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Beta-2 Adrenergic Activation of L-Type Ca^{++} Current in Cardiac Myocytes¹

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ABSTRACT

The whole-cell patch-clamp and intracellular perfusion techniques were used for studying the effects of a *beta*-2 adrenergic receptor activation on the L-type Ca current (I_{Ca}) in frog ventricular myocytes. The *beta*-2 adrenergic agonist zinterol increased I_{Ca} in a concentration-dependent manner with an EC_{50} (i.e., the concentration of zinterol at which the response was 50% of the maximum) of 2.2 nM. The effect of zinterol was essentially independent of the membrane potential. The stimulatory effect of zinterol was competitively antagonized by ICI 118,551, a *beta*-2 adrenergic antagonist. The maximal stimulatory effect of zinterol was comparable in amplitude to the effect of a saturating concentration (1 or 10 μ M) of isoprenaline, a nonselective *beta* adrenergic agonist. Moreover, 3-isobutyl-1-methylxanthine (100 μ M), a nonselective phosphodiesterase inhibitor, or forskolin (10 μ M), a direct activator of adenylyl cyclase, had no additive effects in the presence of 0.1 μ M

zinterol. Zinterol had a long lasting action on frog I_{Ca} because after washout of the drug, I_{Ca} returned to basal level with a time constant of 17 min. An application of acetylcholine (1 μ M) during this recovery phase promptly reduced I_{Ca} back to its basal level suggesting a persistent activation of adenylyl cyclase due to a slow dissociation rate constant of zinterol from its receptor. Zinterol also increased I_{Ca} in rat ventricular and human atrial myocytes, and the maximal effect was obtained at 10 and 1 μ M, respectively. In all three preparations, intracellular perfusion with 20 μ M PKI(15-22), a highly selective peptide inhibitor of cAMP-dependent protein kinase, completely antagonized the stimulatory effect of zinterol on I_{Ca} . We conclude that *beta*-2 adrenergic receptor activation produces a strong increase in I_{Ca} in frog, rat and human cardiac myocytes which is due to stimulation of adenylyl cyclase and activation of cAMP-dependent phosphorylation.

Beta-1 and *beta*-2 adrenergic receptors 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.*, 1991). However, the relative amount of each receptor subtype as well as the postreceptor cellular signaling 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 and Ruffolo, 1991 and refs therein). Competitive radioligand binding studies performed in membranes from homogenized hearts have shown that only 20 to 30% of the total *beta* adrenergic receptors are of the *beta*-2 subtype in adult mammalian ventricular tissue (Stiles

et al., 1984; Brodde, 1991; Hieble and Ruffolo, 1991). This number is even further reduced when purified cardiac myocytes rather than homogenized tissues are used (Freissmuth *et al.*, 1986; Lau *et al.*, 1980; Buxton and Brunton, 1985; Kuznetsov *et al.*, 1995; Cerbai *et al.*, 1995). Yet, selective activation of *beta*-2 adrenergic receptors produces a large increase in the amplitude of contraction in intact mammalian cardiac muscle (Cerbai *et al.*, 1990; Lemoine and Kaumann, 1991; Brodde, 1991) as well as in isolated ventricular myocytes (del Monte *et al.*, 1993; Xiao and Lakatta, 1993; Xiao *et al.*, 1994; 1995; Altschuld *et al.*, 1995; Kuznetsov *et al.*, 1995). When compared to the effect produced by nonselective *beta* adrenergic receptor agonists such as isoprenaline, the *beta* 2-response may represent 25 to 100% of the isoprenaline response (Xiao and Lakatta, 1993; Altschuld *et al.*, 1995). This suggests that the two receptors may differ in their signaling cascade or in the post-receptor amplification mechanisms. In that regard, *beta*-2 adrenergic receptors were shown to be more tightly coupled to the adenylyl cyclase system than *beta*-1 receptors (Waelbroeck *et al.*, 1983; Bris-

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ABBREVIATIONS: ACh, acetylcholine; CGP 20712A, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoro-methyl-2-imidazolyl)phenoxy]-2-propranolol; IBMX, 3-isobutyl-1-methylxanthine; I_{Ca} , L-type calcium current; ICI 118551, erythro(±)-1-[(7-methylindane-4-yl)oxy]-3-isopropylamino-2-butanol; cAMP, cyclic adenosine 3',5'-monophosphate.

tow *et al.*, 1989; Green *et al.*, 1992; Levy *et al.*, 1993). Surprisingly, however, the positive inotropic effect mediated by a *beta*-2 adrenergic receptor agonists is not always correlated with changes in cAMP concentration (Xiao *et al.*, 1994; Altschuld *et al.*, 1995; Kuznetsov *et al.*, 1995), nor is it always accompanied by a positive lusitropic effect that should result from a cAMP-dependent phosphorylation of phospholamban and/or troponin I (Lemoine and Kaumann, 1991; Borea *et al.*, 1992; Xiao *et al.*, 1994). These discrepancies have led the authors to postulate that *beta*-2 adrenergic receptors, unlike *beta*-1 receptors, may be coupled to other mechanisms in addition to the adenylyl cyclase system (Lemoine and Kaumann, 1991; Borea *et al.*, 1992; Xiao and Lakatta, 1993; Xiao *et al.*, 1994; 1995; Kuznetsov *et al.*, 1995). In that regard, *beta*-2 adrenergic receptors have been shown recently to be functionally coupled to pertussis toxin-sensitive G proteins in rat ventricular myocytes (Xiao *et al.*, 1995).

Because the positive inotropic effect of *beta* adrenergic agonists is generally associated with a stimulation of the I_{Ca} (Hartzell, 1988; McDonald *et al.*, 1994), it was of interest to examine the respective contribution of *beta*-1 and *beta*-2 adrenergic receptors in this effect and to compare the cellular mechanisms involved. Selective *beta*-2 adrenergic receptor activation was found to produce a stimulation of I_{Ca} in guinea pig atrial myocytes (Iijima and Taira, 1989), and in rat (Xiao and Lakatta, 1993; Xiao *et al.*, 1994; 1995; Cerbai *et al.*, 1995), guinea pig (Wang and Pelzer, 1995; but see Iijima and Taira, 1989), dog (Altschuld *et al.*, 1995) and frog ventricular myocytes (Skeberdis *et al.*, 1997). The signaling cascade involved in this stimulation has been studied in detail only in rat (Xiao and Lakatta, 1993; Xiao *et al.*, 1994; 1995) and dog ventricular myocytes (Altschuld *et al.*, 1995) using zinterol as a selective *beta*-2 adrenergic agonist (Minneman *et al.*, 1979). It was concluded that stimulation of I_{Ca} by zinterol was not mediated by cAMP-dependent mechanisms. This conclusion was based on phenomenological differences between the effects of zinterol and *beta*-1 adrenergic agonists on I_{Ca} , cytoplasmic Ca^{++} concentration transients, and cell shortening, and their respective correlation and lack of correlation with changes in the concentration of cAMP (Xiao and Lakatta, 1993; Xiao *et al.*, 1994; 1995; Altschuld *et al.*, 1995).

The frog heart is a rather unique preparation in which the *beta* adrenergic receptor population is composed of a majority ($\approx 80\%$) of *beta*-2 subtype (Hancock *et al.*, 1979; Hieble and Ruffolo, 1991). Moreover, a recent competition curve analysis of the effects of various *beta*-1 and *beta*-2 agonists and antagonists on I_{Ca} led to the findings that only *beta*-2 adrenergic receptors are coupled to I_{Ca} in this preparation (Skeberdis *et al.*, 1997). Thus, we anticipated that this preparation might be valuable in getting some additional insights on the coupling mechanisms between these receptors and the L-type Ca^{++} channels. For this reason, we investigated the effects of zinterol on I_{Ca} in whole-cell patch-clamped single frog ventricular myocytes. For comparison, we also examined the effect of zinterol on I_{Ca} in rat ventricular and human atrial myocytes and tested the hypothesis that a cAMP-independent mechanism may be involved in these effects by directly dialyzing the myocytes with a peptide inhibitor of cAMP-dependent protein kinase. Preliminary results have appeared in abstract form (Skeberdis *et al.*, 1996).

The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J no. L358, December 18, 1986) and the French decree no. 87/748 of October 19, 1987 (J Off République Française, October 20, 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 (no. 04226, April 12, 1991). All protocols for obtaining human cardiac tissue were approved by the ethics committee of our institution (GREBB, Hôpital de Bicêtre, Université de Paris-Sud). [h]Experimental Solutions and Drugs

For the preparation of frog ventricular cells, the ionic composition of Ca^{++} -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; 1 mg.ml⁻¹ fatty acid-free bovine serum albumin; 50 I.U.ml⁻¹ penicillin; 50 μ g.ml⁻¹ streptomycin; pH 7.4 maintained with 95% O₂, 5% CO₂. Storage Ringer solution was Ca^{++} -free Ringer solution to which was added 0.9 mM CaCl₂ and 10 μ l ml⁻¹ nonessential and essential amino acid and vitamin solution (minimal essential medium 100x). Dissociation medium was composed of Ca^{++} -free Ringer solution to which was added 0.2 mg.ml⁻¹ trypsin, 0.14 mg.ml⁻¹ collagenase (Yakult, Tokyo, Japan), and 10 μ l.ml⁻¹ M199 medium. For the preparation of rat and human cardiomyocytes, the ionic composition of the Ca^{++} -free Tyrode solution was (mM): NaCl 117; KCl 5.7; $NaHCO_3$ 4.4; KH_2PO_4 1.5; $MgCl_2$ 1.7; HEPES 21.1; creatine 10; D-glucose 11.7; taurine 20; pH adjusted to 7.1 with NaOH. For electrophysiology, the control external solution contained (in mM): NaCl 107; HEPES 10; CsCl 20 (for frog and rat) or 40 (for human); $NaHCO_3$ 4; NaH_2PO_4 0.8; $MgCl_2$ 1.8; CaCl₂ 1.8; D-glucose 5; sodium pyruvate 5; tetrodotoxin 3×10^{-4} (for frog) or 6×10^{-3} (for rat and human); pH 7.4 adjusted with NaOH. Patch electrodes (0.6–2.0 Mohms) 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; CaCl₂ 0.062 (pCa 8.5); HEPES 10; pH 7.1 (frog) or 7.3 (rat and human) adjusted with CsOH. Collagenase type IV and protease type XXIV used for human atrial cells dissociation were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Collagenase type A for rat cardiac myocyte dissociation and fetal calf serum were from Boehringer Mannheim (Germany). Collagenase for frog ventricular myocyte dissociation was from Yakult. Delbecco's minimal essential medium was obtained from Gibco-BRL. Tetrodotoxin was from Latoxan (Rosans, France). Zinterol was a generous gift of Bristol Myers Squibb (Evansville, IN). CGP 20712A was a generous gift from Novartis Pharma AG (Basel, Switzerland). ICI 118551 was from Tocris Cookson (Bristol, UK). All other drugs were from Sigma Chemical Co. (St. Louis, MO). All drugs tested in patch-clamp experiments were solubilized in experimental solutions just before application onto the cell studied, *i.e.*, only fresh solutions were tested.

Frog Ventricular Myocytes

Ventricular cells were enzymatically dispersed from frog (*Rana esculenta*) heart, by a combination of collagenase (Yakult) and trypsin (type III or XIII, Sigma) as described (Fischmeister and 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 hr after dissociation). In some isolations, amino acids were omitted from the dissociation and storage solutions, with no change in the results.

Human Atrial Myocytes

Surgery. Specimens of right atrial appendages were obtained from two patients (one male aged 44, one female aged 73) undergoing heart surgery for coronary artery disease at the Hôpital Marie-Lannelongue, Le Plessis-Robinson, France. Both patients received a pharmacological pretreatment composed of a Ca-channel blocker (diltiazem), a β -adrenergic antagonist (atenolol) and a NO-donor

(molsidomine). In addition to these medications, both patients received sedatives, anesthesia, and antibiotics. Dissociation of the cells was realized immediately after surgery.

Cell dissociation. Myocytes were isolated as described previously (Kirstein *et al.*, 1995; Rücker-Martin *et al.*, 1993). The cell suspension was filtered, centrifuged and the pellet resuspended in DMEM supplemented with 10% fetal calf serum, nonessential amino acids, 1 nM insulin and antibiotics (penicillin, 100 IU/ml and streptomycin, 0.1 μ g/ml). For patch-clamp experiments 100 to 200 μ l of this cell suspension were put in a Petri dish containing control external solution.

Rat Ventricular Myocytes

Rat cardiomyocytes were obtained by retrograde perfusion from hearts of male Wistar rats (180–220 g) as previously described (Pucéat *et al.*, 1990) with slight modifications. Briefly, the rats were subjected to anesthesia by intra-peritoneal injection of urethane and the hearts were rapidly excised. The hearts were perfused retrogradely at a constant flow and at 37°C by an oxygenated Ca-free Tyrode solution during 5 min followed by 1 hr perfusion with the same solution containing 1 mg/ml collagenase A (Boehringer-Mannheim, Indianapolis, IN) and 300 μ M EGTA (free Ca^{++} concentration adjusted to 20 μ M). The ventricles and atria were then separated. Ventricles were chopped finely and agitated gently to dissociate individual cells. The resulting cell suspension was filtered and the cells settled down. The supernatant was discarded and cells resuspended a further four times in Tyrode solution containing a progressively increasing calcium concentration. The myocytes were maintained at 37°C until use.

Electrophysiological Experiments

The whole-cell configuration of the patch-clamp technique was used to record the high-threshold calcium current (I_{Ca}) on Ca^{++} -tolerant frog ventricular, human atrial and rat ventricular myocytes. In the routine protocols the cells were depolarized every 8 sec from a holding potential of -80 to 0 mV for 200 or 400 msec. In human and rat cardiomyocytes, the test pulse to 0 mV was preceded by a short pre-pulse (50 msec) to -50 mV. The pre-pulse and/or the application of tetrodotoxin (0.3 μ M for frog, 6 μ M for human and rat) was used to eliminate fast sodium currents. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+ . For the determination of current-voltage relationships for I_{Ca} (see fig. 2A) and I_{Ca} inactivation curve (see fig. 2B) in frog ventricular myocytes, a double pulse voltage-clamp protocol was used (Argibay *et al.*, 1988). Briefly, every 4 sec, the membrane potential of the cell, which was normally maintained at its holding value of -80 mV, experienced the following sequence of events: different potentials values ranging from -100 to +100 mV for 200 msec, -80 mV for 3 msec and 0 mV for 200 msec (see inset in fig. 2B). In few experiments in rat, the holding potential was maintained at -60 mV with no difference in results. Voltage-clamp protocols were generated by a challenger/09-VM programmable function generator (Kinetic Software, Atlanta, GA). The cells were voltage-clamped using a patch-clamp amplifier (model RK-400; Bio-Logic, Claix, France). Currents were sampled at a frequency of 10 kHz using a 16-bit analogue-to-digital converter (PCL816, Advantech France, Levallois Perret, France) connected to a PC compatible micro computer.

Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 300- μ m inner diameter capillary tubings flowing at a rate of \approx 50 μ l/min (Fischmeister and Hartzell, 1986). Changes in extracellular solutions were automatically achieved using a rapid solution changer (RSC100, Bio-Logic, Claix, France). Drug-containing solutions were applied to the interior of the cell by a system that permitted perfusion of the patch-electrode with different solutions (Fischmeister and Hartzell, 1987). The dead volume of the intracellular perfusion system was such that 30 to 50 sec were needed for an air bubble to travel from one end to

the other end of the system. Perfusion time depended on patch-electrode resistance, access to the cell and the molecular weight of the molecule tested. Typically, with the cAMP-dependent protein kinase inhibitor peptide PKI(15–22) (MW = 2222.4), the beginning of I_{Ca} inhibition occurred 3 to 5 min after the beginning of intracellular perfusion with this compound (see *e.g.*, fig. 7). 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

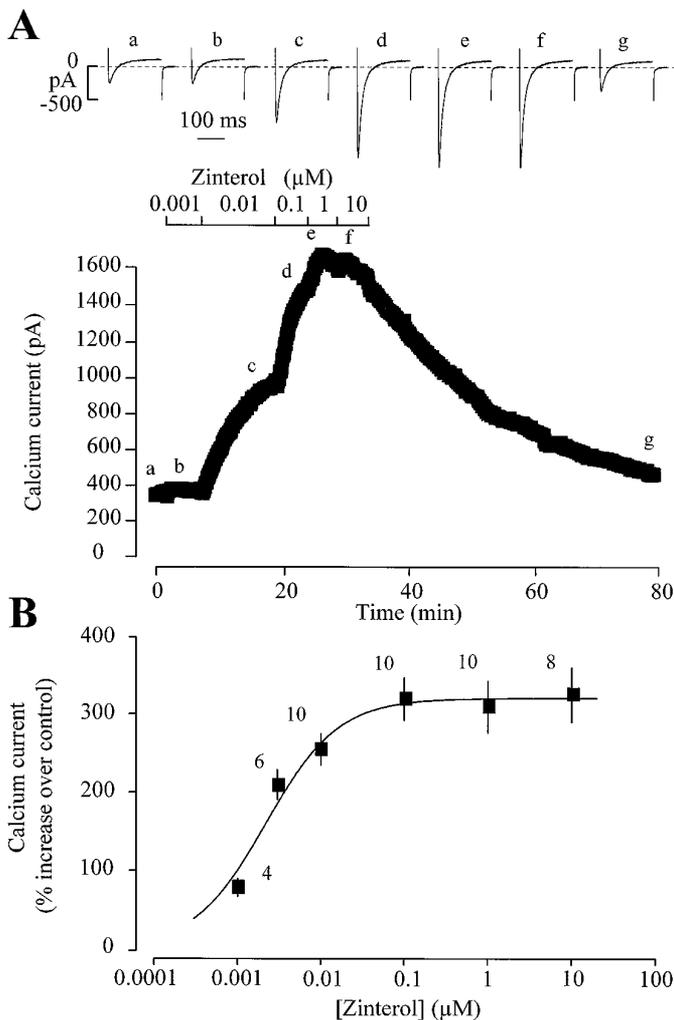
The maximal amplitude of whole-cell I_{Ca} was measured as previously described (Fischmeister and Hartzell, 1986; Argibay *et al.*, 1988). Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was made possible by programming a PC-compatible 486/66 microcomputer in Assembling language (Borland, USA) to determine, for each membrane depolarization, peak and steady-state current values (Fischmeister and Hartzell, 1986). The results are expressed as mean \pm S.E.M. Differences between means were tested for statistical significance by Student's *t* test. In the text, the "basal" condition refers to the absence of *beta* adrenergic agonist. In the case of single applications, the effect of a compound is referred to as the percent variation over the basal amplitude of I_{Ca} .

Results

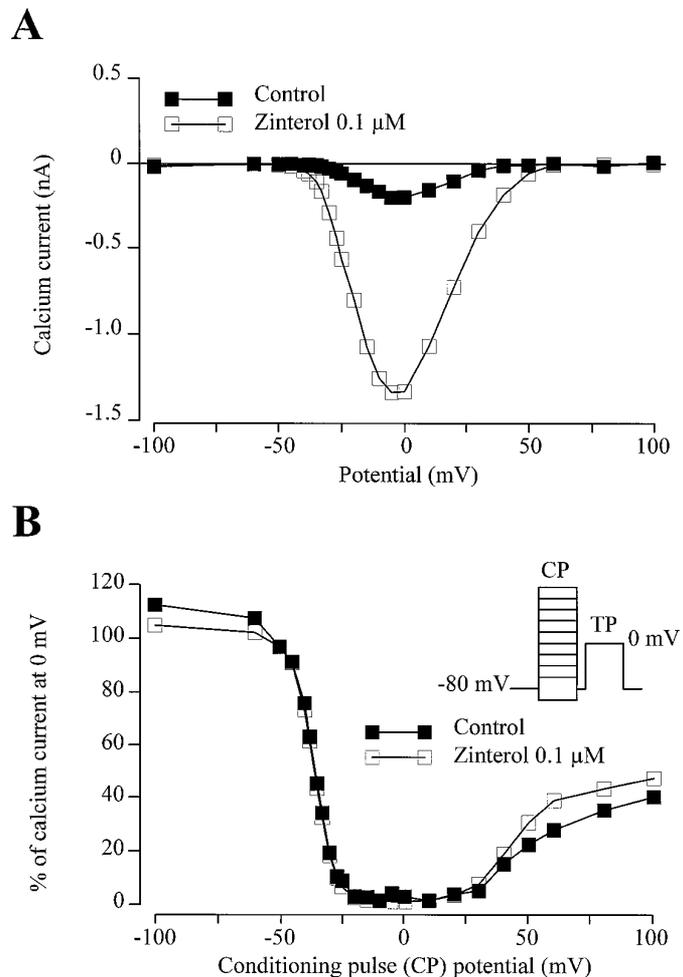
Zinterol stimulates frog I_{Ca} . A typical experiment using zinterol as a selective *beta*-2 adrenergic agonist in a frog ventricular myocyte is shown in figure 1A. I_{Ca} was measured every 8 sec by depolarizing the cell over a period of 200 msec to 0 mV from a holding potential of -80 mV. Zinterol produced a clear increase in I_{Ca} at concentrations > 1 nM. At 10 nM, the current increased more than 2-fold, and a maximal stimulation of \approx 300% was reached between 100 nM and 1 μ M zinterol. Upon washout of the drug, I_{Ca} returned progressively to its basal amplitude. Figure 1B shows the results of several similar experiments as the one shown in figure 1A. The data are presented as a dose-response curve for the effect of zinterol on I_{Ca} . The dose-response curve was fitted using a nonlinear least-mean-squares regression of the means to the Michaelis equation. The concentration of zinterol (EC_{50}) required for half-maximal stimulation of I_{Ca} was derived from this analysis: $\text{EC}_{50} = 2.2$ nM. Thus, zinterol was highly potent in stimulating I_{Ca} in frog ventricular myocytes.

As shown in the experiment of figure 2, which is typical of four similar ones, the stimulatory effect of zinterol was not accompanied by any significant change in the voltage-dependence of peak I_{Ca} amplitude or I_{Ca} inactivation. Indeed, figure 2A shows that zinterol (0.1 μ M) increased I_{Ca} by a similar extent whatever the potential of the depolarizing pulse. Similarly, figure 2B shows that the degree of I_{Ca} inactivation induced by a 200-msec conditioning pulse to membrane potentials ranging from -100 to +100 mV was essentially the same in the absence or presence of zinterol.

Beta-2 adrenergic receptors mediate the stimulatory effect of zinterol on frog I_{Ca} . The large stimulatory effect of zinterol on I_{Ca} in frog cardiac myocytes and the lack of voltage-dependence of this effect is reminiscent of the stimulatory effect of isoprenaline, a nonselective *beta* adrenergic agonist, seen in the same preparation (Fischmeister and Hartzell, 1986). Moreover, zinterol mimics the effects of salbutamol, another selective *beta*-2 adrenergic agonist, on I_{Ca} (Skeberdis *et al.*, 1997). However, to ensure that the stimulatory effect of zinterol on I_{Ca} was mediated by activation of the *beta*-2



subtypes of adrenergic receptors, we examined the effect of ICI 118551, a selective antagonist of these receptors. In the experiment shown in figure 3, 1 μ M ICI 118551 was initially applied to a frog ventricular myocyte which alone produced no apparent change in the basal I_{Ca} amplitude. However, the presence of 1 μ M ICI 118551 blunted the stimulatory response of I_{Ca} to 0.1 μ M zinterol. A progressive reduction in the concentration of ICI 118551 from 1 μ M to 1 nM, in the continuing presence of 0.1 μ M zinterol, unveiled the stimulatory effect of zinterol on I_{Ca} . With 100 nM ICI 118551, I_{Ca} was about half-maximally stimulated by 0.1 μ M zinterol



which corresponded to ≈ 50 -fold increase in the EC_{50} value. Application of competition curve analysis to a total number of six experiments similar to the one shown in figure 3 allowed to determine a dissociation constant for ICI 118551 ranging between 2 and 5 nM. At this concentration, ICI 118551 remains a highly selective antagonist of β_2 adrenergic receptors (O'Donnell and Wanstall, 1980; Bilski *et al.*, 1983). Thus, the stimulatory effect of zinterol on I_{Ca} appeared to be mediated by the activation of β_2 adrenergic receptors.

Zinterol produces a maximal stimulation of frog I_{Ca} . We then examined whether zinterol was capable to produce a maximal stimulation of I_{Ca} in frog ventricular myocytes, *e.g.*, comparable to the maximal effect of isoprenaline or forskolin, a direct adenylyl cyclase activator. To answer this question, we performed five experiments like the one illustrated in figure 4. After stimulation of I_{Ca} with a saturating concentration (1 μ M) of zinterol, 3-isobutyl-1-methylxanthine (IBMX, 100 μ M), a nonselective phosphodiesterase inhibitor,

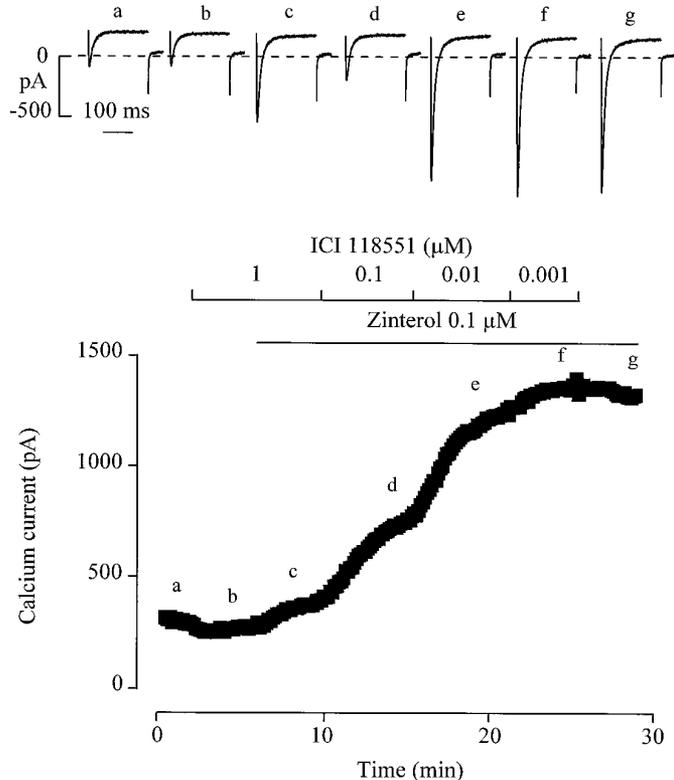


Fig. 3. Antagonist effects of ICI 118551 on the stimulatory effect of zinterol on I_{Ca} in frog ventricular myocytes. A frog ventricular myocyte was initially superfused with control external solution and internally dialyzed with control intracellular solution. Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarizing the cell every 8 sec to 0 mV over a period of 200 msec from a holding potential of -80 mV. During the periods indicated by the horizontal lines, the cell was successively exposed to four decreasing concentrations of ICI 118551 (1, 0.1, 0.01 and 0.001 μ M) and/or to zinterol (0.1 μ M). The current traces shown on top of the graph were recorded in the different experimental conditions, at the times indicated by the corresponding letters on the graph.

or forskolin (10 μ M) was added to the solution containing zinterol to maximally increase the concentration of cAMP within the cell, either by blocking its degradation (IBMX) or by maximally stimulating its synthesis (forskolin). We found that neither IBMX nor forskolin were able to increase I_{Ca} above the level reached in the presence of zinterol alone. In five other experiments, the effect of isoprenaline (1 μ M) was tested after a stimulation of I_{Ca} with 1 μ M zinterol. In these experiments, zinterol alone increased I_{Ca} by $418 \pm 54\%$ above basal level, and the current was not further increased by addition of isoprenaline ($442 \pm 51\%$). Thus, activation of the β_2 -adrenergic receptors with zinterol is sufficient to maximally stimulate I_{Ca} in frog ventricular myocytes.

Long-lasting, adenylyl-cyclase-mediated effects of zinterol on frog I_{Ca} . Although the effects of zinterol on I_{Ca} resemble in their amplitude those of other *beta* adrenergic agonists such as isoprenaline or salbutamol, they differed markedly in their kinetics of action and washout. For example, with isoprenaline, the time for half maximal stimulation of I_{Ca} (t_{on}) was <20 sec and was essentially independent of the concentration used (Méry *et al.*, 1993). This shows that the rate-limiting step for the effect of isoprenaline is beyond agonist binding to the receptor (Frace *et al.*, 1993). However,

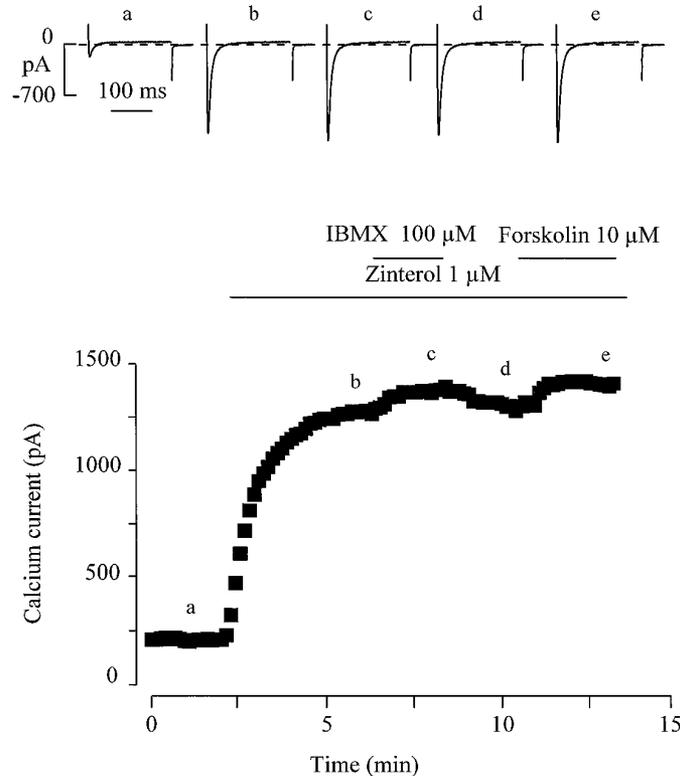


Fig. 4. Effects of IBMX and forskolin on I_{Ca} in the presence of a saturating concentration of zinterol. A frog ventricular myocyte was initially superfused with control external solution and internally dialyzed with control intracellular solution. Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarizing the cell every 8 sec to 0 mV over a period of 200 msec from a holding potential of -80 mV. During the periods indicated by the horizontal lines, the cell was exposed to zinterol (1 μ M) alone or to zinterol in the presence of IBMX (100 μ M) or forskolin (10 μ M). The current traces shown on top of the graph were recorded in the different experimental conditions, at the times indicated by the corresponding letters on the graph.

with zinterol, t_{on} was at least an order of magnitude larger and was strongly dependent on the drug concentration. For instance, t_{on} was 153 ± 22.5 sec ($n = 7$) with 10 nM zinterol and 71.3 ± 10.5 sec with 100 nM zinterol ($n = 5$). Thus, unlike with isoprenaline, binding of the agonist to its receptor was rate-limiting in the case of zinterol action. Moreover, during washout of the drug, I_{Ca} recovered much faster after stimulation with isoprenaline or salbutamol than after stimulation with zinterol. Indeed, the time for 50% recovery from the stimulation of I_{Ca} (t_{off}) was <80 sec with isoprenaline (Méry *et al.*, 1993) as well as with salbutamol (Skeberdis *et al.*, 1997) although t_{off} was an order of magnitude larger with zinterol (16.5 ± 2.1 min, $n = 5$). Actually, figure 1A shows that complete washout of zinterol effect required a period of almost an hour.

The difference in the kinetics of action and washout of zinterol and isoprenaline or salbutamol on I_{Ca} can not be due to different *beta* adrenergic receptor subtypes mediating the effects of these drugs. Indeed, we have shown recently that isoprenaline and salbutamol, like zinterol (fig. 3), mediate their effects on I_{Ca} in frog ventricular myocytes via a single population of *beta* adrenergic receptors and that these receptors correspond to the *beta*-2 subtype (Skeberdis *et al.*, 1997). Thus, the slow kinetics observed in the recovery of I_{Ca} after

zinterol washout might rather reflect a very slow dissociation rate constant of the agonist from its receptor. To test this hypothesis, we performed two series of experiments. In the first series of experiments, ICI 118551 ($1 \mu\text{M}$) was applied to the cell after I_{Ca} had been maximally stimulated by $1 \mu\text{M}$ zinterol. In three such experiments, a 3 to 11 min application of ICI 118551 in the presence of zinterol failed to significantly reduce the stimulatory effect of the β -2 agonist on I_{Ca} ($-5.4 \pm 7.4\%$) although, as shown above (fig. 3), ICI 118551 strongly antagonized the response to zinterol when added before the β -2 agonist. The second series of experiments is illustrated in figure 5. After a frog ventricular myocyte was exposed to $1 \mu\text{M}$ zinterol and I_{Ca} had increased ≈ 6 -fold, the β -2 agonist was washed out and the cell was exposed immediately to $1 \mu\text{M}$ ACh. As shown earlier (Fischmeister and Hartzell, 1986; Jurevičius and Fischmeister, 1996a), ACh has no effect on I_{Ca} in frog cardiomyocytes unless adenylyl cyclase activity is increased. Application of ACh right after washout of zinterol resulted in a rapid decrease in I_{Ca} back to its basal amplitude (fig. 5). This decrease was ≈ 2 orders of magnitude faster than the average time course of recovery of I_{Ca} from zinterol stimulation (indicated by the exponential dotted line using a time constant of 16.5 min). However, upon washout of ACh 2 min later, the current was increased again and reached $\approx 65\%$ of its amplitude in zinterol within 5 min. This increase was followed by a slower decline that now paralleled the average time course. A second

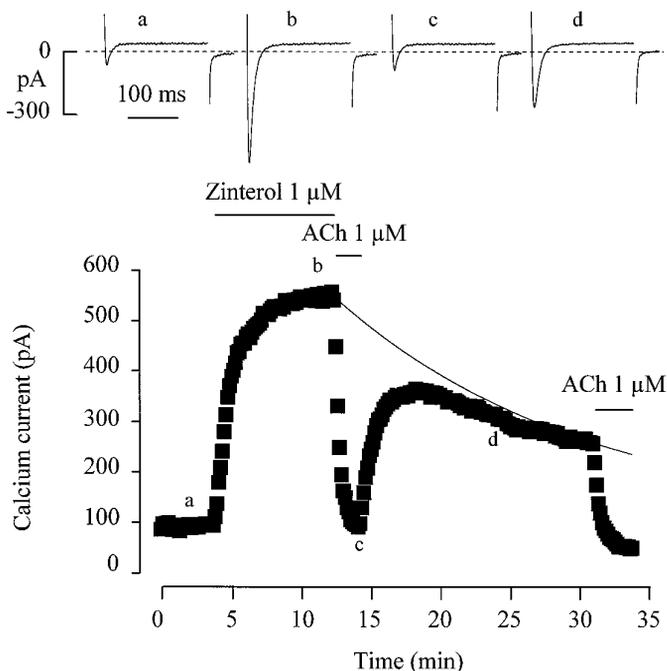


Fig. 5. Long-lasting effect of zinterol on I_{Ca} in frog ventricular myocytes. A frog ventricular myocyte was initially superfused with control external solution and internally dialyzed with control intracellular solution. Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarizing the cell every 8 sec to 0 mV over a period of 200 msec from a holding potential of -80 mV. During the periods indicated by the horizontal lines, the cell was exposed to zinterol ($1 \mu\text{M}$) or to acetylcholine (ACh, $1 \mu\text{M}$). The first application of ACh was obtained right after washout of zinterol. The dotted line is a mono-exponential fit of the time course of recovery of I_{Ca} from zinterol stimulation using a 18-min time constant. The current traces shown on top of the graph were recorded in the different experimental conditions, at the times indicated by the corresponding letters on the graph.

application of ACh 20 min later resulted in a second rapid decrease in I_{Ca} back to its basal level, from a level that was still ≈ 3 -fold larger. This experiment, which is typical of a total number of five similar ones, indicates that, during the whole period of zinterol washout, the activity of adenylyl cyclase was still enhanced. Because, by comparison, application of ACh during the recovery phase of isoprenaline has no effect on I_{Ca} (Li *et al.*, 1994), the simplest explanation of these results is that zinterol is more tightly bound to β -2 adrenergic receptors than isoprenaline or salbutamol and that recovery of I_{Ca} follows the time course of zinterol dissociation from its receptor.

Role of cAMP-dependent phosphorylation in the stimulatory effect of zinterol on I_{Ca} in frog, rat and human cardiomyocytes. Because the stimulatory effect of zinterol on I_{Ca} in frog ventricular myocytes 1) is maximal, 2) is not additive with the stimulatory effects of isoprenaline, forskolin or IBMX and 3) is inhibited by ACh, the most likely hypothesis is that this effect is mediated by activation of adenylyl cyclase and subsequent cAMP-dependent phosphorylation of L-type Ca^{++} channels. However, recent studies indicate that cAMP-independent mechanisms may also participate in the stimulatory effect of zinterol on I_{Ca} in mammalian cardiac preparations (Xiao and Lakatta, 1993; Xiao *et al.*, 1994; 1995; Altschuld *et al.*, 1995). Indeed, in rat (Xiao *et al.*, 1994) and dog ventricular myocytes (Altschuld *et al.*, 1995), the stimulation of I_{Ca} by zinterol as well as the positive inotropic effect of the drug were shown to be independent of cAMP concentration. If there were a cAMP-independent mechanism involved in the effect of zinterol on I_{Ca} , one would predict that an inhibitor of cAMP-dependent protein kinase would not completely block the stimulatory effect of zinterol. However, none of these studies examined the effect of zinterol in the presence of such inhibitors. For this reason, we reexamined the effect of zinterol on I_{Ca} in the presence of an intracellular application of a highly selective cAMP-dependent protein kinase peptide inhibitor, PKI(15-22) (Walsh *et al.*, 1990). These experiments were performed in frog ventricular myocytes as well as in two different mammalian species, rat and human, using an intracellular perfusion system (Fischmeister and Hartzell, 1987). Figure 6A shows a typical experiment performed in frog. After I_{Ca} had been enhanced by extracellular application of $0.1 \mu\text{M}$ zinterol, $20 \mu\text{M}$ PKI(15-22) were added to the intracellular solution which started to dialyze the cell. Few min after addition of PKI(15-22), I_{Ca} decreased dramatically, although zinterol was still present in the extracellular solution. As seen in figure 6A, the current returned to basal level in the presence of zinterol and PKI(15-22). On average, in four cells in which $0.1 \mu\text{M}$ zinterol increased I_{Ca} by $993 \pm 18\%$ over basal level, intracellular application of $20 \mu\text{M}$ PKI(15-22) reduced the stimulatory effect of zinterol by $99.1 \pm 1.1\%$ after 15 to 20 min. The control experiment illustrated in figure 6B shows that the strong reduction in I_{Ca} observed in the presence of PKI(15-22) was not a consequence of rundown or desensitization of the zinterol effect on I_{Ca} because I_{Ca} decreased by less than 25% from its maximal amplitude after a 20 min continuing exposure to $0.1 \mu\text{M}$ zinterol in the absence of PKI(15-22) (on average $-23.5 \pm 2.9\%$ decrease, $n = 4$).

Similar experiments were performed in rat ventricular myocytes (fig. 7) and human atrial myocytes (fig. 8). The protocols used to record I_{Ca} were identical in both prepara-

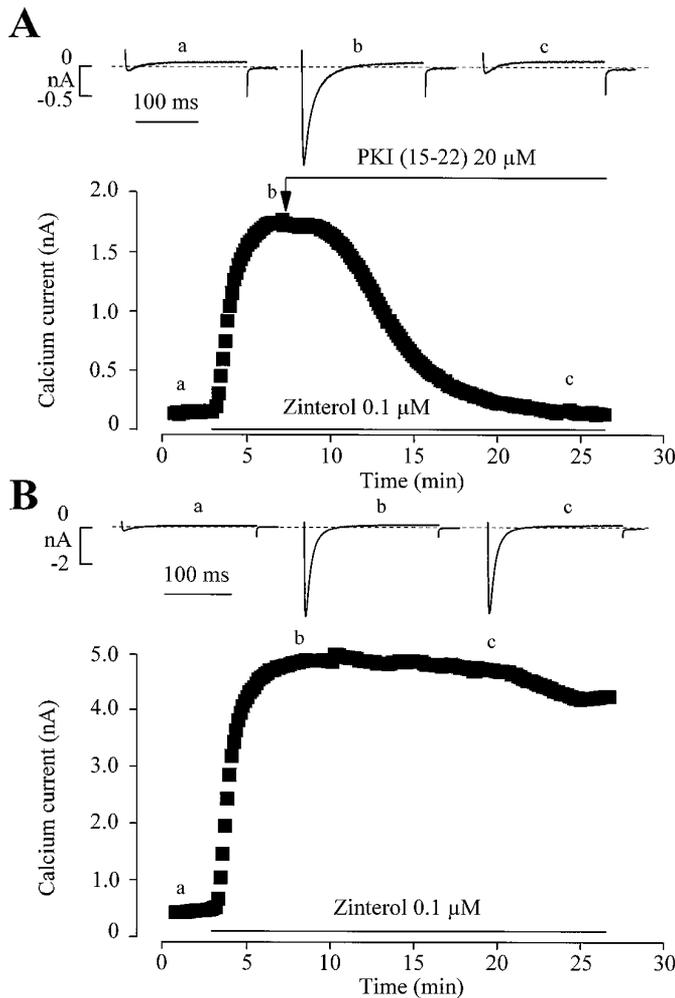


Fig. 6. Effect of intracellular perfusion with PKI(15-22) on the stimulatory effect of zinterol on I_{Ca} in frog ventricular myocytes. In A and B, a frog ventricular myocyte was initially superfused with control external solution and internally dialyzed with control intracellular solution. Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarizing the cell every 8 sec to 0 mV over a period of 200 msec from a holding potential of -80 mV. During the periods indicated, the cells were superfused with zinterol (0.1 μ M). In A, the intracellular solution was changed at the arrow to one containing 20 μ M PKI(15-22) which perfused the cell throughout the rest of the experiment as indicated by the upper horizontal line. In B, the control intracellular solution dialyzed the cell throughout the entire experiment. The current traces shown on top of the graphs in A and B were recorded in the different experimental conditions, at the times indicated by the corresponding letters on the main graphs.

tions. I_{Ca} was measured every 8 sec by depolarizing the cell to -50 mV during 50 msec and then over a period of 400 msec to 0 mV from a holding potential of -80 mV. Unlike in frog and human cardiomyocytes, I_{Ca} was subject to rundown in rat ventricular myocytes (indicated by the dotted lines in fig. 7A and B). However, in both preparations, zinterol produced a large increase in I_{Ca} . In rat ventricular myocytes, 10 μ M zinterol increased I_{Ca} by $62.7 \pm 9.1\%$ ($n = 12$), an effect that was comparable in amplitude to the stimulatory effect of 10 μ M isoprenaline ($78.0 \pm 9.3\%$, $n = 11$). Similarly to what was shown by Xiao and Lakatta (1993) in the same preparation, we found that a lower concentration (1 μ M) of zinterol did not reach maximal effect on I_{Ca} (data not shown). However, despite the large concentration of zinterol used, the effect of the

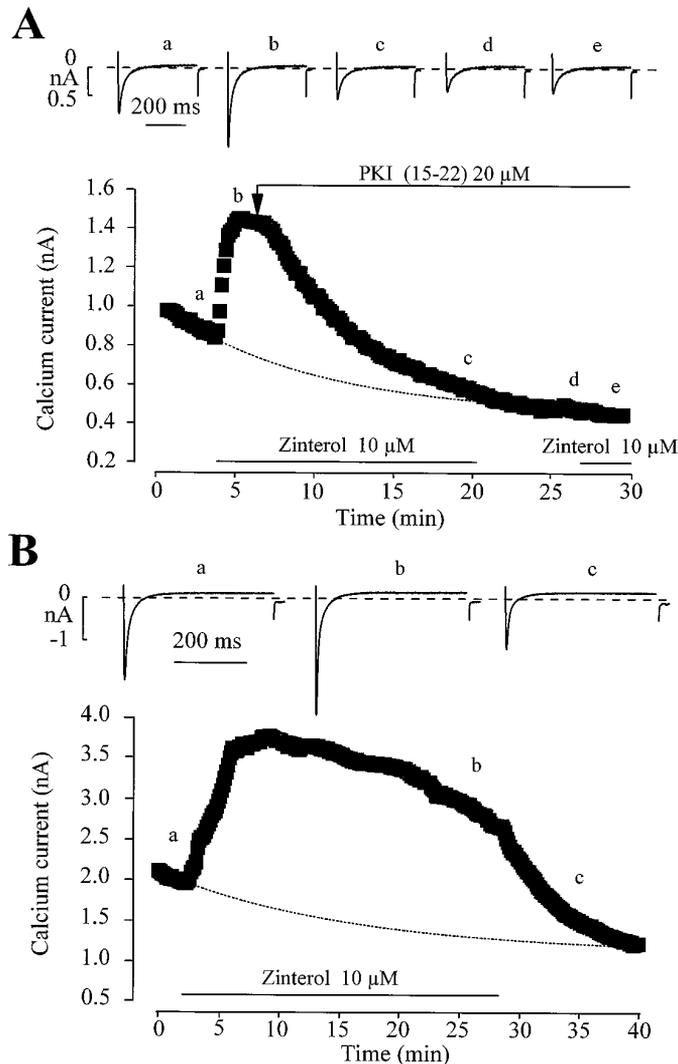


Fig. 7. Effect of intracellular perfusion with PKI(15-22) on the stimulatory effect of zinterol on I_{Ca} in rat ventricular myocytes. In A and B, a rat ventricular myocyte was initially superfused with control external solution and internally dialyzed with control intracellular solution. Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarizing the cell every 8 sec to 0 mV over a period of 400 msec from a holding potential of -80 mV. During the periods indicated, the cells were superfused with zinterol (10 μ M). In A, the intracellular solution was changed at the arrow to one containing 20 μ M PKI(15-22) which perfused the cell throughout the rest of the experiment as indicated by the upper horizontal line. In B, the control intracellular solution dialyzed the cell throughout the entire experiment. The current traces shown on top of the graphs in A and B were recorded in the different experimental conditions, at the times indicated by the corresponding letters on the main graphs. The dotted lines on the main graphs indicate the exponential rundown of I_{Ca} .

drug was likely mediated by activation of β_2 adrenoceptors since application of 0.1 μ M CGP 20712A, a selective β_1 -adrenergic antagonist, had no significant effect on the response of I_{Ca} to zinterol ($+6.6 \pm 2.9\%$, $n = 6$). On the contrary, a 1 μ M concentration of zinterol was sufficient to induce a maximal response in human atrial myocytes, increasing I_{Ca} by $144.2 \pm 47.8\%$ ($n = 7$) an effect that was also comparable in amplitude to the stimulatory effect of isoprenaline (1 μ M, $116.0 \pm 16.7\%$, $n = 17$). Figures 7A and 8A show that, in both rat and human cardiac myocytes, intracellular dialysis with a solution containing 20 μ M PKI(15-22) com-

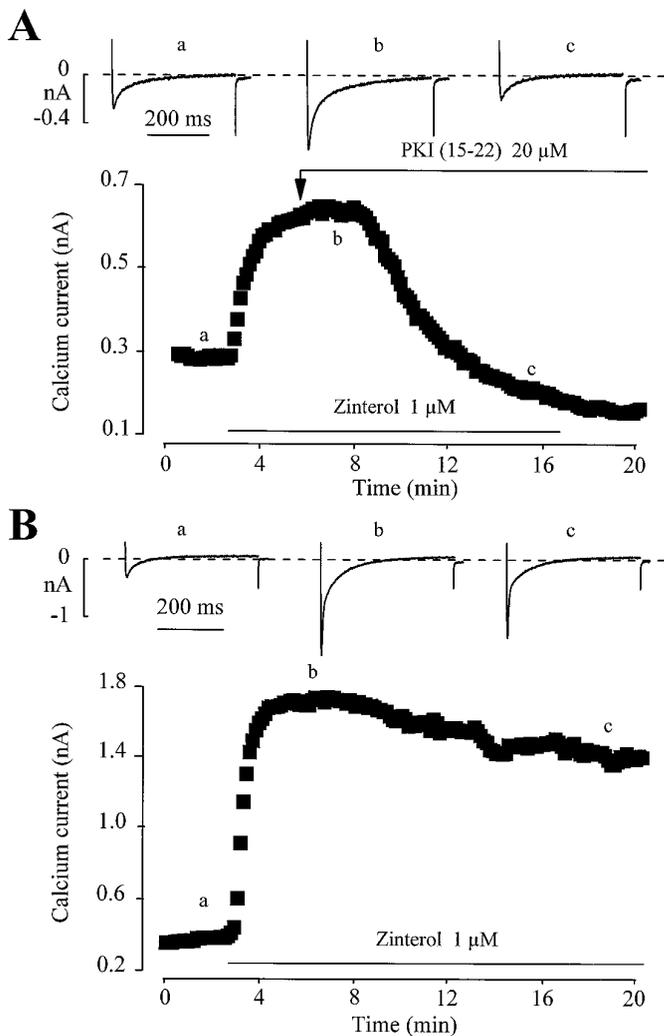


Fig. 8. Effect of intracellular perfusion with PKI(15-22) on the stimulatory effect of zinterol on I_{Ca} in human atrial myocytes. In A and B, a human atrial myocyte was initially superfused with control external solution and internally dialyzed with control intracellular solution. Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarizing the cell every 8 sec to 0 mV over a period of 400 msec from a holding potential of -80 mV. During the periods indicated, the cells were superfused with zinterol (1 μ M). In A, the intracellular solution was changed at the arrow to one containing 20 μ M PKI(15-22) which perfused the cell throughout the rest of the experiment as indicated by the upper horizontal line. In B, the control intracellular solution dialyzed the cell throughout the entire experiment. The current traces shown on top of the graphs in A and B were recorded in the different experimental conditions, at the times indicated by the corresponding letters on the main graphs.

pletely reversed the stimulatory effect of zinterol on I_{Ca} . After 15 min perfusion with PKI(15-22) in the continuing presence of 1 (human) or 10 μ M (rat) zinterol, I_{Ca} returned to a value that was substantially below the basal level in human ($-44.9 \pm 8.6\%$, $n = 4$) but not in rat myocytes ($-6.2 \pm 8.5\%$, $n = 9$, exponential rundown subtracted). The control experiment illustrated in figure 7B and 8B show that the strong reduction in I_{Ca} observed in the presence of PKI(15-22) was not simply a consequence of rundown or desensitization of the zinterol effect on I_{Ca} . Indeed, I_{Ca} decreased by only, respectively, $34.2 \pm 7.3\%$ ($n = 3$) in rat and $35.2 \pm 6.9\%$ ($n = 3$) in human, after 15 min of continuous exposure to 10 μ M and 1 μ M zinterol in the absence of PKI(15-22). More-

over, in two rat ventricular myocytes in which intracellular PKI(15-22) (20 μ M) was applied first and zinterol (10 μ M) was added to the extracellular solution 20 min later, no stimulatory effect of zinterol was observed.

Discussion

In our study, we examined the effect of the β_2 adrenergic receptor agonist zinterol on the L-type Ca^{++} current (I_{Ca}) in frog ventricular myocytes and, to a lesser extent, in rat ventricular and human atrial myocytes. In all three preparations, zinterol produced a large increase in I_{Ca} and the maximal stimulation was comparable to that produced by a saturating concentration of isoprenaline, a nonselective β adrenergic agonist. A precise characterization of the effect of zinterol on I_{Ca} in frog ventricular myocytes demonstrated that this effect was 1) concentration-dependent ($EC_{50} = 2.2$ nM); 2) independent of the membrane potential; 3) long lasting; 4) antagonized by ICI 118551, a β_2 adrenergic receptor antagonist; 5) not additive with the effects of IBMX or forskolin and 6) reversed by ACh. Moreover, in all three preparations tested, the stimulatory effect of zinterol on I_{Ca} was fully antagonized by intracellular perfusion with PKI(15-22), a highly selective peptide inhibitor of the cAMP-dependent protein kinase. We conclude that, in frog, rat and human cardiac myocytes, β_2 adrenergic receptor activation induces an increase in I_{Ca} which is mediated by activation of adenylyl cyclase, and subsequent activation of cAMP-dependent protein kinase and phosphorylation of L-type Ca^{++} channels.

Although initial competitive binding studies concluded to the absence of β_2 adrenergic receptors in purified ventricular myocytes from mammalian hearts (Freissmuth *et al.*, 1986; Lau *et al.*, 1980; Buxton and Brunton, 1985), 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 and Saffitz, 1988), baboons (Cui *et al.*, 1996) and humans (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 and Saffitz, 1988; 59/41 in baboon: Cui *et al.*, 1996; 20/80 in frog: Hancock *et al.*, 1979). 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 and Ruffolo, 1991 and references therein). Finally, in mammals, the proportion of β_2 adrenergic receptors was shown to be somewhat larger in atrial compared to ventricular tissues (Carlsson *et al.*, 1977; Hedberg *et al.*, 1980; Molenaar and Summers, 1987), and more so in human where β_2 adrenergic receptors may account for 35 to 50% of the total number of β adrenergic receptors (Robberecht *et al.*, 1983; Brodde, 1991; Hieble and Ruffolo, 1991). The latter finding may explain why β_2 adrenergic agonists exert preferentially positive chronotropic rather than inotropic effects in humans (Brodde, 1991).

Selective agonists of β_2 adrenergic receptors were shown earlier to increase I_{Ca} in guinea pig atrial myocytes

(Iijima and Taira, 1989), as well as in rat (Xiao and Lakatta, 1993; Cerbai *et al.*, 1995), dog (Altschuld *et al.*, 1995), guinea pig (Wang and Pelzer, 1995; but see Iijima and Taira, 1989) and frog ventricular myocytes (Skeberdis *et al.*, 1997). The maximal stimulatory effect of these agonists on I_{Ca} varied from 30% in guinea pig (Wang and Pelzer, 1995) to 100% in rat ventricular myocytes (Xiao and Lakatta, 1993) of the effect of isoprenaline. Here we found that a selective activation of β -2 adrenergic receptors with zinterol accounts for 100% of the isoprenaline response in frog and rat ventricular and human atrial myocytes. Although both subtypes of β receptors can increase cardiac I_{Ca} and force of contraction, some difference may exist in the mechanisms involved. First, Xiao and Lakatta (1993) showed in rat ventricular myocytes a marked prolongation of the I_{Ca} inactivation phase upon application of zinterol which was not found upon activation of the β -1 adrenoceptors. This phenomenon, however, was not observed in another study in the same preparation (Cerbai *et al.*, 1995). Although we did not study in details the kinetics of I_{Ca} in our experiments, we found no drastic change in the inactivation phase of I_{Ca} in none of the three preparations examined (frog, rat and human, data not shown). Second, unlike isoprenaline or noradrenaline, a more selective β -1 adrenergic agonist, zinterol did not abbreviate the twitch relaxation or the cytosolic Ca^{++} transient in rat, canine and human isolated cardiomyocytes (Xiao and Lakatta, 1993; Xiao *et al.*, 1994; Altschuld *et al.*, 1995; Kuznetsov *et al.*, 1995). Third, unlike the effects of noradrenaline, the positive inotropic effect of zinterol as well as the increase in Ca^{++} transient were found to be poorly correlated with the increase in cAMP concentration and cAMP-dependent phosphorylation of phospholamban in the same preparations (Lemoine and Kaumann, 1991; Borea *et al.*, 1992; Xiao *et al.*, 1994; Altschuld *et al.*, 1995; Kuznetsov *et al.*, 1995). Finally, unlike the effects of noradrenaline, the stimulatory effects of zinterol on I_{Ca} , Ca^{++} transient and cell shortening were strongly potentiated by a pertussis toxin pre-treatment in rat ventricular myocytes (Xiao *et al.*, 1995).

Altogether, these studies concluded to the presence of a cAMP-independent mechanism in the stimulatory effects of β -2, but not β -1, adrenergic receptor agonists. However, this conclusion was not supported by our current findings. Indeed, we found that when cAMP-dependent phosphorylation is blocked by PKI(15–22), a highly selective peptide inhibitor of cAMP-dependent protein kinase (Walsh *et al.*, 1990), zinterol does not anymore stimulate I_{Ca} in frog, rat and human cardiomyocytes. On the contrary, we propose that all of the stimulatory effect of β -2 adrenergic receptor activation on I_{Ca} is actually mediated by cAMP-dependent phosphorylation. Such a conclusion is consistent with previous studies on I_{Ca} regulation in frog, rat and rabbit cardiomyocytes in response to the nonselective agonist isoprenaline (Hartzell *et al.*, 1991; Hartzell and Fischmeister, 1992; Tanaka *et al.*, 1996) and with recent contractile and biochemical studies performed in human atrium (Kaumann *et al.*, 1996). This conclusion is not necessarily at variance with the aforementioned lack of correlation between the effect of β -2 adrenergic receptor activation and measured cAMP levels. Indeed, we have shown recently that the response of I_{Ca} in frog ventricular myocytes to isoprenaline is mainly due to a local rise in cAMP (Jurevičius and Fischmeister, 1996b). Because the β adrenergic receptor population is composed

of $\approx 80\%$ of β -2 subtype in frog cardiomyocytes (Hancock *et al.*, 1979; Hieble and Ruffolo, 1991) and only β -2 adrenergic seem to be coupled to L-type Ca^{++} channels in this preparation (Skeberdis *et al.*, 1997), the possibility exists that the cAMP generated by β -2 adrenergic receptor activation may be more localized, and hence less visible in biochemical assays, than the cAMP generated by activation of β -1 adrenoceptors. Although both pools of cAMP would be efficiently coupled to L-type calcium channels that are sarcolemmal membrane proteins, the pool of cAMP generated by β -2 adrenoceptor activation may be less efficiently coupled to more distant proteins, such as phospholamban and/or troponin I.

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