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Pharmacological characterization of the receptors involved in the β-adrenoceptor-mediated stimulation of the L-type Ca^{2+} current in frog ventricular myocytes

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1 The whole-cell patch-clamp was used for studying the effects of various β_1- and β_2-adrenoceptor agonists and antagonists on the L-type Ca current (I_{Ca}) in frog ventricular myocytes.

2 Dose-response curves for the effects of isoprenaline (non selective β-agonist), salbutamol (β_2-agonist), dobutamine (β_1-agonist) on I_{Ca} were obtained in the absence and presence of various concentrations of ICI 118551 (β_2-antagonist), metoprolol (β_1-antagonist) and xamoterol (partial β_1-agonist) to derive EC_{50} (i.e. the concentration of β-agonist at which the response was 50% of the maximum) and E_{max} (the maximal response) values by use of a Michaelis equation. Schild regression analysis was performed to examine whether the antagonists were competitive and to determine the equilibrium dissociation constant (K_B) for the antagonist-receptor complex.

3 Isoprenaline increased I_{Ca} with an EC_{50} of 20.0 nM and an E_{max} of 597%. ICI 118551 and metoprolol competitively antagonized the effect of isoprenaline with a K_B of 3.80 nM and 207 nM, respectively.

4 Salbutamol increased I_{Ca} with an EC_{50} of 290 nM and an E_{max} of 512%. ICI 118551 and metoprolol competitively antagonized the effect of salbutamol with a K_B of 1.77 nM and 456 nM, respectively.

5 Dobutamine increased I_{Ca} with an EC_{50} of 2.40 μM and an E_{max} of 265%. ICI 118551 and metoprolol competitively antagonized the effect of dobutamine with a K_B of 2.84 nM and 609 nM, respectively.

6 Xamoterol had no stimulating effect on I_{Ca}. However, xamoterol competitively antagonized the stimulating effects of isoprenaline, salbutamol and dobutamine on I_{Ca} with a K_B of 58–64 nM.

7 We conclude that a single population of receptors is involved in the β-adrenoceptor-mediated regulation of I_{Ca} in frog ventricular myocytes. The pharmacological pattern of the response of I_{Ca} to the different β-adrenoceptor agonists and antagonists tested suggests that these receptors are of the β_2-subtype.

Keywords: Ca^{2+} current; frog heart; β_1-adrenoceptor agonists and antagonists; β_2-adrenoceptor agonists and antagonists; dobutamine; metoprolol; ICI 118551; xamoterol; salbutamol; isoprenaline

Introduction

β_1- and β_2-adrenoceptors coexist in the heart of various animal species, including man. Both receptors are positively coupled to the adenylyl cyclase system and participate in the mediation of the positive chronotropic and inotropic effects of catecholamines (for reviews, see Stiles et al., 1984; Brodde, 1991). However, the relative amount of each receptor subtype as well as the post-receptor cellular signalling pathways may differ significantly depending on the cardiac tissue, the animal species, the pathophysiological state, the age or the developmental stage (for reviews, see Stiles *et al*., 1984; Brodde, 1991; 1993; Hieble & Ruffolo, 1991 and references therein). Competitive radioligand binding studies performed in membranes from homogenized hearts have shown that only 20–30% of the total β-adrenoceptors are of the β_2-subtype in adult mammalian ventricular tissue (Stiles *et al*., 1984; Brodde, 1991; Hieble & Ruffolo, 1991). This number is even further reduced when purified cardiac myocytes rather than homogenized tissues are used (Lau *et al*., 1980; Buxton & Brunton, 1985; Freissmuth *et al*., 1986; Kuznetsov *et al*., 1995; Cerbai *et al*., 1995). Yet, selective activation of β_2-adrenoceptors produces a large increase in the amplitude of contraction in intact mammalian cardiac muscle (Cerbai *et al*., 1990; Lemoine & Kaumann, 1991; Brodde, 1991) as well as in isolated ventricular myocytes (del Monte *et al*., 1993; Xiao & Lakatta, 1993; Xiao *et al*., 1994; 1995; Altschuld *et al*., 1995; Kuznetsov *et al*., 1995). Moreover, selective β_2-adrenoceptor activation was found to produce a stimulation of the L-type Ca^{2+} channel current (I_{Ca}) in guinea-pig atrial myocytes (Iijima & Taira, 1989), and in rat (Xiao & Lakatta, 1993; Xiao *et al*., 1994; 1995; Cerbai *et al*., 1995), guinea-pig (Wang & Pelzer, 1995; but see Iijima & Taira, 1989) and dog ventricular myocytes (Altschuld *et al*., 1995). When compared to the effect produced by non-selective β-adrenoceptor agonists such as isoprenaline, the β_2-response may represent 25–100% of the isoprenaline response (Xiao & Lakatta, 1993; Altschuld *et al*., 1995). This suggests that the two receptors may differ in their signalling cascade (Lemoine & Kaumann, 1991; Borea *et al*., 1992; Xiao & Lakatta, 1993; Xiao *et al*., 1994; 1995; Kuznetsov *et al*., 1995; but see Skeberdis *et al*., 1996) or in the post-receptor amplification mechanisms (Waelbroeck *et al*., 1983; Bristow *et al*., 1989; Green *et al*., 1992; Levy *et al*., 1993).

Unlike in the mammalian heart, the β-adrenoceptor population in the frog heart is composed of a majority ($\approx$ 80%) of β_2-receptors (Stene-Larsen & Helle, 1978; Hancock *et al*., 1979; Port *et al*., 1992; Hieble & Ruffolo, 1991). Thus, one may question the functional role of β_1-adrenoceptors in this preparation and their contribution to the sympathetic control of heart function. For this reason, we have undertaken a pharmacological characterization of the β-adrenoceptor regulation of I_{Ca} in whole-cell patch-clamped single frog ventricular myocytes. Most particularly, we quantified the contribution of β_1- and β_2-receptor subtypes in the stimulating effect of the non-selective β-adrenoceptor agonist isoprenaline on I_{Ca}, by use of, respectively, metoprolol and ICI 118551 as selective antagonists of β_1- and β_2-receptors (Bilski *et al*., 1983; Bylund

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et al., 1994). We also investigated the effect of selective β_1- and β_2-receptor activation on I_{Ca} by use of, respectively, dobutamine and salbutamol as selective agonists of these receptors (Bylund *et al*., 1994). Finally, we examined the effect of xamoterol, a partial β_1-adrenoceptor agonist (Brodde, 1991; Bylund *et al*., 1994) on I_{Ca}. All our results converge towards the participation of a single population of receptors in the β-adrenoceptor-mediated regulation of I_{Ca} in frog ventricular myocytes. These receptors essentially resemble the pharmacologically defined β_2-adrenoceptors of mammalian preparations.

A preliminary account of this work was presented at the 41st Annual Meeting of the Biophysical Society, New Orleans, U.S.A., March 1997 (Skeberdis *et al*., 1997).

Methods

The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J n°L358, 18 December 1986) and the French decree n°87/748 of October 19, 1987 (J Off République Française, 20 October 1987, pp. 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Forêt (n°04226, April 12, 1991).

Cell dissociation

Ventricular cells were enzymatically dispersed from frog (*Rana esculenta*) heart, by a combination of collagenase and trypsin as described by Fischmeister & Hartzell (1986). Frogs were decapitated and double pithed. The isolated cells were stored in storage Ringer solution, and kept at 4°C until use (2–48 h following dissociation).

Electrophysiological experiments

The whole-cell configuration of the patch-clamp technique was used to record the high-threshold calcium current (I_{Ca}) on Ca^{2+}-tolerant frog ventricular myocytes. In the routine protocols the cells were depolarized every 8 s from a holding potential of −80 vV to 0 mV for 200 ms. Application of tetrodotoxin (0.3 μM) was used to eliminate fast sodium currents. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+. Voltage-clamp protocols were generated by a challenger/09-VM programmable function generator (Kinetic Software, Atlanta, GA). The cells were voltage-clamped by use of a patch-clamp amplifier (model RK-400; Bio-Logic, Claix, France). Currents were sampled at a frequency of 10 kHz by a 16-bit analogue-to-digital converter (PCL816, Advantech France, Levallois Perret, France) connected to a PC compatible 486/66 micro computer.

Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 300 μm inner diameter capillary tubings flowing at a rate of $\approx$ 50 μl min^{-1} (Fischmeister & Hartzell, 1986). Changes in extracellular solutions were automatically achieved by a rapid solution changer (RSC100, Bio-Logic, Claix, France). All experiments were done at room temperature (19–25°C), and the temperature did not change by more than 1°C in a given experiment.

Data analysis

The maximal amplitude of whole-cell I_{Ca} was measured as previously described (Fischmeister & Hartzell, 1986; Argibay *et al*., 1988). Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was made possible by programming a PC-compatible 486/66 microcomputer in Assembling language (Borland, U.S.A.) to determine, for each membrane depolarization, peak and steady-state current values (Fischmeister & Hartzell, 1986).

Cumulative dose-response curves were obtained by testing 4 or 5 successively increasing concentrations of a β-adrenoceptor agonist on I_{Ca} in the presence and absence of a β-adrenoceptor antagonist. For each concentration of agonist, a percentage increase in I_{Ca} amplitude with respect to its 'basal' level in the absence of agonist was calculated: (% increase in I_{Ca}) = 100[(I_{Ca} with agonist) − (basal I_{Ca})]/(basal I_{Ca}). For each individual experiment, the results obtained were fit to the Michaelis equation: (% increase in I_{Ca}) = E_{max}[agonist]/([agonist] + EC_{50}). The E_{max} values from several similar experiments are given in the text and/or figure legends as means ± s.e.mean. The results of a given experiment were then normalized by dividing each individual '% increase in I_{Ca}' value by E_{max}. The normalized values from several similar experiments were then averaged and are represented as percentage of maximal response by means (symbols) ± s.e.mean (vertical lines) in the corresponding dose-response curves. These average values were fit to the Michaelis equation again to derive an average EC_{50} value, i.e. the concentration of agonist required to produce 50% of the maximal increase in I_{Ca}. In the absence of antagonist, EC_{50} was EC_{50}^0; in the presence of a concentration [X] of antagonist, EC_{50} was EC_{50}^x. Antagonist potency was determined by Schild regression (Kenakin, 1993). The logarithms of dose-ratios minus 1, i.e. $\log(EC_{50}^x/EC_{50}^0 - 1)$, were plotted as a function of the logarithms of molar concentrations of the antagonist, i.e. log[X], and fit to a straight line by use of a least-means squares regression analysis. The Schild coefficient was determined from the slope of the line. The Schild coefficient was considered to be equal to unity if the slope was within a 95% confidence limit range of 0.9–1.2 (Kenakin, 1993). If this was the case, the equilibrium dissociation constant (K_B) for the antagonist-receptor complex was calculated for each dose-response curve as: $\log(K_B) = \log[X] - \log(EC_{50}^x/EC_{50}^0 - 1)$. K_B values given in the text are the means ± s.e. mean of 3 or 4 values, depending on the number of antagonist concentrations tested.

Materials

For the preparation of frog ventricular myocytes, the ionic composition of Ca^{2+}-free Ringer solution was (mM): NaCl 88.4, KCl 2.5, $NaHCO_3$ 23.8, NaH_2PO_4 0.6, $MgCl_2$ 1.8, creatine 5, D-glucose 10 and 1 mg ml^{-1} fatty acid-free bovine serum albumin, 50 i u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin; pH 7.4 maintained with 95% O_2, 5% CO_2. Storage Ringer solution was Ca^{2+}-free Ringer solution to which was added 0.9 mM $CaCl_2$ and 10 μl ml^{-1} non-essential and essential amino acid and vitamin solution (MEM 100×). Dissociation medium was composed of Ca^{2+}-free Ringer solution to which was added 0.2 mg ml^{-1} trypsin type XIII (Sigma, St. Louis, U.S.A.), 0.14 mg ml^{-1} collagenase (Yakult, Tokyo, Japan), and 10 μl ml^{-1} M199 medium (Sigma).

For electrophysiology, the control external solution contained (in mM): NaCl 107, HEPES 10, CsCl 20, $NaHCO_3$ 4, NaH_2PO_4 0.8, $MgCl_2$ 1.8, $CaCl_2$ 1.8, D-glucose 5, sodium pyruvate 5 and tetrodotoxin 3×10^{-4}, pH 7.4 adjusted with NaOH. Patch electrodes (0.6–2.0 MΩ) were filled with control internal solution which contained (mM): CsCl 119.8, EGTA (acid form) 5, $MgCl_2$ 4, creatine phosphate disodium salt 5, Na_2ATP 3.1, Na_2GTP 0.42, $CaCl_2$ (pCa 8.5) 0.062 and HEPES 10; pH 7.1 adjusted with CsOH.

Tetrodotoxin was from Latoxan (Rosans, France). The β-adrenoceptor agonists and antagonists used were: isoprenaline (Sigma), salbutamol (Sigma), xamoterol (Tocris Cookson, Bristol, U.K.), dobutamine (Tocris Cookson), ICI 118551 (erythro-1-(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol; Tocris Cookson), and metoprolol (ICN Biomedicals Inc., Aurora, Ohio, U.S.A.). All other drugs were from Sigma. Salbutamol was solubilized in ethanol and all other β-adrenoceptor compounds were dissolved in distilled water. Each day, fresh 1–10 mM stock solutions were prepared and stored

at 4°C. Immediately before being applied to the cell, the drug was dissolved at the desired final concentration in control external solution, i.e. only fresh solutions were tested. In the case of salbutamol, an appropriate amount of ethanol was added to each solution so that the same ethanol concentration as that present in the solution containing the highest concentration of the drug was present in all solutions tested.

Results

Regulation of I_{Ca} by the non-selective β-adrenoceptor agonist isoprenaline

Figure 1a shows a typical experiment with isoprenaline as a non-selective β-adrenoceptor agonist to stimulate I_{Ca} in a frog ventricular myocyte. I_{Ca} was measured every 8 s by depolarizing the cell over a period of 200 ms to 0 mV from a holding potential of −80 mV. As shown earlier (Fischmeister & Hartzell, 1986; Hartzell *et al*., 1991), isoprenaline produced a dose-dependent increase in I_{Ca}. A cumulative dose-response curve for the effect of isoprenaline on I_{Ca} is presented in Figure 1c (■). The data are normalized to the maximal stimulation of I_{Ca} (E_{max}) which was derived from the fit of the experimental points to the Michaelis equation (see Methods). Thus, isoprenaline increased I_{Ca} with an EC_{50} value of 20.0 nM and E_{max} was 597 ± 105% (n = 9).

To examine the participation of β_2-adrenoceptors in the stimulating effect of isoprenaline on I_{Ca}, the same type of experiment was repeated in the presence of different concentrations of ICI 118551, a selective β_2-adrenoceptor antagonist. As shown in the typical experiment of Figure 1b, ICI 118551 (used at 100 nM in this experiment) was applied first to the myocyte which was then exposed to increasing concentrations of isoprenaline. ICI 118551 alone had no effect on I_{Ca} at any of the concentrations used (1 nM to 1 μM, data not shown). However, in the presence of the drug, larger concentrations of isoprenaline were required to stimulate I_{Ca} (Figure 1b, c). The mean data points in Figure 1c show the response of I_{Ca} to isoprenaline in the presence of 1 nM, 10 nM, 100 nM and 1 μM ICI 118551. The dose-response curve for the response of I_{Ca} to isoprenaline was progressively shifted to higher concentrations of the agonist when increasing concentrations of the β_2-antagonist were used. A Schild regression analysis (see Methods) of the five curves presented in Figure 1c allowed us to determine a Schild coefficient close to 1 (1.08) and a K_B of 3.80 ± 0.90 nM (n = 4) for the inhibitory effect of ICI 118551 on the response of I_{Ca} to isoprenaline. This suggests that, in the range of concentrations used (1 nM to 100 μM), isoprenaline activates I_{Ca} through a single population of receptors which bind ICI 118551 in the low nanomolar range of concentrations.

We then examined the participation of β_1-adrenoceptors in the stimulating effect of isoprenaline on I_{Ca}. Thus, the same type of experiment as above was repeated in the presence of different concentrations of metoprolol, a selective β_1-adrenoceptor antagonist. As shown in the typical experiment of Figure 2a, a frog myocyte was exposed at first to metoprolol (used at 10 μM in this experiment) and subsequently to increasing concentrations of isoprenaline in the presence of metoprolol. Metoprolol alone had no effect on I_{Ca} at any of the concentrations used (10 nM to 10 μM, data not shown). However, in the presence of the drug, larger concentrations of isoprenaline were required to stimulate I_{Ca} (e.g. compare Figure 1a and 2a). The mean data points in Figure 2b show the response of I_{Ca} to isoprenaline alone (■, taken from Figure 1c) or in the presence of 10 nM, 100 nM, 1 μM and 10 μM metoprolol. The dose-response curve for the response of I_{Ca} to isoprenaline was progressively shifted to higher concentrations of the agonist when increasing concentrations of the β_1-antagonist were used. A competition curve analysis of the five curves of Figure 2b allowed us to determine a Schild coefficient

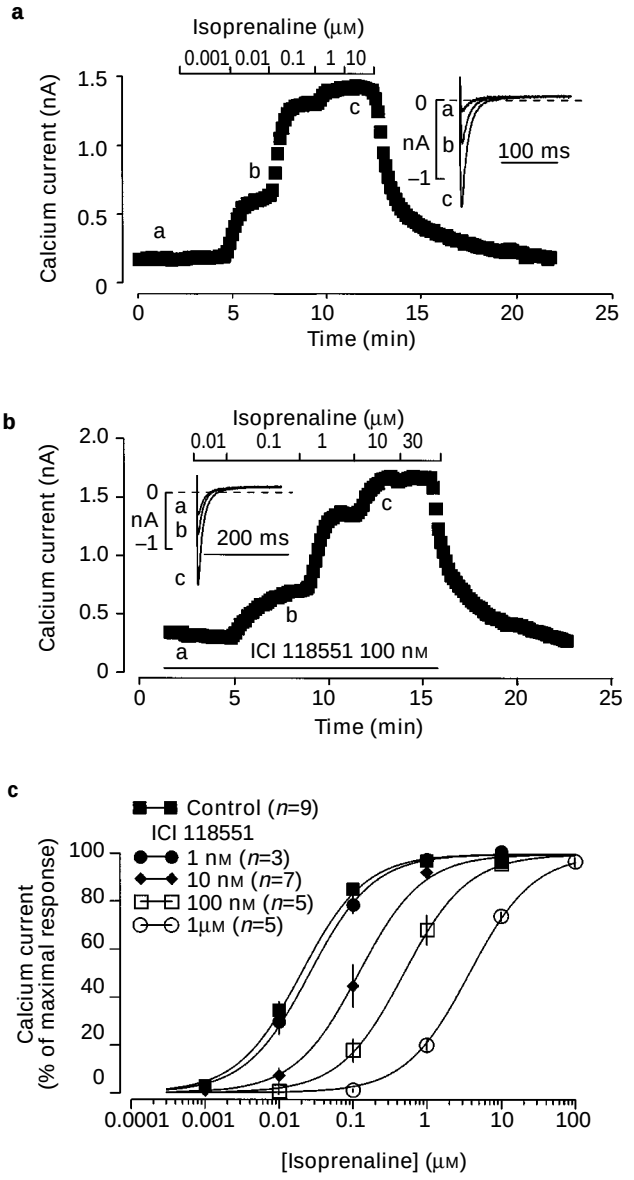

Figure 1 Effect of isoprenaline and ICI 118551 on I_{Ca} in frog ventricular cells. (a) A frog ventricular myocyte was initially superfused with control external solution and internally dialysed with control intracellular solution. Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarizing the cell every 8 s to 0 mV over a period of 200 ms from a holding potential of −80 mV. During the periods indicated by the horizontal lines, the cell was successively exposed to five increasing concentrations of isoprenaline (0.001, 0.01, 0.1, 1 and 10 μM). (b) The same experiment as in (a) but in the presence of 100 nM ICI 118551. After application of the β_2-antagonist, the cell was successively exposed to five increasing concentrations of isoprenaline (0.01, 0.1, 1, 10 and 30 μM). The current traces shown in the insets of (a) and (b) were recorded at the times indicated by the corresponding letters on the main graphs. (c) Concentration-response curve for the stimulating effect of isoprenaline on I_{Ca}. The effect of increasing concentrations of isoprenaline on I_{Ca} were obtained in several experiments performed in the absence of ICI 118551 as in (a) (control) or in the presence of 1 nM, 10 nM, 100 nM (as in (b) or 1 μM ICI 118551. The points show the mean and vertical lines s.e.mean of the number of cells indicated. The continuous line was derived from a non-linear least-mean-squares regression of the means to the Michaelis equation (see Methods). The concentration of isoprenaline (EC_{50}) required for half-maximal stimulation of I_{Ca} and the maximal effect of isoprenaline (E_{max}) were derived from this analysis: EC_{50} and E_{max} values were: 20.0 nM and 597% in control; 25.8 nM and 1237% with 1 nM ICI 118551; 120 nM and 435% with 10 nM ICI 118551; 470 nM and 322% with 100 nM ICI 118551; 3.70 μM and 744% with 1 μM ICI 118551. The data were normalized to E_{max} and are presented as percentage of the maximal response to isoprenaline.

close to 1 (1.06) and a K_B of 207±51.9 nM ($n=3$) for the inhibitory effect of metoprolol on the response of I_{Ca} to isoprenaline. This suggests again that isoprenaline activates I_{Ca} through a single population of receptors. These receptors are sensitive to metoprolol in the high nanomolar range of concentrations.

Regulation of I_{Ca} by the selective β_2-adrenoceptor agonist salbutamol

We have shown earlier that zinterol and salbutamol, two selective β_2-adrenoceptor agonists, stimulate I_{Ca} in frog ventricular myocytes and produce a similar maximal effect as isoprenaline (Skeberdis *et al*., 1996). We have now characterized in more detail the response of I_{Ca} to salbutamol. As shown in the typical experiment of Figure 3a, salbutamol produced a dose-dependent increase in I_{Ca}. A cumulative dose-response curve for the effect of salbutamol on I_{Ca} is presented in Figure 3c (■). Salbutamol increased I_{Ca} with an EC_{50} value of 290 nM and E_{max} was 512±69% ($n=6$). To examine the participation of β_2- and β_1-adrenoceptors in the stimulating effect of salbutamol on I_{Ca}, the same type of experiment was repeated in the presence of different concentrations of ICI 118551 (Figure 3b and c) or metoprolol (Figure 4). As shown in the typical experiments of Figure 3b and Figure 4a, ICI 118551 (used at 10 nM in Figure 3b) and metoprolol (used at 0.1 μM in Figure 4a) reduced the sensitivity of I_{Ca} to salbutamol, since larger concentrations of the drug were required to stimulate I_{Ca}. The mean data points in Figure 3c show the response of I_{Ca} to salbutamol in the presence of 1 nM, 10 nM and 100 nM ICI

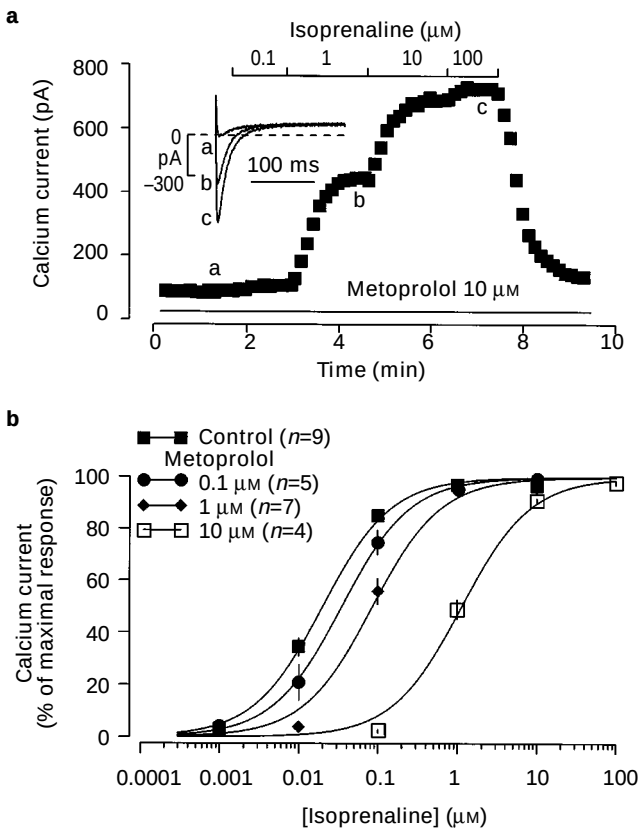

Figure 2 Effect of isoprenaline and metoprolol on I_{Ca}. (a) A frog ventricular myocyte was initially superfused with control external solution in the presence of 10 μM metoprolol. During the periods indicated, the cell was successively exposed to four increasing concentrations of isoprenaline (0.1, 1, 10 and 100 μM). The current traces shown in the inset were recorded at the times indicated by the corresponding letters on the main graph. (b) Concentration-response curve for the stimulating effect of isoprenaline on I_{Ca}. The effects of increasing concentrations of isoprenaline on I_{Ca} were obtained in several experiments performed in the absence of metoprolol (Control, same data as in Figure 1c) or in the presence of 100 nM, 1 μM or 10 μM metoprolol (as in (a)). The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values were: 20.0 nM and 597% in control; 34.9 nM and 287% with 100 nM metoprolol; 85.1 nM and 240% with 1 μM metoprolol; 1.13 μM and 715% with 10 μM metoprolol. The data were normalized to E_{max} and are presented as percentage of the maximal response to isoprenaline.

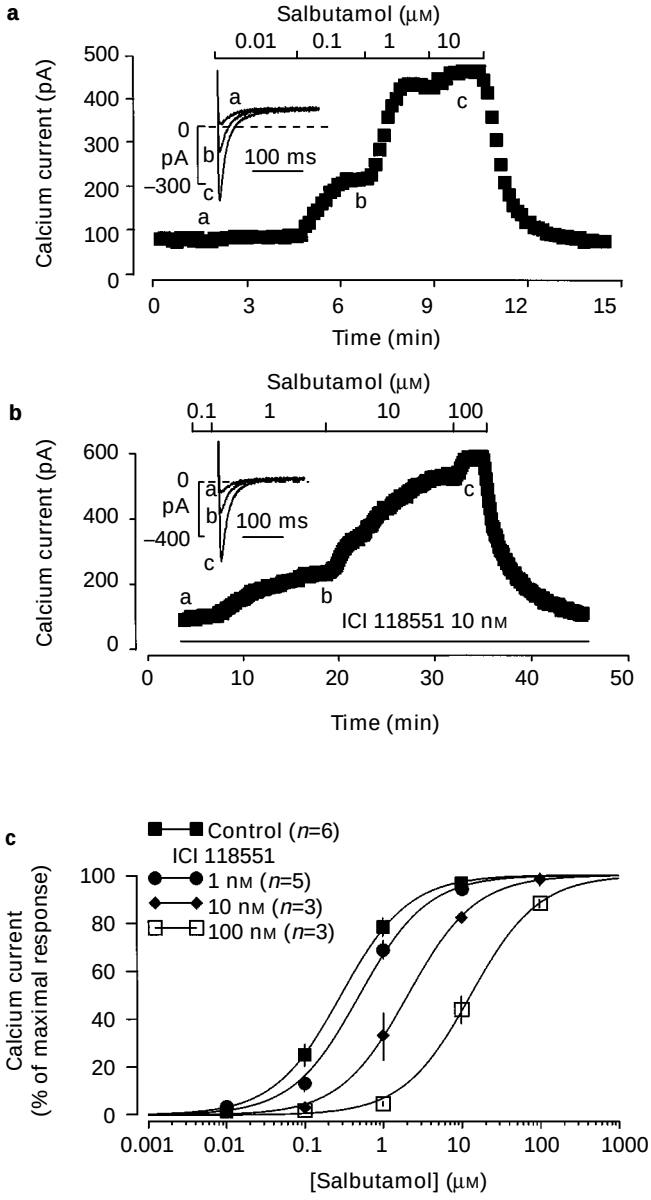

Figure 3 Effect of salbutamol and ICI 118551 on I_{Ca}. (a) A frog ventricular myocyte was initially superfused with control external solution. During the periods indicated, the cell was successively exposed to four increasing concentrations of salbutamol (0.01, 0.1, 1 and 10 μM). (b) The same experiment as in (a) but in the presence of 10 nM ICI 118551. After application of the β_2-antagonist, the cell was successively exposed to four increasing concentrations of salbutamol (0.1, 1, 10 and 100 μM). The current traces shown in the insets of (a) and (b) were recorded at the times indicated by the corresponding letters on the main graphs. (c) Concentration-response curve for the stimulating effect of salbutamol on I_{Ca}. The effect of increasing concentrations of salbutamol on I_{Ca} were obtained in several experiments performed in the absence of ICI 118551 as in (a) (Control) or in the presence of 1 nM, 10 nM (as in (b)) or 100 nM ICI 118551. The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values for the effect of salbutamol on I_{Ca} were: 290 nM and 512% in control; 0.51 μM and 531% with 1 nM ICI 118551; 2.06 μM and 837% with 10 nM ICI 118551; 13.0 μM and 559% with 100 nM ICI 118551. The data were normalized to E_{max} and are presented as percentage of the maximal response to salbutamol.

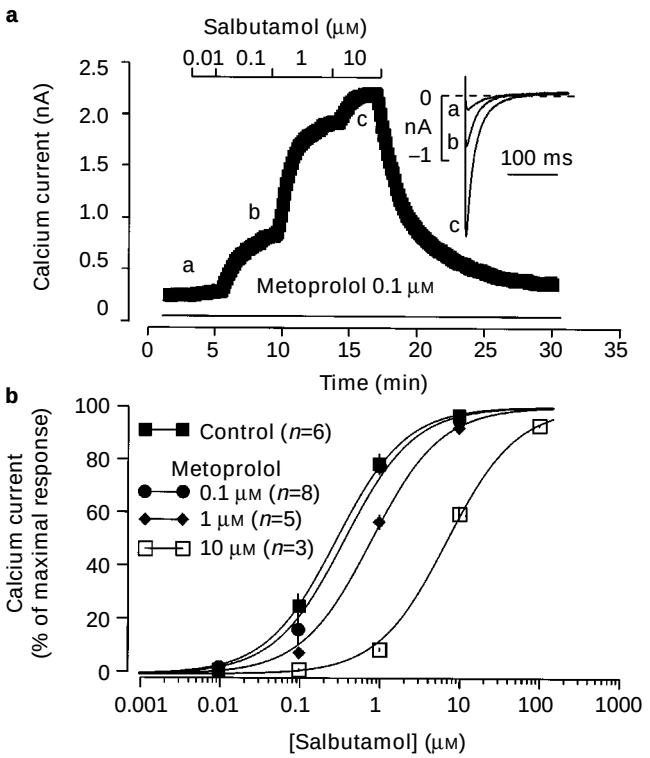

Figure 4 Effect of salbutamol and metoprolol on I_{Ca}. (a) A frog ventricular myocyte was initially superfused with control external solution in the presence of 100 nM metoprolol. During the periods indicated, the cell was successively exposed to four increasing concentrations of salbutamol (0.01, 0.1, 1 and 10 μM). The current traces shown in the inset were recorded at the times indicated by the corresponding letters on the main graph. (b) Concentration-response curve for the stimulating effect of salbutamol on $I_{Ca.}$ The effect of increasing concentrations of salbutamol on I_{Ca} were obtained in several experiments performed in the absence of metoprolol (Control, same data as in Figure 3c) or in the presence of 0.1 μM (as in (a)), 1 μM or 10 μM metoprolol. The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values were: 290 nM and 512% in control; 367 nM and 631% with 0.1 μM metoprolol; 800 nM and 802% with 1 μM metoprolol; 7.13 μM and 532% with 10 μM metoprolol. The data were normalized to E_{max} and are presented as percentage of the maximal response to salbutamol.

118551. The mean data points in Figure 4b show the response of I_{Ca} to salbutamol alone (■, taken from Figure 3c) or in the presence of 100 nM, 1 μM and 10 μM metoprolol. It appears that the dose-response curve for the response of I_{Ca} to salbutamol was progressively shifted to higher concentrations of the β_2-agonist when increasing concentrations of ICI 118551 (Figure 3c) or metoprolol (Figure 4b) were used. A Schild regression analysis (see Methods) of the curves in Figures 3c and 4b allowed us to determine Schild coefficients close to 1 (1.12 in Figure 3c, 1.02 in Figure 4b) and K_B values of 1.77 ± 0.28 nM ($n=3$) for the inhibitory effect of ICI 118551 and 456 ± 57.8 nM ($n=3$) for the inhibitory effect of metoprolol on the response of I_{Ca} to salbutamol. This suggests that, in the range of concentrations used (10 nM to 100 μM), salbutamol activates I_{Ca} through a single population of receptors which bind ICI 118551 in the low nanomolar range of concentrations and metoprolol in the high nanomolar range of concentrations.

Regulation of I_{Ca} *by the selective* β_1*-adrenoceptor agonist dobutamine*

The same strategy was applied to examine the effect of a selective β_1-adrenoceptor agonist, dobutamine, on I_{Ca} in frog ventricular myocytes. As shown in the experiment of Figure 5a, dobutamine produced a dose-dependent increase in I_{Ca}. A cumulative dose-response curve for the effect of dobutamine on I_{Ca} is presented in Figure 5c (■). Dobutamine increased I_{Ca} with an EC_{50} value of 2.4 μM and E_{max} was $265 \pm 47\%$ ($n=7$). However, we found that increasing the concentration of dobutamine above 100 μM produced severe inhibitory effects on I_{Ca} and a complete block of the current appeared at a concentration of 1 mM. Thus, in the following, only the ef-

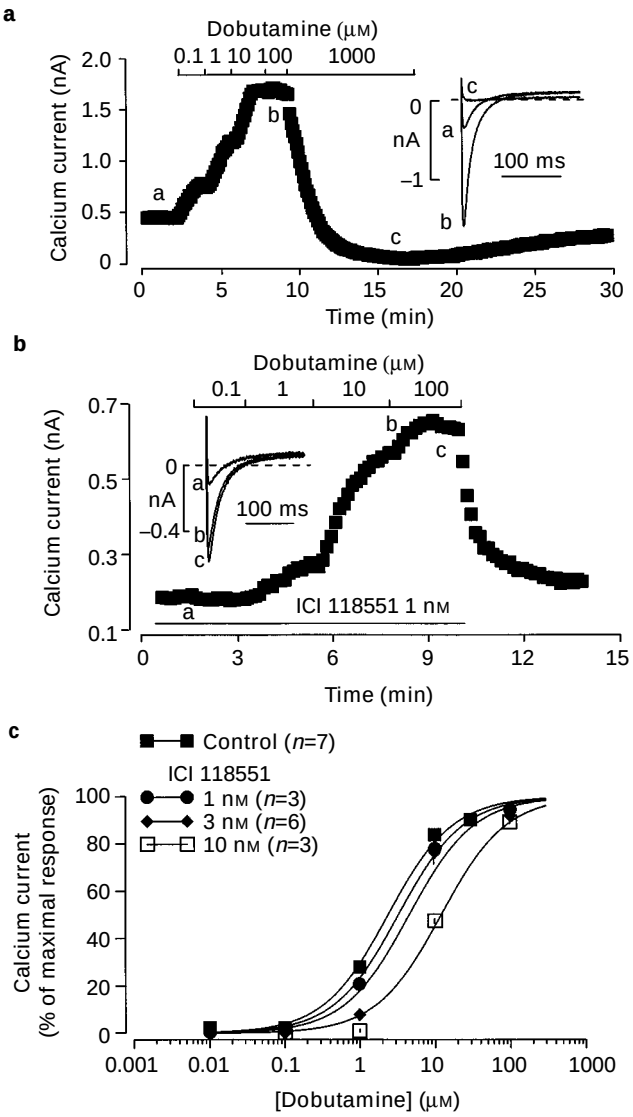

Figure 5 Effect of dobutamine and ICI 118551 on I_{Ca}. (a) A frog ventricular myocyte was initially superfused with control external solution. During the periods indicated, the cell was successively exposed to five increasing concentrations of dobutamine (0.1, 1, 10, 100 and 1000 μM). Note the dramatic inhibitory effect on I_{Ca} of the highest concentration of dobutamine. (b) The same experiment as in (a) but in the presence of 1 nM ICI 118551. After application of the β_2-antagonist, the cell was successively exposed to four increasing concentrations of dobutamine (0.1, 1, 10 and 100 μM). The current traces shown in the insets of (a) and (b) were recorded at the times indicated by the corresponding letters on the main graphs. (c) Concentration-response curve for the stimulating effect of dobutamine on I_{Ca}.The effect of increasing concentrations of dobutamine on I_{Ca} were obtained in several experiments performed in the absence of ICI 118551 as in (a) (Control) or in the presence of 1 nM (as in (b)), 3 nM or 10 nM ICI 118551. The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values for the effect of salbutamol on I_{Ca} were: 2.40 μM and 265% in control; 3.28 μM and 318% with 1 nM ICI 118551; 4.57 μM and 203% with 3 nM ICI 118551; 12.2 μM and 108% with 10 nM ICI 118551. The data were normalized to E_{max} and are presented as percentage of the maximal response to dobutamine.

fects of lower concentrations of dobutamine ($\leqslant 100$ μM) were investigated. To examine the participation of β_2- and β_1-adrenoceptors in the stimulating effect of dobutamine on I_{Ca}, the same type of experiment was repeated in the presence of different concentrations of ICI 118551 (Figure 5b and c) or metoprolol (Figure 6). As shown in the typical experiments of Figure 5b and Figure 6a, ICI 118551 (used at 1 nM in Figure 5b) and metoprolol (used at 0.1 μM in Figure 6a) reduced the sensitivity of I_{Ca} to dobutamine, since larger concentrations of the drug were required to stimulate I_{Ca}. The mean data points in Figure 5c show the response of I_{Ca} to dobutamine in the presence of 1 nM, 3 nM and 10 nM ICI 118551. The mean data points in Figure 6b show the response of I_{Ca} to dobutamine alone (■, taken from Figure 5c) or in the presence of 100 nM, 1 μM and 10 μM metoprolol. It appears that the dose-response curve for the response of I_{Ca} to dobutamine was progressively shifted to higher concentrations of the β_1-agonist when increasing concentrations of ICI 118551 (Figure 5c) or metoprolol (Figure 6b) were used. A Schild regression analysis (see Methods) of the curves in Figures 5c and 6b allowed us to determine Schild coefficients close to 1 (0.98 in Figure 5c, 1.11 in Figure 6b) and K_B values of 2.84 ± 0.26 nM ($n = 3$) for the inhibitory effect of ICI 118551 and 609 ± 84.1 nM ($n = 3$) for the inhibitory effect of metoprolol on the response of I_{Ca} to dobutamine. This suggests that, in the range of concentrations used (10 nM to 100 μM), dobutamine activates I_{Ca} through a single population of receptors which bind ICI 118551 in the low nanomolar range of concentrations and metoprolol in the high nanomolar range of concentrations.

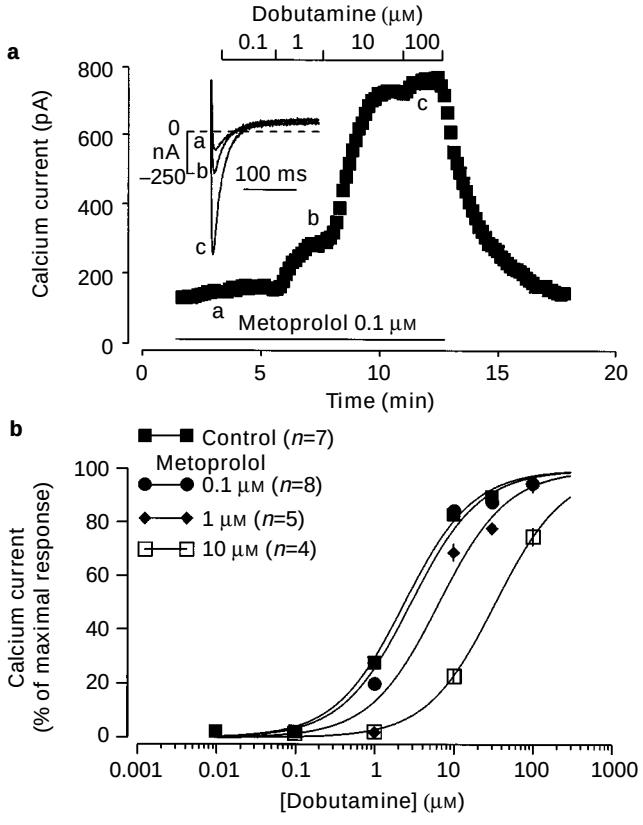

Figure 6 Effect of dobutamine and metoprolol on $I_{Ca.}$ (a) A frog ventricular myocyte was initially superfused with control external solution in the presence of 0.1 μM metoprolol. During the periods indicated, the cell was successively exposed to four increasing concentrations of dobutamine (0.1, 1, 10 and 100 μM). The current traces shown in the inset were recorded at the times indicated by the corresponding letters on the main graph. (b) Concentration-response curve for the stimulating effect of dobutamine on I_{Ca}. The effect of increasing concentrations of dobutamine on I_{Ca} were obtained in several experiments performed in the absence of metoprolol (Control, same data as in Figure 5c) or in the presence of 100 nM (as in (a)), 1 μM or 10 μM metoprolol. The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values were: 2.40 μM and 265% in control; 2.93 μM and 260% with 100 nM metoprolol; 6.54 μM and 91% with 1 μM metoprolol; 33.7 μM and 754% with 10 μM metoprolol. The data were normalized to E_{max} and are presented as percentage of the maximal response to dobutamine.

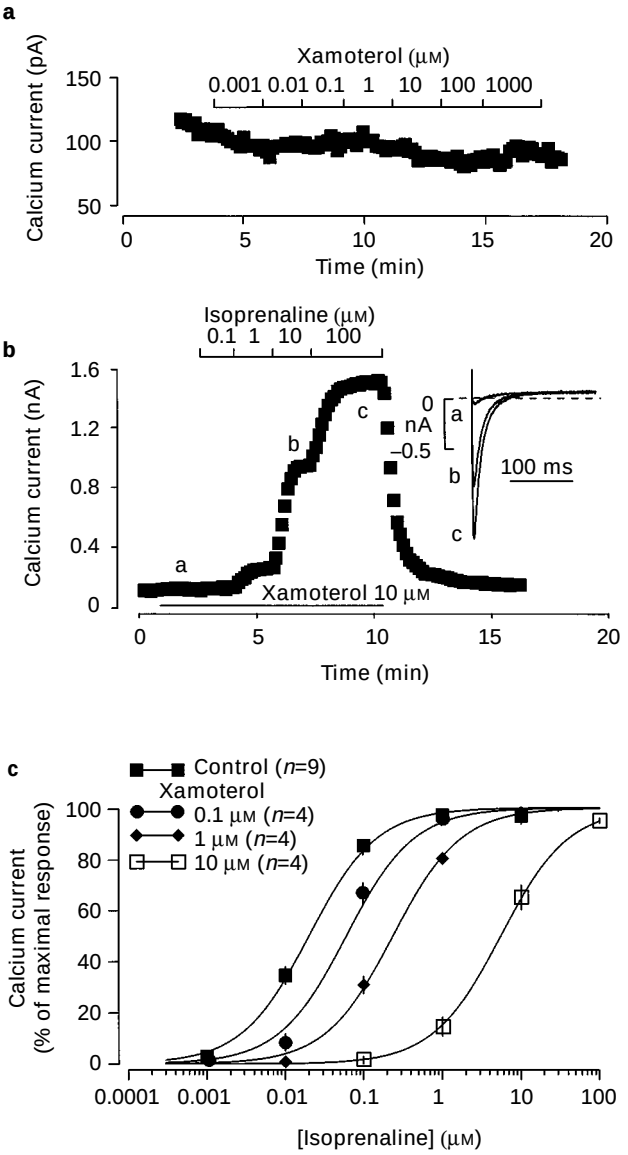

Figure 7 Effect of xamoterol and isoprenaline on $I_{Ca.}$ (a) A frog ventricular myocyte was initially superfused with control external solution. During the periods indicated, the cell was successively exposed to seven increasing concentrations of xamoterol (0.001, 0.01, 0.1, 1, 10, 100 and 1000 μM). (b) A frog ventricular myocyte was initially superfused with control external solution. During the periods indicated, the cell was successively exposed 10 μM xamoterol and, then, to four increasing concentrations of isoprenaline (0.1, 1, 10 and 100 μM). The current traces shown in the inset of (b) were recorded at the times indicated by the corresponding letters on the main graphs. (c) Concentration-response curve for the stimulating effect of isoprenaline on I_{Ca}. The effect of increasing concentrations of isoprenaline on I_{Ca} were obtained in several experiments performed in the absence of xamoterol (Control, same data as in Figure 1c) or in the presence of 0.1 μM, 1 μM or 10 μM xamoterol (as in (b)). The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values were: 20.0 nM and 597% in control; 60.0 nM and 1098% with 0.1 μM xamoterol; 240 nM and 730% with 1 μM xamoterol; 5.51 μM and 905% with 10 μM xamoterol. The data were normalized to E_{max} and are presented as percentage of the maximal response to isoprenaline.

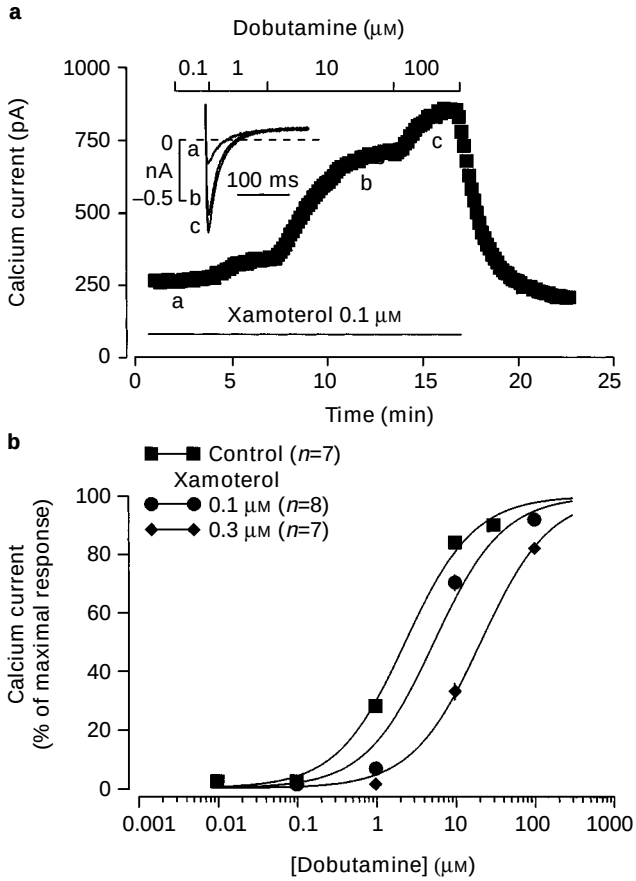

Figure 8 Effect of dobutamine and xamoterol on $I_{Ca.}$ (a) A frog ventricular myocyte was initially superfused with control external solution in the presence of 100 nM xamoterol. During the periods indicated, the cell was successively exposed to four increasing concentrations of dobutamine (0.1, 1, 10 and 100 μM). The current traces shown in the inset were recorded at the times indicated by the corresponding letters on the main graph. (b) Concentration-response curve for the stimulating effect of dobutamine on I_{Ca}. The effect of increasing concentrations of dobutamine on I_{Ca} were obtained in several experiments performed in the absence of metoprolol (Control, same data as in Figure 5c). or in the presence of 0.1 μM (as in (a)) or 0.3 μM xamoterol. The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values were: 2.40 μM and 265% in control; 5.52 μM and 191% with 0.1 μM xamoterol; 21.2 μM and 211% with 0.3 μM xamoterol. The data were normalized to E_{max} and are presented as percentage of the maximal response to dobutamine.

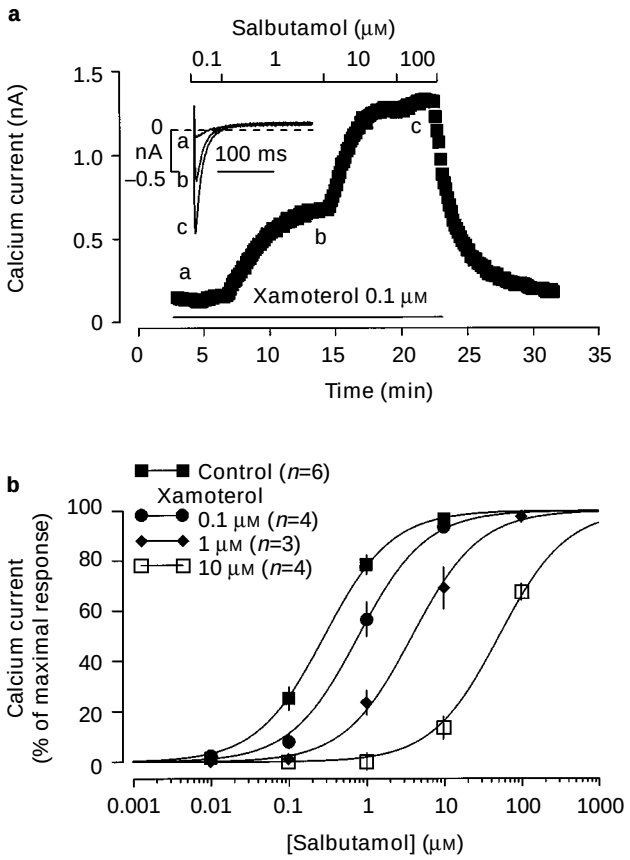

Figure 9 Effect of salbutamol and xamoterol on $I_{Ca.}$ (a) A frog ventricular myocyte was initially superfused with control external solution in the presence of 100 nM xamoterol. During the periods indicated, the cell was successively exposed to four increasing concentrations of salbutamol (0.1, 1, 10 and 10 μM). The current traces shown in the inset were recorded at the times indicated by the corresponding letters on the main graph. (b) Concentration-response curve for the stimulating effect of salbutamol on I_{Ca}. The effect of increasing concentrations of salbutamol on I_{Ca} were obtained in several experiments performed in the absence of metoprolol (Control, same data as in Figure 3c) or in the presence of 0.1 μM (as in (a)), 1 μM or 10 μM xamoterol. The points show the mean and vertical lines s.e.mean of the number of cells indicated. EC_{50} and E_{max} values were: 290 nM and 512% in control; 0.80 μM and 697% with 0.1 μM xamoterol; 3.91 and 542% with 1 μM xamoterol; 51.8 and 360% with 10 μM xamoterol. The data were normalized to E_{max} and are presented as percentage of the maximal response to salbutamol.

Regulation of I_{Ca} *by the* β_1*-adrenoceptor partial agonist xamoterol*

We also examined the effects of a β_1-adrenoceptor partial agonist, xamoterol, on I_{Ca} in frog ventricular myocytes. Surprisingly, xamoterol (from 1 nM to 1 mM), unlike dobutamine (Figure 5), did not produce any stimulating effect on I_{Ca} (Figure 7a). On the contrary, xamoterol antagonized the stimulating effect of the three other β-adrenoceptor agonists tested. Indeed, the individual experiments in Figures 7b, 8a and 9a show that the presence of xamoterol (10 μM in Figure 7b, 0.1 μM in Figure 8a, 0.1 μM in Figure 9a) reduced the sensitivity of I_{Ca} to isoprenaline (compare Figures 7b and 1a), dobutamine (compare Figures 8a and 5a) and salbutamol (compare Figures 9a and 3a). The mean data points in Figure 7c show the response of I_{Ca} to isoprenaline alone (■, taken from Figure 1c) or in the presence of 100 nM, 1 μM and 10 μM xamoterol. The mean data points in Figure 8b show the response of I_{Ca} to dobutamine alone (■, taken from Figure 5c) or in the presence of 100 nM and 300 nM xamoterol. The mean data points in Figure 9b show the response of I_{Ca} to salbutamol alone (■, taken from Figure 3c) or in the presence of 100 nM, 1 μM and 10 μM xamoterol. In all cases, the dose-response curves for the response of I_{Ca} to a β-adrenoceptor agonist were progressively shifted to higher concentrations of the agonist when increasing concentrations of xamoterol were used. A Schild regression analysis (see Methods) of the curves in Figures 7c and 9b allowed us to determine Schild coefficients of, respectively, 0.92 and 0.99, and K_B values of 59.1 ± 16.4 ($n=3$) and 64.4 ± 7.84 nM ($n=3$), for the inhibitory effect of xamoterol on the response of I_{Ca} to isoprenaline (Figure 7c) and salbutamol (Figure 9b). Since dobutamine produced deleterious effects on I_{Ca} at concentrations >100 μM, we were unable to obtain sufficient data to perform a satisfactory Schild regression analysis with dobutamine in the presence of high concentrations of xamoterol (Figure 8b). However, if we assume that there is competitive interaction between dobutamine and xamoterol (i.e. a Schild coefficient of 1), then the dose-response curves of Figure 8b lead to a K_B value of 57.7 ± 13.6 nM ($n=2$) for the inhibitory effect of xamoterol on the response of I_{Ca} to dobutamine. Since this value is very

similar to the K_B values determined from the inhibitory effect of xamoterol on the response of I_{Ca} to isoprenaline (Figure 7) and salbutamol (Figure 9), our data suggest that xamoterol binds to a single population of β-adrenoceptors with an affinity of $\approx$ 60 nM. Binding of xamoterol resulted in an antagonistic effect on the β-adrenoceptor regulation of I_{Ca}.

Discussion

In the present study, we examined the effect of isoprenaline and several selective β_1- and β_2-adrenoceptor agonists and antagonists on the L-type Ca^{2+} current (I_{Ca}) in frog ventricular myocytes. With the exception of xamoterol, which is a partial β_1-agonist, all the agonists used (isoprenaline (non-selective), salbutamol (β_2-selective), dobutamine (β_1-selective)) produced a dose-dependent stimulation of I_{Ca}. In a previous study of ours, we have shown that zinterol, another β_2-adrenoceptor agonist, also stimulates frog I_{Ca} in a dose-dependent manner (Skeberdis *et al*., 1996). While salbutamol and zinterol (Skeberdis *et al*., 1996) increased I_{Ca} with a similar efficacy to isoprenaline, the efficacy of dobutamine was only half that of isoprenaline. EC_{50} values were calculated from the dose-response curves for the stimulating effects of the agonists on I_{Ca} which allowed us to determine the potency rank order for these agonists: zinterol (2.2 nM, Skeberdis *et al*., 1996) > isoprenaline (20 nM) > salbutamol (290 nM) > dobutamine (2.4 μM) >> xamoterol. Whatever the agonist used to stimulate I_{Ca}, their effect was antagonized by ICI 118551 (β_2-antagonist) and metoprolol (β_1-antagonist), as well as by xamoterol. Competition curve analysis by use of the Schild equation yielded a Schild coefficient of 1 (0.9 – 1.2) in all experimental conditions. This suggests that all the β-adrenoceptor agonists and antagonists tested competed on a single population of receptors. Equilibrium dissociation constants (K_B) for the receptor-antagonist complex were calculated from the Schild equation in each experimental condition. This allowed us to determine a rank order of potency for the different antagonists: ICI 118551 (2 – 4 nM) > xamoterol (60 nM) > metoprolol (200 – 600 nM). We conclude that a single population of receptors is involved in the β-adrenoceptor regulation of I_{Ca} in frog ventricular myocytes. The pharmacological profiles obtained indicate that this population of receptors fits the criteria set for the β_2-adrenoceptor subtype in mammalian tissue.

Although initial competitive binding studies concluded that β_2-adrenoceptors are not present in purified ventricular myocytes from mammalian hearts (Lau *et al*., 1980; Buxton & Brunton, 1985; Freissmuth *et al*., 1986), more recent studies have clearly established the presence of these receptors in ventricular myocytes from several mammals, such as rats (Kuznetsov *et al*., 1995; Cerbai *et al*., 1995), dogs (Murphree & Saffitz, 1988), baboons (Cui *et al*., 1996) and man (del Monte *et al*., 1993). However, the β_1/β_2 ratio may vary somewhat from one study to the other in a given animal species (e.g. 80/20 to 92/8 in rat myocytes: Cerbai *et al*., 1995; Kuznetsov *et al*., 1995; Cui *et al*., 1996) and from one species to the other (e.g. 85/15 in dog: Murphree & Saffitz, 1988; 59/41 in baboon: Cui *et al*., 1996). The β_1/β_2 ratio may also vary depending on the pathophysiological state (Brodde, 1993; Ihl-Vahl *et al*., 1996), the age (White *et al*., 1994; Cerbai *et al*., 1995) or the developmental stage (Kuznetsov *et al*., 1995; for reviews, see Stiles *et al*., 1984; Brodde, 1991; 1993; Hieble & Ruffolo, 1991 and references therein). Finally, the proportion of β_2-adrenoceptors was shown to be somewhat larger in atrial compared to ventricular tissues (Carlsson *et al*., 1977; Hedberg *et al*., 1980; Molenaar & Summers, 1987), and more so in human where β_2-adrenoceptors may account for 35 – 50% of the total number of β-receptors (Robberecht *et al*., 1983; Brodde, 1991; Hieble & Ruffolo, 1991). The latter finding may explain why β_2-adrenoceptor agonists exert preferentially positive chronotropic rather than inotropic effects in man (Brodde, 1991).

Unlike in mammals, the β-adrenoceptors in the frog heart are composed of a majority of β_2-subtype. Radioligand binding experiments and competition curve analysis in frog myocardial membrane preparations have shown that the proportion of β_2-receptors may vary from 50% to 100% of the total population of β-receptors depending on the specific antagonist used to displace the radiolabelled ligand and the individual cell preparation (Hancock *et al*., 1979; Port *et al*., 1992). Measurements of force and rate of spontaneous contractions in the isolated heart (Lands *et al*., 1969) or in isolated auricles (Stene-Larsen & Helle, 1978) of the frog have shown that the inotropic and chronotropic responses to various sympathomimetic amines are essentially mediated by β_2-adrenoceptors. This suggests that, although present in the sarcolemmal membrane, β_1-adrenoceptors may not be functionally coupled to the effectors involved in the contractile and electrical activities of the frog heart. Since the L-type Ca^{2+} channel current (I_{Ca}) is one of the main actors in the excitation-contraction coupling of the frog heart, it was of interest to examine how this current is regulated by the two populations of β-adrenoceptors receptors.

Selective agonists of β_2-adrenoceptors were shown earlier to increase I_{Ca} in mammalian cardiac myocytes, such as guinea-pig atrial myocytes (Iijima & Taira, 1989), rat (Xiao & Lakatta, 1993; Cerbai *et al*., 1995), dog (Altschuld *et al*., 1995) and guinea-pig ventricular myocytes (Wang & Pelzer, 1995; but see Iijima & Taira, 1989). The maximal stimulating effect of these agonists on I_{Ca} varied from 30% in guinea-pig (Wang & Pelzer, 1995) to 100% in rat ventricular myocytes (Xiao & Lakatta, 1993) of the effect of isoprenaline. In frog cardiomyocytes, we found that selective activation of β_2-adrenoceptors with salbutamol (this study) or with zinterol (Skeberdis *et al*., 1996) accounted for 100% of the isoprenaline response in frog ventricular myocytes. By comparison, the maximal stimulant effect of the β_1-agonist dobutamine on I_{Ca} was only about half that of isoprenaline, while xamoterol had no stimulant effect at all. Thus, the agonists acting more selectively on the β_2-adrenoceptors (zinterol, salbutamol) had a higher potency and efficacy in stimulating frog I_{Ca} than the agonists acting on the β_1-receptors (dobutamine, xamoterol). Moreover, the stimulating effect of dobutamine on I_{Ca} was probably mediated by activation of β_2-, and not β_1-, receptors. Indeed, dobutamine increased I_{Ca} with an EC_{50} of 2.4 μM, which is two orders of magnitude higher than the concentration at which it binds selectively to mammalian β_1-receptors (Brodde, 1991). Moreover, the stimulating effect of dobutamine on I_{Ca} was strongly antagonized by ICI 118551 (K_B = 2.8 nM) while it was only weakly antagonized by metoprolol (K_B = 609 nM). While at a concentration of 2.8 nM ICI 118551 remains a highly selective antagonist of β_2-receptors (Bylund *et al*., 1994), metoprolol is not selective for β_1-receptors anymore when its concentration approaches the micromolar range (Bylund *et al*., 1994). Since ICI 118551 and metoprolol antagonized the stimulant effects of isoprenaline, dobutamine and salbutamol on I_{Ca} with the same K_B values (2 – 4 nM for ICI 118551, 200 – 600 nM for metoprolol) and the same Schild coefficient (equal to 1), it is likely that these agonists and antagonists produced their effects by binding exclusively to β_2-adrenoceptors. However, the frog β_2-receptor may differ somewhat from its mammalian counterpart since salbutamol increased I_{Ca} with an EC_{50} of 290 nM, which was about 10 fold higher than the concentration required to activate β_2-receptors in mammalian preparations (Bylund *et al*., 1994). On the contrary, the frog cardiac β_2-receptor appears to be about 50 times more sensitive to zinterol than the mammalian cardiac β_2-receptor (EC_{50} = 2.2 nM in frog myocytes, Skeberdis *et al*., 1996; EC_{50} = 1 μM in rat myocytes, Xiao & Lakatta, 1993). Another peculiarity of the frog cardiac β_2-receptor is its relatively high sensitivity to xamoterol. While xamoterol alone had no effect on I_{Ca}, this partial β_1-agonist antagonized the stimulant effect of isoprenaline, dobutamine and salbutamol with a similar K_B value ($\approx$ 60 nM) independently of which agonist was used to stimulate I_{Ca}. The Schild coefficient was equal to 1 in all cases, demonstrating that xamoterol competed with the agonist on a single population of

receptors. Thus, xamoterol is an antagonist of frog β_2-receptors and binds to these receptors with an affinity which is comparable to that at which it binds to mammalian β_1-receptors (Brodde, 1991; Bylund *et al*., 1994).

The major finding of our study is that the β-adrenoceptor regulation of I_{Ca} in frog cardiac myocytes is exclusively mediated by β_2-adrenoceptors. This observation is consistent with earlier functional data obtained on whole heart (Lands *et al*., 1969) or multicellular cardiac preparations of the frog (Stene-Larsen & Helle, 1978). This finding is also consistent with the fact that the sympathetic nerves carry adrenaline in the frog (Loewi, 1936) and the β_2-receptor is, by definition, an 'adrenaline receptor' (Stene-Larsen & Helle, 1978). However, there is evidence for the presence of β_1-receptors in frog cardiac myocytes (Hancock *et al*., 1979; Port *et al*., 1992), and their relative proportion is comparable to that of β_2-receptors in mammalian cardiac myocytes (Hieble & Ruffolo, 1991). Interestingly, β_2-receptors have been shown to be more tightly coupled to the adenylyl cyclase than β_1-receptors (Waelbroeck *et al*., 1983; Bristow *et al*., 1989; Green *et al*., 1992; Levy *et al*., 1993). This may explain why β_2-adrenoceptors, although present in a minority in mammalian cardiac myocytes, are efficiently coupled to I_{Ca} and contraction in these preparations (Xiao & Lakatta, 1993; Xiao *et al*., 1994; 1995; Altschuld *et al*., 1995; Kuznetsov *et al*., 1995; Skeberdis *et al*., 1996). In frog myocytes where they are present in a majority, the coupling of β_2-receptors to adenylyl cyclase may overwhelm that of β_1-receptors and nullify their contribution to the stimulation of I_{Ca}. Alternatively, β_2-receptors may lead to an elevation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in a compartment more efficiently coupled to L-type Ca^{2+} channels than β_1-receptors (Jurevičius & Fischmeister, 1996; Skeberdis *et al*., 1996). Further experiments are needed to examine these hypotheses and to determine the function of β_1-receptors in the frog heart.

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