Effects of clenbuterol on contractility and Ca\(^{2+}\) homeostasis of isolated rat ventricular myocytes

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Clenbuterol, a compound classified as a \(\beta_2\)-adrenoceptor (AR) agonist, has been employed in combination with left ventricular assist devices (LVADs) to treat patients with severe heart failure. Previous studies have shown that chronic administration of clenbuterol affects cardiac excitation-contraction coupling. However, the acute effects of clenbuterol and the signaling pathway involved remain undefined. We investigated the acute effects of clenbuterol on isolated ventricular myocyte sarcomere shortening, Ca\(^{2+}\) transients, and L-type Ca\(^{2+}\) current and compared these effects to two other clinically used \(\beta_2\)-AR agonists: fenoterol and salbutamol. Clenbuterol (30 \(\mu\)M) produced a negative inotropic response, whereas fenoterol showed a positive inotropic response. Salbutamol had no significant effects. Clenbuterol reduced Ca\(^{2+}\) transient amplitude and L-type Ca\(^{2+}\) current. Selective \(\beta_2\)-AR blockade did not affect the action of clenbuterol on sarcomere shortening but significantly reduced contractility in the presence of fenoterol and salbutamol (\(P < 0.05\)). Incubation with 2 \(\mu\)g/ml pertussis toxin significantly reduced the negative inotropic effects of 30 \(\mu\)M clenbuterol. In addition, overexpression of inhibitory G protein (Gi) by adenoviral transfection induced a stronger clenbuterol-mediated negative inotropic effect, suggesting the involvement of the Gi protein. We conclude that clenbuterol does not increase and, at high concentrations, significantly depresses contractility of isolated ventricular myocytes, an effect not seen with fenoterol or salbutamol. In its negative inotropism, clenbuterol predominantly acts through Gi, and the consequent downstream signaling pathways activation may explain the beneficial effects observed during chronic administration of clenbuterol in patients treated with LVADs.

\(\beta\)-adrenoceptor; G proteins; heart failure; cardiac myocytes

CHRONIC HEART FAILURE (HF) is a clinical syndrome with large incidence and very poor prognosis (21). Heart transplantation is the most effective treatment for this disease but is hindered due to an inadequate availability of donor organs. Left ventricular (LV) assist devices (LVADs) have been shown to be a suitable alternative (30). In a few patients, LVAD treatment induces substantial functional improvement that allows explantation of the device without the need of transplantation (12). However, the rate for explantation of the LVAD is low, with an incidence of only 4–5% (24, 25). In addition, the functional improvement observed during LVAD treatment seems to be a transient phenomenon (25).

In a recent study, our group (1) has shown that a combination of mechanical unloading and pharmacological therapy leads to a substantially improved explantation rate in patients with dilated cardiomyopathy. Together with other drugs, the \(\beta_2\)-adrenoceptor (\(\beta_2\)-AR) agonist clenbuterol has been included in the protocol with the aim to prevent unloading-induced atrophy and consequent myocardial dysfunction. Based on its structural and functional properties, clenbuterol is classified as a \(\beta_2\)-AR agonist (23). Clenbuterol is structurally similar to salbutamol and was previously used as a bronchodilator in the treatment of asthma (32). Clenbuterol is also known for enhancing regeneration of peripheral nerves in rodents (13, 44), for inducing redistribution of white blood cells in the circulation (33), and decreasing body fat (46). Chronic clenbuterol administration is known to produce skeletal (16) and cardiac hypertrophy and to affect excitation-contraction coupling and cellular metabolism (34), gene expression, and myocardial function (27, 36, 37) in normal hearts. Clenbuterol treatment during mechanical unloading of normal hearts improves \(\beta\)-AR responsiveness and gene expression [sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2a and \(\beta\)-myosin heavy chain] and reduces myocardial apoptosis (35).

Despite the several reported studies and the use of clenbuterol in treatment of patients with HF in combination with LVADs (1, 41, 42), the acute effects of clenbuterol on cardiac contractility and its downstream signaling pathway are unknown. The main aims of this study were, therefore: 1) to determine the acute effects of clenbuterol on cardiomyocyte contractility and electrolyphysiology; 2) to compare these effects with other clinically used \(\beta_2\)-AR agonists; 3) to investigate which \(\beta\)-AR subtypes (\(\beta_1\), \(\beta_2\), \(\beta_3\)) are involved; 4) to elucidate the downstream signaling pathway; and 5) to observe if the effects of clenbuterol are different in failing cardiomyocytes.

We found that acute administration of clenbuterol not only does not increase, but, at high concentrations, significantly depresses contraction of isolated ventricular myocytes. This effect was not seen in cells exposed to similar concentrations of salbutamol or fenoterol, suggesting a distinctive mode of action for clenbuterol. Results with \(\beta\)-AR-selective antagonists indicate that the observed differences among these drugs may be attributable to their differential activation of \(\beta\)-AR subtypes and their signaling to different G proteins. The study shows
that clenbuterol at high concentrations predominantly activates the inhibitory G (Gᵢ) protein.

METHODS

The protocol was approved by the Ethical Review Committee of the Heart Science Center and by the UK Home Office under the terms of the Animals (Scientific Procedures) Act 1986.

**Cell isolation.** Sprague-Dawley rats (150–250 g) were anesthetized with 5% isoflurane-95% O₂ and then killed by cervical dislocation. Hearts were rapidly removed and placed in Tyrode solution [normal Tyrode (NT)] [in mM: 140 NaCl, 6 KCl, 10 glucose, 10 HEPES (free acid), 1 MgCl₂, 1 CaCl₂; pH 7.4], where any excess tissue was removed. Following aerobic cannulation to the Langendorff setup, the hearts were perfused with Tyrode solution for 2–3 min, then with low Ca²⁺ solution [in mM: 120 NaCl, 5.4 KCl, 5 MgSO₄, 5 sodium pyruvate, 20 glucose, 20 taurine, 10 HEPES (free acid), 5 nitritroacetic acid, and 0.04 CaCl₂; pH 6.96] for 5 min, and finally for 9 min with solution containing collagenase (1 mg/ml, Worthington) and hyaluronidase (0.6 mg/ml; Sigma-Aldrich) solved in buffer solution [in mM: 120 NaCl, 5.4 KCl, 5 MgSO₄, 5 sodium pyruvate, 20 glucose, 20 taurine, 10 HEPES (free acid), and 0.2 CaCl₂; pH 7.4]. The LV was then removed, cut into small pieces, resuspended in collagenase/hyaluronidase solution, and shaken in a water bath at 37°C for 5 min twice. Then the cells were filtered through a 200-μm nylon mesh and centrifuged at 500 counts/min for 1 min. The cells used for experiments were resuspended and stored in buffer solution at room temperature.

Failing cardiomyocytes were obtained after 8 wk from left coronary artery ligation, as previously reported (10). Briefly, the anesthetized rats were intubated with a 16-G plastic cannula and mechanically ventilated (Harvard Apparatus, Kent, UK) at 2.5 ml tidal volume and 70 breaths/min. A left-sided thoracotomy at the fourth intercostal space followed by pericardectomy provided access to the heart. The left coronary artery was identified and permanently ligated at the level of the left atrial appendage using a 6–0 suture Prolene (Ethicon) to cause myocardial infarction and subsequent HF. The diagnosis of HF was based on ejection fraction measured by using a 15-MHz probe on an Acuson Sequoia 256 System (Siemens Medical Systems). Transmural echocardiography was performed to obtain parasternal short-axis views at the midpapillary muscle level. Ejection fraction was calculated from the systolic and diastolic two-dimensional cross-sectional LV areas, and ejection fraction <30% was taken as HF. Cardiomyocytes were isolated from the viable LV of these hearts, as described above.

**Overexpression of Gᵢ.** Cardiomyocytes overexpressing Gᵢ₉₂ were obtained by adenoviral transfection [as previously described by Gong et al. (18)]. They were cultured with E1-deficient-type adenovirus constructed to express the human Gᵢ₉₂ + green fluorescent protein (AdvGᵢ₉₂,GFP). The control cells were transfected with GFP-only adenovirus (Adv.GFP). Cells were cultured in complete culture medium (67% DMEM, 16% medium 199, ~10% horse serum, 4% fetal bovine serum, 2% 1 M HEPES, 1% penicillin/streptomycin) at 37°C for 48 h. Adenovirus expressing Gᵢ protein was initially added to each well, containing 2 × 10⁶ cardiomyocytes in 2 ml medium at 3.5 × 10⁷ plaque-forming units for Adv.Gᵢ₉₂,GFP or 10⁷ for Adv.GFP. Gᵢ protein overexpression was confirmed by immunoblotting techniques, as previously described (17).

**Sarcomere shortening.** Cells were superfused at a rate of 2–3 ml/min with Krebs solution (in mM: 120 NaCl, 25 NaHCO₃, 4.7 KCl, 0.97 MgSO₄, 1.2 KH₂PO₄, 11 glucose, 1 CaCl₂; equilibrated with 95% O₂ and 5% CO₂ to produce pH 7.4) at 37°C and field stimulated at a constant frequency of 1 Hz using a pair of platinum electrodes. The cell image was directed to an intensified charge-coupled device camera (Ionoptix Myocam CCD 100M) and acquired at 240 frames/s. The sarcomere pattern was digitized by the software (Ionwizard, Ionoptix), using fast Fourier transformation into a frequency power spectrum. Cardiomyocytes selected to the experiments had appropriate morphological appearance (rod-shaped with clean edges, clear striations, and no large blebs), resting sarcomere length >1.70 μm, and no spontaneous contraction.

**Concentration-response curves.** After an equilibration period of 5–15 min of superfusion with Krebs solution, the contracting cells were superfused with increasing concentrations of the β₂-AR agonists fenoterol, salbutamol, or clenbuterol (0.01–300 μM). Superfusion with each agonist concentration continued until a new steady state of contraction was established. At this point, the signal traces were analyzed. Each cell served as its own control based on the attainment of an initial steady state before agonist exposure. A final washout was performed with Krebs solution for 2–4 min to assess the reversibility of any induced effect.

The experimental protocol was repeated in the presence of β₁-AR antagonist CGP 20712A (0.3 μM), with or without other β-AR antagonists: β₂, ICI 118,551 (0.05 or 1 μM); nonselective, carvedilol (1 μM); and β₁, SR 59230A (1 μM), as well as a muscarinic receptors antagonist, atropine (1 μM), each administered according to the specified experimental protocol. A final washout was performed with Krebs solution containing appropriate antagonists. Some experiments performed in the presence of CGP 20712A (0.3 μM) also had the Gᵢ protein blocker pertussis toxin (PTX; 2 μg/ml). Cells were incubated with PTX for 3 h at 37°C (control cells to this group were incubated in the same conditions with buffer solution only).

**Cytoplasmic Ca²⁺ measurements.** Cytoplasmic Ca²⁺ was monitored using Ca²⁺-sensitive, single-excitation, single-emission fluorescent dye fluo 4-AM (acetoxyethyl ester form; Invitrogen; 10 μM) [as described before by Farkasfalvi et al. (10)]. Briefly, the cells were loaded for 10 min with the dye. Supernatant was then discarded, and cells were resuspended in buffer solution and used for experiments after 1 h to allow deesterification of the indicator. During experiments, the cells were superfused with NT solution at 37°C. For analysis of Ca²⁺ transients, 10–15 events were averaged with reference to the field stimulation signal. Peak amplitude was calculated from the field stimulation signal baseline using pClamp software (version 9.0, Axon Instruments).

**Electrophysiological parameters.** Cells were studied using a MultiClamp 700A (Axon Instruments) in whole cell patch configuration. The pipette resistance was ~2–3 MΩ, and the pipette-filling solution contained the following (in mM): 115 cesium-aspartate, 20 tetraethylammonium-chloride, 10 EGTA, 10 HEPES, and 5 MgATP, pH 7.2. The external solution contained the following (in mM): 140 NaCl, 10 glucose, 10 HEPES, 1 CaCl₂, 1 MgCl₂, 6 CsCl, pH 7.4. Current-voltage relationships for L-type Ca²⁺ current were built using 450-ms depolarization steps from a holding potential of ~ 40 mV (range: −40 mV to + 40 mV, in 5-mV increments) at 1 Hz. Cd²⁺ (200 μM) was applied, and the protocol repeated. Subtracted currents obtained were normalized to cell capacitance. All experiments were conducted at 37°C.

**Induction of detubulation using formamide.** Freshly isolated cardiomyocytes were incubated with formamide (1.5 M) for 15 min to cause detubulation, as previously described (9). The cells were then washed and incubated with 4-[β-(2-di-n-butylamino)-6-naphthyl]vinylpyridinium (Molecular Probes, Eugene, OR) (10 μM for 10 min). To visualize the t-tubule structure of cardiomyocytes, a z-series stack of images 1 μm apart was obtained with confocal microscopy (Zeiss LSM 510). During experiments, the cells were superfused with NT solution at 37°C. The degree of detubulation was assessed by a custom-written macro using ImageJ (29), which calculates the relative area stained for t-tubules from the fluorescent signal through the midsection of the z-series stack (28).

**Western blotting.** Cardiomyocytes were snap frozen and stored at −80°C, either immediately after isolation or after culture for 48 h, with or without clenbuterol (1 μM). The frozen cell pellet was thawed in homogenizing buffer [1% sodium dodecyl sulfate, 1 mM NaF, 1 mM NaVO₄, and 1 tablet of protease inhibitor cocktail (Roche, Switzerland) per 10-ml buffer] and homogenized, and supernatant was collected. Total protein concentration was estimated using a bicincho-
nic acid protein kit (Pierce). Proteins were separated using 10% SDS-PAGE gels, and 40 μg of total protein were loaded per lane. Proteins were blotted onto a nitrocellulose membrane (Hybond C-Super, Amersham). Membranes were blocked in 3% (wt/vol) nonfat, dried milk in phosphate-buffered saline contained 0.05% Tween 20 overnight. Blots were then incubated in primary antibody for G\textsubscript{i} (1:300, mouse IgG2b, catalog no. 612702; BD Biosciences), followed by exposure to horseradish peroxidase-conjugated, species-specific, secondary antibody (1:1,000; Dako). Positivity was detected using enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce). Densitometry was performed using Quantity One software (BioRad). The level of expression of G\textsubscript{i} protein was standardized to total protein reactivity on the same blot.

Chemicals. Fenoterol, salbutamol, clenbuterol, CGP 20712A, ICI 118,551, atropine, and PTX were from Sigma-Aldrich; SR 59230A from Tocris; and carvedilol was a gift from GSK. All chemicals used for solutions were purchased from VWR, except nitrilotriacetic acid, which was from Sigma-Aldrich. The drugs were dissolved in AnalAR water, except for carvedilol, which was dissolved in DMSO (final concentration of DMSO in Krebs solution was <0.1% vol/vol).

Statistical analysis. To assess statistical differences, a one-way ANOVA, a repeated-measures two-way ANOVA with the Bonferroni posttest, or a Student t-test was performed as appropriate, using Prism 4.0 software (GraphPad, San Diego, CA). Results are expressed as means ± SE of the mean (n = number of cells). All of the experiments were performed using a minimum of three animals, unless otherwise stated. P < 0.05 was interpreted as being statistically significant.

RESULTS

Effects of clenbuterol on cardiomyocyte contractility and Ca\textsuperscript{2+} transient amplitude: comparison with other β\textsubscript{2}-AR agonists. Superfusion of the cardiomyocytes with clenbuterol did not induce a positive inotropic effect (Figs. 1A and 2). A reduction of sarcomere shortening was observed with concentrations ≥10 μM. This effect was reversible on washout. The time course of sarcomere shortening was not affected by clenbuterol [time to peak (in ms): control, 0.049 ± 0.004, n = 10; clenbuterol 30 μM, 0.048 ± 0.004, n = 10; clenbuterol 100 μM, 0.048 ± 0.005, n = 9, P = not significant (NS); time to 90% relaxation (in ms): control, 0.075 ± 0.008, n = 8; clenbuterol 30 μM, 0.089 ± 0.010, n = 8; clenbuterol 100 μM, 0.093 ± 0.007, n = 8].

Fig. 1. Clenbuterol (CLEN) reduced sarcomere length (representative trace shown in A) and Ca\textsuperscript{2+} transients amplitude (B) in a concentration-dependent manner in rat cardiomyocytes (CMs). #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. control [one-way ANOVA with Dunnett’s posttest for 6 cells from 3 animals (A) and 5 cells from 2 animals (B)].
inotropic effect elicited by clenbuterol. To test whether the reduced shortening was due to a reduction in Ca$^{2+}$ transient amplitude, cells were loaded with fluo 4-AM and field stimulated at 1 Hz. Clenbuterol at concentrations of 30 μM (74.55 ± 6.105, n = 5 from 2 animals, P < 0.05) and 100 μM (59.54 ± 9.388, n = 5 from 2 animals, P < 0.01) reduced Ca$^{2+}$ transient amplitude (Fig. 1B).

The effects observed with clenbuterol were different from those observed when other β2-AR agonists were used. Fenoterol induced a concentration-dependent positive inotropic response (Fig. 2), and salbutamol had no significant effect on cardiomyocyte contractility (Fig. 2).

**Influence of β-AR antagonists and atropine on the effects of clenbuterol, fenoterol, and salbutamol on cardiomyocyte contractility.** To investigate the selectivity of clenbuterol for the β-AR subtypes, we inhibited the β1, β2, and β3-ARs using CGP 20712A (300 nM), ICI 118,551 (50 nM and 1 μM), and SR 59230A (1 μM), respectively. We also used the nonselective β-AR antagonist carvedilol (1 μM) and the muscarinic receptor antagonist atropine (1 μM). The negative inotropic effect of clenbuterol was not inhibited by β-AR antagonists: β1, CGP 20712A (300 nM), in the presence of which the curve was even shifted to the left (Fig. 3A); or β2, ICI 118,551 (50 nM: Fig. 3A; 1 μM: Fig. 4A). On the contrary, the positive inotropic effect of fenoterol was strongly reduced in the presence of β2-AR antagonist CGP 20712A (300 nM) and even more by the addition of β2-AR antagonist ICI 118,551 (50 nM) (Fig. 3B). The dose-response curve obtained with salbutamol was not affected by CGP 20712A (300 nM) and became negative in the presence of ICI 118,551 (50 nM) (Fig. 3C). Moreover, the effects of clenbuterol, elicited in the presence of CGP 20712A, were not affected either by the nonselective β-AR antagonist carvedilol (1 μM) or the β3-AR antagonist SR 59230A (1 μM) (Fig. 4, B and C). The muscarinic receptor antagonist atropine (1 μM) had no influence on the dose-response curve elicited by clenbuterol (Fig. 4D).

**Involvement of the inhibitory G protein in the negative inotropic effect elicited by clenbuterol.** Some β2-AR agonists have been previously shown to signal to the G1 protein (38). To investigate whether clenbuterol signals through this pathway, which could explain the negative inotropic response at high concentrations, cardiomyocytes were pretreated with the G1 protein inhibitor PTX (2 μg/ml). The negative inotropic effect of clenbuterol, elicited in the presence of 300 nM CGP 20712A, was abolished in PTX-treated cardiomyocytes (Fig. 5A). L-type Ca$^{2+}$ current was also recorded in these cardiomyocytes using the whole cell configuration of the patch-clamping technique. The Ca$^{2+}$ current-voltage relationship in Fig. 5B shows that clenbuterol inhibits the Ca$^{2+}$ current (P < 0.001). However, the L-type Ca$^{2+}$ current inhibition caused by clenbuterol was significantly smaller in PTX-treated cardiomyocytes (Fig. 5, C and D).

Moreover, cardiomyocytes overexpressing G1 using adenoviral transfection (Adv.G1α2,GFP) were used after 48-h culture. Adv.G1α2,GFP myocytes showed a larger diastolic sarcomere length without significant changes in the sarcomere shortening under baseline conditions (diastolic sarcomere length: (in μm) Adv.GFP, 1.777 ± 0.017, n = 7; Adv.G1α2,GFP, 1.824 ± 0.010, n = 8, P < 0.05; sarcomere shortening (in μm): Adv.GFP, 0.115 ± 0.026, n = 7; Adv.G1α2,GFP, 0.082 ± 0.009, n = 8, P = NS]. Adv.G1α2,GFP myocytes responded to clenbuterol perfusion with a strong, negative inotropic effect (Fig. 5E). However, a surprising finding was that control GFP-only cardiomyocytes (Adv.GFP) cultured for 48 h did not respond to clenbuterol with a negative inotropic effect, as observed in freshly isolated cardiomyocytes.

To explain the lack of the negative inotropic effect of clenbuterol in normal cardiomyocytes cultured for 48 h, further studies were performed. One hypothesis was that reduction in
t-tubules, clearly occurring during culture (19), could disrupt the signaling involved during the application of clenbuterol. With the use of confocal microscopy, the effects of clenbuterol on cardiomyocyte contractility were compared within three groups of cardiomyocytes: 1) freshly isolated cells used as a control; 2) freshly isolated cells incubated with formamide to cause detubulation; and 3) cells cultured for 48 h (Fig. 6A). The degree of detubulation was similar in the formamide-treated and 48-h culture cell groups, which was a significant reduction in t-tubular structures compared with control (Fig. 6B). However, a lack of response to clenbuterol of the cardiomyocyte contractility was observed only in the 48-h culture group (Fig. 6C).

Another hypothesis was that the lack of the negative inotropic effect of clenbuterol in cardiomyocytes cultured for 48 h was caused by a decrease of G_i protein expression. With the use of Western blotting, we measured the level of G_i protein in three groups of cardiomyocytes: 1) freshly isolated cells used as a control; 2) cells cultured for 48 h; and 3) cells cultured for 48 h in the presence of clenbuterol (1 μM). We did not observe any significant difference in the level of G_i protein expression within the three groups (Fig. 7). These results suggest that the negative inotropic response to clenbuterol is not related to the integrity of the t-tubular structure or the level of G_i protein expression.

Influence of clenbuterol on failing cardiomyocyte contractility. It is known that β-2-ARs and G_i proteins change their relative expression, compared with β-1-AR and stimulatory G (G_s) protein, respectively, in HF (3, 20), and in failing tissue the effects of clenbuterol may be quantitatively different, eliciting a different inotropic response. We tested this hypothesis using cardiomyocytes isolated from a chronic rat model of postinfarction cardiomyopathy. In these myocytes, clenbuterol still induced a strong, negative inotropic effect in a concentration-dependent manner, and this response was not different from that elicited in normal cardiomyocytes (Fig. 8). These effects were not inhibited by the selective blockade of the β-1-AR in either group.

DISCUSSION

The present study shows that acute administration of clenbuterol does not increase contractility via activation of the G_s pathway and, at high concentrations, significantly depresses contractility of isolated rat ventricular myocytes via G_i activation. This effect was not seen in cells exposed to similar concentrations of salbutamol or fenoterol, suggesting a distinctive mode of action for clenbuterol.

Unlike salbutamol and fenoterol, clenbuterol produces a negative inotropic effect, which is largely unaffected by β-1-AR and β-2-AR-G_s blockade, implying that it mediates its effects...
through a \( \beta_1/\beta_2 \)-AR-Gs independent mechanism. While similar findings have been reported with some \( \beta \)-AR blockers, these occur only in the failing heart (18). This is the first study to demonstrate the significant negative inotropic effects of a \( \beta_2 \)-AR agonist in cardiomyocytes from a normal heart.

The present study does not provide the demonstration that \( \beta_2 \)-AR-Gs is activated by clenbuterol. ICI 118,551 (even at 1 \( \mu \)M concentration) or carvedilol does not produce a shift in clenbuterol’s concentration-response curve, and this may indicate that \( G_i \) is activated either via a \( \beta_2 \)-AR-independent path-

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way or by a separate population of β2-AR-Gi [as previously proposed (20)], which may be ICI insensitive. While the latter hypothesis remains speculative, we tested the hypothesis that other receptors known to activate Gi were involved. It has been shown that 3-AR can reduce amplitude of the action potential, inhibit L-type Ca2+ channels, and induce negative inotropic effects in rat and human normal and failing cardiomyocytes via a Gi-mediated pathway (15, 31, 45). However, in our study, the specific antagonist SR 59230A failed to affect the contractile response to clenbuterol, suggesting that the 3-AR is not involved. Moreover, blocking the muscarinic receptors, which are also known to cause negative inotropic effect in the heart (4), had no effects on the clenbuterol-elicited contractile response.

Comparison with other clinically used β2-AR agonists. The predominantly negative inotropic response elicited by clenbuterol at high concentrations was not observed when fenoterol or salbutamol were used. In agreement with previous studies, the positive inotropic effects of fenoterol were demonstrated to involve both β1-AR and possibly β2-AR. β1-AR is assumed to have a greater functional role in mediating these effects, as illustrated by more significant reductions in the inotropic responses that occurred following β2-AR blockade. This would be an expected response in light of the well-ascribed role of the β1-AR in producing positive inotropic effects (3). The β1-AR is coupled to Gs (22), and its activation results in increased cyclic AMP levels. This results in the activation of downstream PKA-dependent pathways to phosphorylate a host of intracellular proteins, including L-type Ca2+ channels (14). The increased Ca2+ entry raises the level of cytosolic Ca2+, a factor principally governing cellular contraction. In this way, blockade would be expected to impede these events and produce the observed reductions in cell contractility.

The involvement of β2-AR-Gs in the response to salbutamol and fenoterol is suggested by two findings. First, additional β2-AR-Gs blockade with ICI 118,551 produces a greater reduction in the inotropic effect of each drug beyond that seen with β1-AR blockade alone. Second, a pure β1-AR activation would be expected to cause a 3–4 log shift in the concentration-response curves of each drug; the smaller shift otherwise observed implies a mixed response (8).

The involvement of Gs in the actions of fenoterol and salbutamol has been described before (39). Salbutamol, in particular, signals via this pathway in a way that can be revealed when Gs is inhibited with PTX. On the other hand, fenoterol seems to be PTX insensitive (39).

The role of Gs in the negative inotropic response to clenbuterol. In the present study, we show that the mechanisms mediating the negative response of high concentrations of clenbuterol involve the Gs pathway. This was done by two
separate lines of evidence: the use of PTX and the overexpression of Gi in cultured cardiomyocytes. A study addressing the negative inotropic effects of ICI 118,551 in failing human heart implicates the involvement of β2-AR-Gi as one possibility (18). The authors showed that ICI 118,551 produced negative inotropy by acting as an agonist at the β2-AR-Gi-coupled receptor, as was demonstrated by sensitivity to PTX (18). While no significant effect was observed in normal hearts, the depression of contraction by ICI 118,551 was seen after up-regulation of Gi protein using adenoviral vectors in rat myocytes, or in myocytes from failing human hearts (in which there is a greater functional role of the β2-AR and Gi) (20).

Activation of β2-AR-Gi has been shown to result in negative inotropic and anti-apoptotic effects (7, 11, 39, 47). β2-AR-Gi signaling disrupts β2-AR-Gα-stimulated increases in cAMP, and this leads to reduced activation of PKA, which results in smaller protein phosphorylation and hence decreases contractile response (38). It has been shown that treatment with PTX enables β2-AR agonists to induce full contractile response in cardiomyocytes (39). Moreover, activation of β2-AR-Gi reduces Ca2+ current. It has been shown that β2-AR agonists activate only Ca2+ channels located in the immediate surrounding membrane, but blockade of Gi with PTX permits β2-AR to stimulate L-type Ca2+ channels throughout the cell membrane (5). Our study shows that clenbuterol influences Ca2+ homeostasis; it reduces Ca2+ transients and L-type Ca2+ current in rat cardiomyocytes. If there is a constitutive activity of β-ARs, our data fit well with the hypothesis that Gi activation by clenbuterol would decrease cAMP activation and thus reduce the L-type Ca2+ current, Ca2+ transients, and contraction. The L-type Ca2+ current may not be the only mechanism responsible for changes in Ca2+ homeostasis, and investigation of other Ca2+ regulatory mechanisms is warranted.

Lack of effects of clenbuterol in cultured cells. An unexpected result was the lack of effect of clenbuterol on contractility in cardiomyocytes cultured for 48 h. Changes in β-AR regulation of ion transporters have been observed in cardiomyocytes in culture before; however, the mechanisms involved are unknown (26). It has been shown that disruption of cytoskeletal integrity can impair Gi-mediated signaling due to displacement of Gi proteins (2). It was found that muscarinic inhibition of L-type Ca2+ current was absent in cardiomyocytes with defective Gi protein coupling (2). Previously, it has been also shown that subunits of cardiac L-type Ca2+ channels are expressed and colocalized in intact cardiomyocytes along cell (5) or even t-tubule membranes (14). In our study, we tested the hypothesis that detubulation could be the cause for this lack of effect. We produced a degree of detubulation using formamide (9), similar to the levels obtained with 48-h culture. However, response to clenbuterol (30 μM) was decreased only in 48-h cultured cells, but not in the formamide-treated group. This suggests that disruption in the t-tubular structure is not responsible. We have also measured the level of Gi protein in cardiomyocytes that were either freshly isolated or cultured, with or without clenbuterol, and we did not find any difference between the groups. Other factors, including the Gi-dependent localization of the signaling pathway, may be important during culture and need further studies.

Role of clenbuterol in patients with HF treated with LVADs. The finding that clenbuterol acts predominantly via β1-AR-independent pathways, which include Gi, should be considered when studying the role of clenbuterol in patients with HF treated with LVADs, in light of the cardiotoxic effects elicited by β1-AR stimulation and by the prosurvival and anti-apoptotic pathway elicited by Gi stimulation (7, 11, 47). In vivo and in vitro models have shown that, while chronic β1-AR stimulation is proapoptotic, β2-AR stimulation, because of their activation of Gi, protects cardiomyocytes from apoptotic insults.

Fig. 7. The expression of Gi protein, assessed with the use of Western blotting (representative band shown in A), was similar in freshly isolated CMs, CM cultured for 48 h (CCM), and CM cultured for 48 h with 1 μM CLEN (CLEN-CCM) (B). P = not significant (one-way ANOVA with Newman-Keuls posttest for CMs from 5 animals in each group).

Fig. 8. Effects of CLEN on sarcomere shortening were similar in normal (N) and failing [heart failure (HF)] CMs (HF: 4 wk after coronary artery ligation), with and without the presence of β1-AR antagonist CGP 20712A. *P < 0.05 and **P < 0.01 vs. CLEN; ###P < 0.001 vs. control (two-way ANOVA with Bonferroni posttest for 11–16 cells from 3 animals in each group).
(6). Thus, while the pathways stimulated by clenbuterol depress contractility in an acute scenario, the same pathways may also afford protection for the failing heart in a chronic phase. This may be beneficial for LVAD patients and may promote reverse remodeling of the myocardium. The possible anti-apoptotic effect may also, at least in part, provide a mechanistic basis for the ability of clenbuterol to ameliorate ventricular remodeling in experimental models of HF (40). The chronic effects of stimulating this pathway may also contribute to beneficial changes in cardiac gene expression and metabolism. Our laboratory has previously shown that Ca\(^{2+}\) transients were increased upon chronic stimulation with clenbuterol, and this was associated with increased expression of sarco(endoplasmic reticulum Ca\(^{2+}\)-ATPase 2a and increased oxidative carbohydrate utilization (27, 34). An important limitation of our study is the observation of a high-concentration range for the activation of G\(_i\); while the lack of activation of G\(_i\) could be seen throughout the concentration-response curves, even in the nanomolar range [the reported serum levels in a small series of patients was \(~100\, \text{nM}\) (43)], the activation of G\(_i\) could not be detected at below micromolar range. However, one should consider that, in the present study, we use the reduction in contractility as a biological assay, and this may not be very sensitive. G\(_i\) may still be activated at lower doses but not capable to produce a significant reduction in contractility. It is possible that the effects of G\(_i\) activation are less efficient on the mechanisms of excitation-contraction coupling than those required for other G\(_i\) actions, such as activation of genomic, prosurvival, and anti-apoptotic pathways. More experiments are required to investigate this specific point. Another limitation of our results in relation to the clinical observations during LVAD treatment is given by the species differences related to \(\beta\)-AR activation. More studies are required to establish the role and signaling pathway of clenbuterol in failing human heart tissue.

In conclusion, acute exposure of isolated rat cardiomyocytes to clenbuterol does not activate the G\(_i\) pathway and, at high concentrations, significantly depresses contractility, amplitude of Ca\(^{2+}\) transients, and L-type Ca\(^{2+}\) current, an effect not seen with fenoterol or salbutamol. Clenbuterol predominantly acts through the PTX-sensitive G\(_i\) protein, but a direct link to the \(\beta\_2\)-AR could not be demonstrated. Activation of the downstream signaling G\(_i\) pathway may explain the beneficial effects observed during chronic administration of clenbuterol in patients treated with LVADS.

GRANTS
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