relevant characteristics of the myocytes. Membrane capacitance (C_m) was significantly higher in myocytes isolated from 22-month-old rats. Membrane capacitance increases with cell size and it is well known that cardiac myocytes from senescent rats show a certain degree of hypertrophy (Fraticelli *et al.*, 1989). Membrane capacitance is a measure of all the sarcolemmal membrane which, in mammalian cardiac cells, is higher infolded in T-tubules and caveolae. In Table 1, the 'estimated cell area' indicates the external or surface sarcolemma calculated according to literature data (Walker *et al.*, 1993) in which, for the same cells, membrane capacitance and geometrical measurements were reported. Those data showed that the external sarcolemma was approximately 40% of the total sarcolemmal membrane, the value for which can be derived from membrane capacitance measurements by assuming that 1 pF corresponds to 100 μm^2 (Hille, 1994).

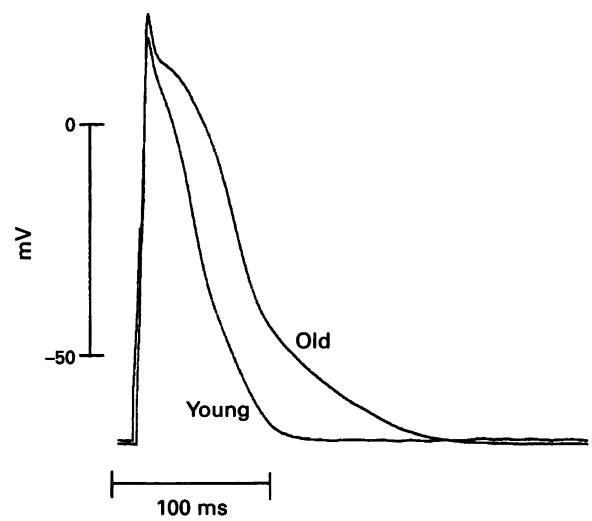

Figure 1 Superimposed action potentials recorded from left ventricular myocytes of 3- (young) and 22-month-old (old) rats.

Table 1 Electrophysiological characteristics of left ventricular myocytes isolated from 3- (3 months) or 22-month-old (22 months) rats

Groups	C_m (pF)	*Estimated cell area* (μm^2)	$I_{Ca,L}$ (pA)	$I_{Ca,L}$ density (pA pF^{-1})	*OS* (mV)	*MDP* (mV)	APD_{-20} (ms)	APD_{-60} (ms)
3 months	164.3 ± 14.6	6570 ± 601	2299 ± 191	15.9 ± 1.9	15.0 ± 2.2	−72.1 ± 1.2	26.2 ± 3.1	87.1 ± 6.9
22 months	271.7 ± 20.2*	10868 ± 810	3657 ± 279*	14.2 ± 1.4	16.7 ± 2.0	−72.0 ± 1.0	41.7 ± 3.6*	154.4 ± 17.7*

Numbers represent mean ± s.e.mean. C_m = membrane capacitance; Estimated cell area = external or surface sarcolemma estimated as 40% of total sarcolemma (external sarcolemma plus T-tubules plus caveolae) derived from C_m (1 pF = 100 μm^2); $I_{Ca,L}$ = peak L-type calcium current measured at 0 mV; OS and MDP = overshoot and maximum diastolic potential of the action potential, respectively; APD_{-20} and APD_{-60} = action potential duration at –20 and –60 mV, respectively. n = 12 to 22; *P < 0.01 vs 3-month-old rats.

The functional effect of β_2-adrenoceptor stimulation was evaluated on the L-type calcium current ($I_{Ca,L}$) which was measured in all the cells as absolute value and as density. The absolute value of $I_{Ca,L}$ was higher in hypertrophied aged myocytes in agreement with previous results (Cerbai *et al.*, 1994a). When normalised for membrane capacitance, $I_{Ca,L}$ density was not different in myocytes isolated from old and young rats (Table 1). As previously observed (Walker *et al.*, 1993; Cerbai *et al.*, 1994a), this finding rules out a major role of $I_{Ca,L}$ in the prolongation of the action potential duration observed in myocytes from old rats (Figure 1). On the whole these data demonstrate that the myocytes we have isolated from young and old rats have the expected characteristics.

To demonstrate the presence of functionally operative β_2AR, myocytes were exposed to the selective β_2-adrenoceptor agonist, zinterol (Minneman *et al.*, 1979; Bristow *et al.*, 1989; Xiao & Lakatta, 1993) or to isoprenaline in the presence of the selective β_1-antagonist, CGP 20712A (Borea *et al.*, 1992). As shown in Figure 2, zinterol (10 μM) consistently increased $I_{Ca,L}$ amplitude in both 3- (a) and 22-month-old (b) myocytes. That the effect of zinterol was due to β_2-adrenoceptor stimulation is confirmed by a series of experiments, one of which is shown in Figure 3. When β_1AR were blocked by the compound CGP 20712A, the non selective agonist, isoprenaline, was able to cause an increase of $I_{Ca,L}$ amplitude (Figure 3a), an effect which was fully antagonized by the selective β_2AR antagonist, ICI 118,551 (Bilski *et al.*, 1980) (Figure 3b).

The effect of β_2-adrenoceptor stimulation was fully reversible and was repeated in the same cell either with zinterol or isoprenaline in the presence of selective β_1AR blockade with CGP 20712A. The typical experiment shown in Figure 4 shows the effect of zinterol followed by that of isoprenaline alone and in the presence of the β_1AR antagonist, CGP 20712A. Zinterol (10 μM) caused the usual increase of $I_{Ca,L}$; the effect was reversed by washout and $I_{Ca,L}$ amplitude returned to a value similar to that before exposure to the drug. Addition of 1 μM isoprenaline caused, as expected, a much greater increase of $I_{Ca,L}$ amplitude; also the effect of isoprenaline was fully reversible. Addition of the β_1-adrenoceptor antagonist, CGP 20712A (0.1 μM) caused a marked reduction of $I_{Ca,L}$ amplitude. The reduction was similar in 3- or 22-month-old rats, the resulting amplitude being 67.2±4.0% ($n=6$, $P<0.01$) and 73.0±6.4% ($n=7$, $P<0.05$) of the control value, respectively.

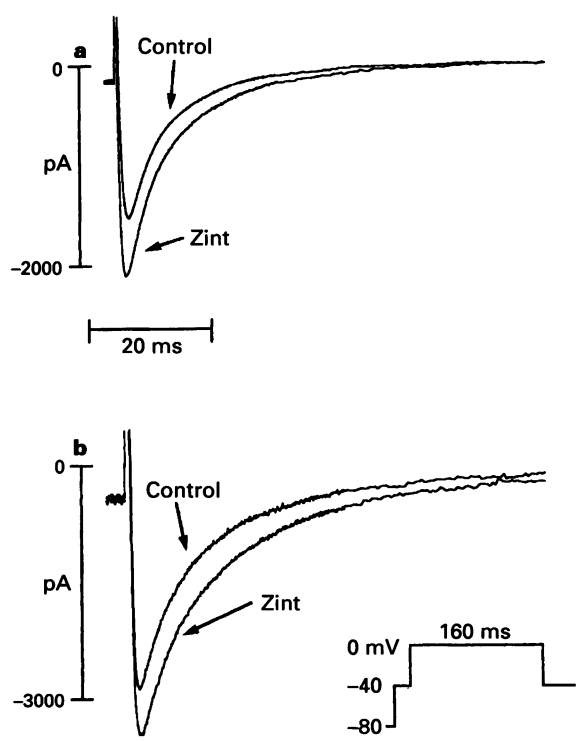

Figure 2 Effect of β_2-adrenoceptor (β_2AR) stimulation by zinterol (10 μM) on the amplitude of $I_{Ca,L}$ recorded from myocytes of 3- (a) and 22-month-old rats (b). Each panel shows superimposed current tracings evoked by a depolarising step to 0 mV (see voltage protocol in the bottom right corner), in control (Control) and when the maximal effect caused by zinterol is reached (Zint).

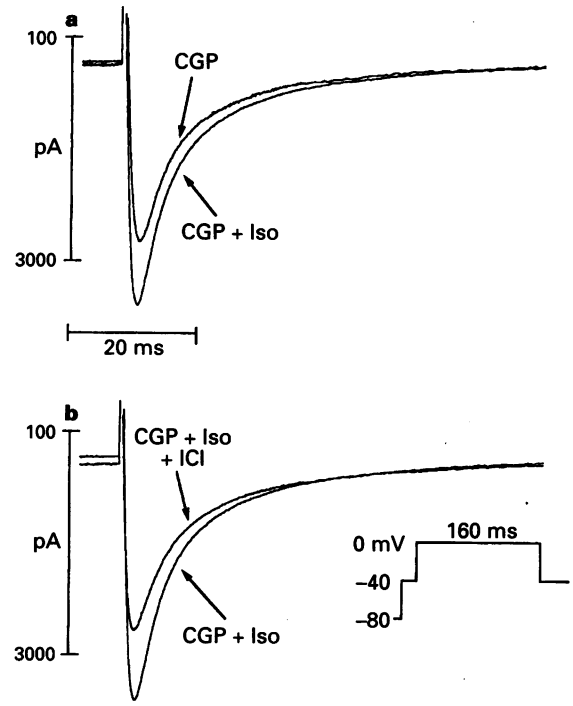

Figure 3 Effect of β_2-adrenoceptor (β_2AR) stimulation by isoprenaline on the amplitude of $I_{Ca,L}$. (a) Superimposed current recordings showing $I_{Ca,L}$ in the presence of the selective β_1AR antagonist, CGP 20712A (0.1 μM) (CGP), and after addition of 1 μM isoprenaline (CGP + Iso). (b) This effect (CGP + Iso) was completely abolished by adding the selective β_2AR antagonist, ICI 118,511 (10 nM) (CGP + Iso + ICI). Bottom right: voltage protocol.

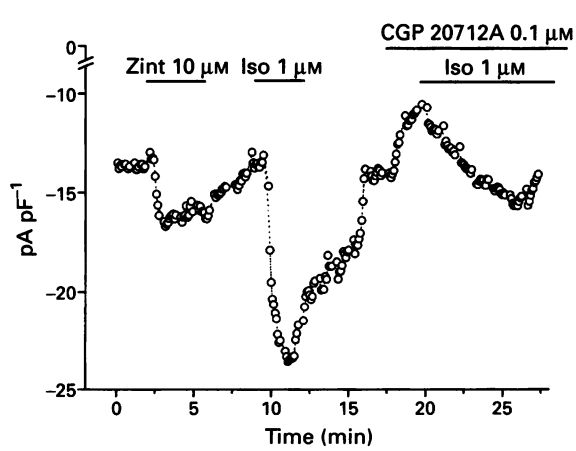

Figure 4 Representative experiment demonstrating the coexistence of both β-adrenoceptor (βAR) subtypes in the same rat ventricular myocyte. Each point represents the value of $I_{Ca,L}$ density measured at the time indicated on the abscissa scale; beginning and duration of superfusion with each drug is shown by the lines. β_2AR stimulation was achieved either by adding zinterol (Zint) or isoprenaline (Iso) in the presence of CGP 20712A. Stimulation of both β_1AR and β_2AR was obtained by isoprenaline alone. See text for explanations.

Figure 5 summarises the results obtained with isoprenaline alone, zinterol and isoprenaline plus CGP 20712A on $I_{Ca,L}$ density in cardiac myocytes of 3- and 22-month-old rats. The drugs were used at concentrations able to exert a similar increase of $I_{Ca,L}$ through β_2AR stimulation (see Figure 4). It is apparent that isoprenaline alone (that is, the stimulation of both β_1AR and β_2AR) causes a significant increase in $I_{Ca,L}$ density. The percentage increase is much smaller in old rats (58.4 ± 12.1%, $n=13$) than in young ones (95.3 ± 8.1%, $n=6$) even though the difference between the two groups did not reach statistical significance ($P=0.067$). Our results also demonstrate that in both young and old groups of myocytes, β_2AR stimulation, no matter how achieved, caused a statistically significant increase in $I_{Ca,L}$ density. The percentage increase is similar in both 3- and 22-month-old rats, either with zinterol (26.9 ± 3.6%, $n=13$ and 24.2 ± 2.8%, $n=9$, respectively) or isoprenaline plus CGP 20712A (30.4 ± 3.7%, $n=6$ and 22.4 ± 4.1, $n=7$, respectively).

Thus, our data do not reveal any clear-cut difference in the effect of β_2AR stimulation on $I_{Ca,L}$ as a function of age, but clearly demonstrate that these receptors are operative in both young and old animals.

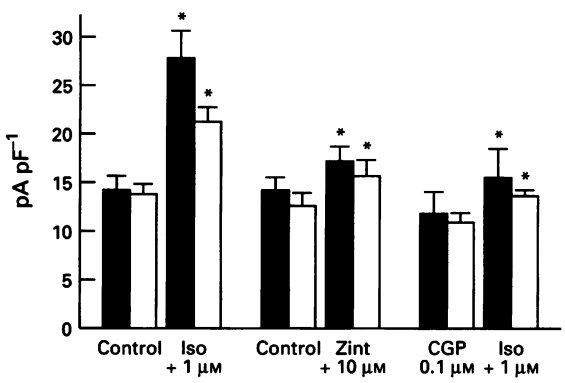

Figure 5 Graph summarising the effects of stimulation of both β_1-adrenoceptor (β_1AR) and β_2AR by isoprenaline (Iso) and the effects of selective β_2AR stimulation by either zinterol (Zint) or isoprenaline in the presence of CGP 20712A (CGP), on peak $I_{Ca,L}$ density recorded from left ventricular myocytes of 3- (solid columns) or 22-month-old rats (open columns). Results are expressed as means ± s.e.mean; $n=6$ to 13; $^*P<0.05$ vs controls.

Binding experiments

Since the electrophysiological data suggest that both β_1AR and β_2AR coexist on the surface of rat ventricular cardiomyocytes at 3 or 22 months of age, we decided to determine whether their number and/or, their density were affected by age.

Saturation binding assays of the non selective βAR antagonist, [^{3}H]-CGP 12177 (0.03–5 nM) exhibited a $K_D = 0.38 \pm 0.15$ nM and 0.32 ± 0.04 nM and a $B_{max} = 33.7 \pm 3.4$ and 26.5 ± 2.0 fmol mg^{-1} protein in 3- and 22-month-old rats respectively. These values correspond to a number of βAR mg^{-1} protein of 1989.4 ± 189.5 for 3- and of 1580.7 ± 161.5 for 22-month-old rats.

To determine the relative proportion of β_1- and β_2-receptor subtypes on intact ventricular cardiomyocytes, competition binding assays were carried out by employing the β_1AR-selective antagonist, CGP 20712A and the β_2AR-selective antagonist, ICI 118,551 as competitor for [^{3}H]-CGP 12177 binding to intact ventricular myocytes. Competition for [^{3}H]-CGP 12177 binding by CGP 20712A resulted in a biphasic curve demonstrating two classes of βAR binding sites (Figure 6). From these experiments, the densities of the two binding sites, expressed as percentages of total βAR density, and their relative affinities were obtained in 3- and 22-month-old rats (Figure 6a and b respectively): 80.4 ± 2.2% ($K_i = 6.6 \pm 1.3$ nM) β_1AR and 19.6 ± 2.2% ($K_i = 6.9 \pm 2.2$ μM) β_2AR were found in myocytes derived from 3-month-old rats while in myocytes from 22-month-old rats the β_1AR/β_2AR ratio was markedly and significantly ($P<0.01$) reduced. In these myocytes, 66.1 ± 1.2% ($K_i = 8.3 \pm 1.1$ nM) β_1AR and 33.9 ± 1.2% ($K_i = 5.2 \pm 0.6$ μM) β_2AR were present. To obtain further confirmation that the lower affinity binding site, observed in inhibition experiments using the β_1AR antagonist CGP 20712A, corresponded to a β_2AR subtype, we carried out experiments with the β_2 AR antagonist, ICI 118,551 obtaining comparable results (data not shown).

The relative proportion of β_1AR and β_2AR in ventricular myocytes was derived from the total number of βAR per cell ($12.6 \pm 1.2 \times 10^4$ and $13.7 \pm 1.4 \times 10^4$, in 3- and 22-month-old rats, respectively) obtained from saturation binding experiments, and the percentage of receptor subtypes obtained from inhibition experiments (Figure 6). We reasoned that a com-

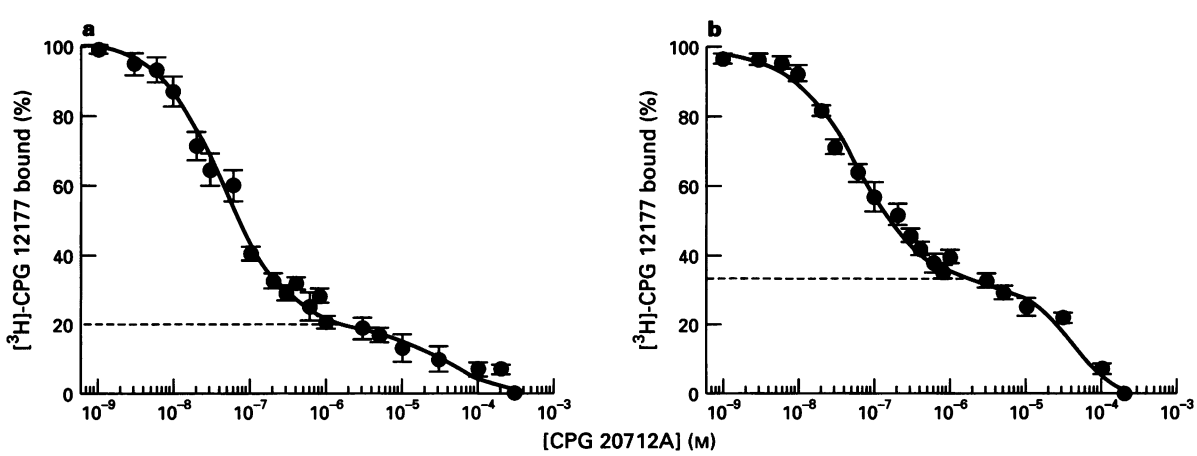

Figure 6 Competition binding experiments showing the relative proportion of the two β-adrenoceptor (βAR) subtypes in left ventricular myocytes from 3-(a) and 22-month-old rats (b). Competition for [^{3}H]-CGP 12177 binding by the selective β_1AR antagonist, CGP 20712A, resulted in a biphasic curve demonstrating two classes of βAR binding sites in both groups of rats. The two binding site densities, expressed as percentages of total βAR density and delimited by the dashed line, and their relative affinities were: 80.4 ± 2.2% ($K_i = 6.6 \pm 1.3$ nM) β_1AR and 19.6 ± 2.2% ($K_i = 6.9 \pm 2.2$ μM) β_2AR (a) and 66.1 ± 1.2% ($K_i = 8.3 \pm 1.1$ nM) β_1AR and 33.9 ± 1.2% ($K_i = 5.2 \pm 0.6$ μM) β_2AR (b). The β_1AR/β_2AR ratio was significantly decreased ($P<0.05$) in old rats. Results are expressed as mean ± s.e.mean of eight experiments performed in duplicate.

Table 2 Densities of β-adrenoceptor (βAR) subtypes in 3- (3 months) and 22-month-old (22 months) rats

	β_1AR		β_2AR	
	Receptors per cell ($\times 10^4$)	*Receptors* μm^{-2}	*Receptors per cell* ($\times 10^4$)	*Receptors* μm^{-2}
3 months	10.1 ± 1.0	15.4 ± 3.7	2.5 ± 0.2	3.8 ± 0.7
22 months	9.1 ± 0.9	8.4 ± 2.0†	4.6 ± 0.5*	4.2 ± 1.1

Densities of β_1AR and β_2AR are expressed as number ± s.e.mean of receptors per cell, and as number ± confidence limits (95%) of receptors per μm^2. †$P < 0.05$; *$P < 0.01$ vs 3-month-old rats.

parison of βAR subtypes during aging must take into account the marked difference in cell size, as suggested by the increase in protein content and in membrane capacitance. The external cell surface was estimated, as previously described, from membrane capacitance measurements. Table 2 summarizes the number of β_1AR and β_2AR per cell and per μm^2. It is worth noting that β_1AR number per cell remains constant but – due to the increase in cell surface – their density decreases significantly, suggesting a dissociation between cell growth and β_1AR synthesis. A completely different situation seems to occur for β_2AR: their number per cell increases significantly during aging, but the density remains unchanged at both 3 and 22 months. This suggests that the synthesis of β_2AR closely parallels cell growth during aging.

Discussion

In this study we show that both β_1AR and β_2AR are functionally present in rat ventricular myocytes isolated from young and senescent animals. The functional consequence of β_2AR stimulation, i.e., the increase in peak $I_{Ca,L}$, is not altered by aging. This effect is reflected by the presence of an unmodified number of β_2AR per surface unit as a consequence of an increase in the number of β_2AR per cell which parallels the increase in cell size. The response to isoprenaline, that is, to β_1AR and β_2AR stimulation, is however markedly reduced during aging. This phenomenon appears to be the consequence of a selective reduction in the cellular density of β_1AR. Our results demonstrate in fact that the reduction of the total number of βAR from 33.7 ± 3.4 (3-month-old rats) to 26.5 ± 2.0 fmol mg^{-1} protein (22-month-old rats) is attributable to a selective statistically significant reduction in the cellular density of β_1AR, while, as already stated, that of β_2AR remains constant. This finding suggests that somehow during aging a discrepancy between cellular growth and synthesis of β_1AR occurs. It can also be speculated that to achieve the same functional effect, i.e. the same increase in peak $I_{Ca,L}$ or eventually contractility mediated through the stimulation of βAR, the cells needs the same density of receptors i.e. a larger cell needs more receptors in order to be able to perform the same function.

Furthermore our data (see Figure 4) demonstrate that β_2AR coexist in the same cell with β_1AR and that their stimulation either with isoprenaline (in the presence of CGP 20712A) or with zinterol reaches the same maximal effect on $I_{Ca,L}$. The reduction of $I_{Ca,L}$, amplitude caused by the β_1AR antagonist, CGP 20712A, suggests that empty β_1AR are operative in rat cardiomyocytes. A similar effect has been reported to occur in guinea-pig and human myocytes exposed to β-blocking drugs (Mewes *et al.*,1993); the phenomenon appears to be due to the fact that agonist-free βAR (empty receptors) are functionally active and can stimulate $I_{Ca,L}$; this stimulating effect can be blocked by β-blocking drugs.

It has been clearly documented that the response of cardiac tissues to catecholamines is diminished by aging (Vestal *et al.*, 1974; Guarnieri *et al.*, 1980; Dobson *et al.*, 1990; White *et al.*, 1991; Stratton *et al.*,1992). However, the basis for this βAR subsensitivity has not been completely elucidated. The finding that the increase in $I_{Ca,L}$ density is smaller in old rats than in young ones (58.4 ± 12.1% vs 95.3 ± 8.1%) is in full agreement with previous results showing a decrease in βAR responsiveness of ventricular myocytes with aging (Sakai *et al.*, 1992). The observation that an age-associated reduction of contractile response to βAR stimulation is present in single cardiac myocytes suggests that the underlying alteration is intrinsic to the cardiac cell. Thus changes in the myocyte number (Anversa *et al.*, 1986) or in the extracellular matrix of the myocardium (Anversa *et al.*, 1989) occuring during senescence cannot totally account for the reduced response to catecholamines of the senescent myocardium. It has been hypothesized that the attenuated response to βAR stimulation is due to an attenuated production of cyclic AMP (Sakai *et al.*, 1992). Obviously an attenuation in cyclic AMP production could be due to several events; a reduction in the number of βAR is the most likely candidate. The general belief that cardiac βAR do not change with aging (Guarnieri *et al.*, 1980; Zitnik & Roth, 1981; Abrass *et al.*, 1982; Scarpace & Abrass, 1986) derives from binding studies performed in rat cardiac membrane preparations looking at the total βAR population. As reviewed by Muntz *et al.* (1994), several technical aspects limit the interpretation of βAR measurement in the heart. First of all the heart is composed of several tissue types, including blood vessels, muscle, connective tissue, nerves, all of which may contain both βAR subtypes. Even more, with senescence the cardiac myocytes increase in size (Anversa *et al.*, 1986; Fraticelli *et al.*, 1989); hypertrophy makes it difficult to distinguish real down-regulation from a simple dilution in the plasma membrane. Finally measurement of total βAR population without looking at the β_1- and the β_2-subtypes may further confound the results, since β_1AR and β_2AR may be differently regulated (Muntz *et al.*, 1994). The rat has been considered until recently to be without β_2AR on ventricular myocytes (Buxton & Brunton, 1985). The unequivocal recent demonstration (Xiao & Lakatta, 1993) of the functional presence of β_2AR in rat ventricular myocytes has allowed the rat to be included among the species containing both βAR subtypes (Harding *et al.*, 1994). More recently the presence of β_2AR in rat ventricular myocytes has been confirmed functionally as well as with radioligand binding experiments (Kuznetsov *et al.*, 1995). The latter experiments were performed on broken cell preparations of adult ventricular myocytes and nonenzymatically resuspended cultured neonatal ventricular myocytes using [^{125}I]-iodocyanopindolol as a ligand. There is a quite good agreement between their and our data on 3-month-old rat myocytes: interestingly the number of β_2AR per cell is practically the same (28094 ± 2859 vs ~25000 ± 2000) representing 16.9 ± 0.9% (Kuznetsov *et al.* data) and 19.6 ± 2.2% (present data) of the total βAR population. Our experiments were performed in intact cells using a hydrophilic radioligand, [^{3}H]-CGP 12177. This was done because one of the most common flaws in studies in intact cells arises from the fact that trapping phenomena can occur when the binding of lipophilic radioligands (like [^{125}I]-iodocyanopindolol) is measured. In addition, hydrophobic ligands can detect the component of βAR population which is sequestered and consequently undetectable with hydrophilic ligands (Homcy *et al.*, 1991) which would only identify sites on the plasma membrane even using intact cells. Due to the use of [^{3}H]-CGP 12177, which may have a higher affinity for β_1AR (2.7 in rat ventricular microsomes, Nanoff *et al.*, 1987), the population of β_1AR and β_2AR may be overestimated and underestimated, respectively. The error in relative percentage of β_1AR and β_2AR is lowered by using relatively high concentrations of the radioligand (2 nM in the present experiments) even if this procedure implies a major error in measurements of affinities toward the two receptor subtypes (McGonigle *et al.*, 1986; Nanoff *et al.*, 1987) which on the other hand were not affected by aging. However, by using this approach, we were able to show for the first time that the

number of β_1AR per unit of cell surface significantly decreases with senescence, while that of β_2AR remain constant. A proper comparison of βAR expression in adult and senescent myocytes must take into account the marked differences in size and surface area between these cells (Fraticelli *et al.*, 1989). We are aware that our measurement of cell surface is imperfect since it derives from an electrophysiological measure (membrane capacitance) which is a rough index of cell size. However, the astonishing similarities of our results with those reported by Kuznetsov *et al.*, (1995), who measured the cell dimensions under the microscope and estimated a density of 20 βAR per μm^2 of cell surface in adult rats, make us quite confident of our approach. In fact we estimated that in myocytes isolated from the heart of rats of simular age (3-month-old rats), the number of β_1AR and of β_2AR was 15.4 ± 3.7 and 3.8 ± 0.75 per μm^2 respectively, which together give exactly the same density of βAR reported by Kuznetsov *et al.*, (1995).

β_1AR and β_2AR undergo a different regulation as the cardiac myocytes grow; in fact the number of β_1AR per cell remains constant and that of β_2AR increases. The final result is that the density (number of receptors μm^{-2}) of β_1AR is significantly reduced and that of β_2AR remains constant. This may explain why the functional response to β_2AR stimulation is not influenced by aging. We found in fact that either zinterol or isoprenaline in the presence of the highly selective β_1AR antagonist, CGP 20712A, was able to increase to the same extent the peak of $I_{Ca,L}$ density in both adult and senescent myocytes. The response to isoprenaline alone was instead markedly reduced in the senescent myocytes compared to that observed in the younger ones. The attenuation of the response to isoprenaline can be reasonably ascribed to its β_1AR stimulating component, since the β_2AR-mediated response remains constant. If so, it can be speculated that the phenomenon is due to the reduced density of β_1AR in the senescent myocyte. In fact the functional response of the myocyte to βAR stimulation (increase of $I_{Ca,L}$ density) is the consequence of the stimulation of both βAR subtypes coexisting in the same cell, as demonstrated by the experiment shown in Figure 4. Since the cellular density of the β_2AR and their contribution to the βAR-mediated response do not change with senescence, it is likely that the reduced density of β_1AR in the senescent myocyte plays a major role in the subsensitivity of the senescent myocardium to β-AR stimulation.

It has been clearly demonstrated that there are differences in the $[Ca^{2+}]_i$ responses to β_1AR and β_2AR stimulation in cardiac myocytes (Xiao & Lakatta, 1993). Regarding this, we have demonstrated that β_1AR stimulation is more arrhythmogenic than β_2AR stimulation (Cerbai *et al.*,1990). Thus the fact that the ratio β_1AR/β_2AR changes in ventricular myocytes during senescence could have important physiological consequences in the heart.

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