Coregulation of Calcium Channels and Beta-adrenergic Receptors in Cultured Chick Embryo Ventricular Cells

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Abstract

To examine mechanisms whereby the abundance of functional Ca channels may be regulated in excitable tissue, Ca channel number was estimated by binding of the dihydropyridine (DHP) antagonist ^{3}H(+)PN200-110 to monolayers of intact myocytes from chick embryo ventricle. Beta adrenergic receptor properties were studied in cultured myocytes using [^{3}H]-CGP12177, an antagonist ligand. Physiological correlates for alterations in DHP binding site number included ^{45}Ca uptake and contractile response to ($\pm$)BAYk 8644, a specific L-type Ca channel activator. All binding and physiological determinations were performed in similar intact cell preparations under identical conditions. 4-h exposure to 1 μM isoproterenol reduced cell surface β-adrenergic receptor number from 44$\pm$3 to 17$\pm$2 fmol/mg ($P < 0.05$); DHP binding sites declined in number from 113$\pm$25 to 73$\pm$30 fmol/mg ($P < 0.03$). When protein kinase A was activated by a non–receptor-dependent mechanism, DHP binding declined similarly to 68% of control. Exposure to diltiazem, a Ca channel antagonist, for 18–24 h had no effect on number of DHP binding sites. After 4-h isoproterenol exposure, ^{45}Ca uptake stimulated by BAYk 8644 declined from 3.3$\pm$0.2 nmol/mg to 2.9$\pm$0.3 nmol/mg ($P < 0.01$) and BAYk 8644-stimulated increase in amplitude of contraction declined from 168$\pm$7 to 134$\pm$11% ($P = 0.02$). Thus, elevation of [cAMP] in myocytes is associated with a time-dependent decline in Ca channel abundance as estimated by DHP binding and a decline in physiological responses that are in part dependent on abundance of Ca channels. Binding of a directly acting Ca channel antagonist for 18–24 h does not modulate the number of DHP binding sites.

Introduction

Cardiac myocytes contain voltage-sensitive, L-type calcium channels that play a pivotal role in regulation of excitation-contraction coupling. (1, 2). Calcium channels are known to be present in the sarcolemma, although the exact locus relative to t-tubules and sarcoplasmic reticulum in heart is uncertain. It is not known whether there are also intracellular pools of calcium channels. Among the important gaps in our understanding of the role of calcium channels in control of excita-

tion-contraction coupling and excitation-secretion coupling is the limited understanding of factors that determine how many functional calcium channels are present in the sarcolemma. Compared with membranes from skeletal muscle, calcium channels in heart are of low abundance, which has hampered studies on processes that regulate channel expression and function. Previous work from this laboratory has demonstrated that during cardiac ontogeny, the number of calcium channels as determined by dihydropyridine (DHP)[1] calcium channel antagonist binding increases. In the developing chick heart, these changes in expression of DHP binding sites occur over the course of days (3). In contrast, for channels present in the sarcolemma at any one moment, gating properties of L-type channels can be modulated over the course of milliseconds to seconds by membrane potential and by drugs (1, 4). It is now well established that β-adrenergic agonist occupancy of the cardiac beta receptor rapidly produces calcium channel phosphorylation and alterations in gating properties through the adenylate cyclase-protein kinase A pathway (5). It is not known whether expression of DHP binding sites and ensemble function of L-type calcium channel in heart can be regulated by tonic exposure to indirect (β-adrenergic) and direct calcium channel effectors. This study investigates those questions.

Previous work on developing skeletal muscle myotubes demonstrated that over the course of days, alterations in β-adrenergic stimulation could alter expression of calcium channels (6). However, the functional role of calcium channels in skeletal muscle cells is poorly understood. It is highly likely that < 1 in 1,000 channels actually participate in calcium flux; the majority may serve as voltage transducers (7). This investigation therefore studied calcium channel regulation in a cardiac model system where the function of Ca channels is more clearly delineated. A cultured chick ventricular myocyte preparation was used that has previously been well characterized with respect to β receptor and calcium channel properties (2, 8–10). We now report that under some conditions in intact heart cells, expression of cell surface β-adrenergic receptors, DHP binding sites, and calcium channel functional properties can be coregulated.

Methods

Tissue culture. Primary monolayer cultures of spontaneously contracting chick embryo ventricular cells were prepared as previously described (8). Briefly, fragments of embryonic chick ventricles 10 d in ovo were dissociated by repeated cycles of trypsinization. The resulting cell suspensions were plated in 35-mm multiwell culture dishes and grown in a culture medium consisting of bicarbonate-buffered physiologic salt solution containing 40% medium 199 (Gibco Laboratories, Grand Island, NY), 6% FCS (Hyclone Laboratories, Logan, UT), and

1. *Abbreviations used in this paper:* DHP, dihydropyridine; IBMX, isobutylmethylxanthine.

54% balanced salt solution containing glucose. Final millimolar concentrations in the culture medium were Na^+, 144; K^+, 4.0; Ca^{2+}, 0.97; HCO_3^-, 18; Mg^{2+}, 0.8; and Cl^-, 131. Cultures were incubated in a humidified 5% CO_2-95% air atmosphere at 37°C. Spontaneously and synchronously contracting confluent monolayers were present by 3 d in culture. For contractility and ion flux experiments, cells were grown on 25-mm circular glass coverslips.

Contractile response measurements. To determine the contractile response of cultured heart cells to (±)-BAYk 8644, measurements of the amplitude of contraction of individual cells in a spontaneously contracting monolayer at 3 d in culture were conducted with the phase-contrast microscope-video motion detector system previously described in detail (8). The medium that superfused cells during contractility measurements was a Hepes-buffered solution containing (in millimeter): Hepes, 5.0; $CaCl_2$, 0.9; KCl, 4.0; NaCl, 140; $MgCl_2$, 0.5; and 2% FCS. Flow rate was 1 ml/min. After a 10-min equilibration period, cells were superfused with 0.1 μM (±)-BAYk 8644. Previous experiments have demonstrated that this concentration produces the peak contractile response in this system (11). The amplitude of contraction was recorded and measured as described by Marsh et al. (8). Previous studies from our own (9, 12) and other laboratories (13, 14) have demonstrated that changes in amplitude and velocity of cell motion can be used to quantify the effects of a variety of interventions on cell contractility.

Loading conditions for contraction caused by attachment of the cell to the coverslip and other cells, as well as the viscoelastic properties of each cell, may vary from cell to cell. However, by expressing the contractile response of the cell as a percentage of its maximal or basal response, these differences in loading conditions are taken into account and each cell can serve as its own control (12). Previous studies from this laboratory have demonstrated that this method of assessing the inotropic state of cells in monolayer culture correlates closely with other physiologic parameters expected to relate to inotropic state, such as alterations in monovalent and divalent cation fluxes (9). The contractility measurements were made on only one cell per coverslip. Several coverslips were used from one plating; all experimental points comprise data from two or more platings.

CGP 12177 binding. Ligand binding experiments using [^{3}H]CGP 12177 were conducted on intact spontaneously contracting myocardial cells identical to those used for contractility measurements. Before ligand binding experiments, culture medium was aspirated and 2 ml of prewarmed Hepes-buffered physiological salt solution, with composition identical to that for contractility studies except that FCS was omitted, was added to the cells. This medium also contained 1 mM ascorbate to inhibit oxidation of isoproterenol. To initiate ligand binding, prewarmed buffer containing appropriate concentrations of [^{3}H]-CGP 12177 were added to the cells with or without graded concentrations of 2 μM±propranolol. Binding was terminated by rapidly washing the wells with buffer at 4°C. Control experiments confirmed that ascorbate was without intrinsic effect on ligand binding or contractile response.

For all binding experiments, at the conclusion of the wash step, cells were lysed and harvested by adding 2 ml of SDS and 1% NaOH to each well, incubating at 37°C for 2 h, and taking aliquots for scintillation counting and protein determination (Lowry method). This procedure was also conducted on wells without cells to correct for ligand binding to the plastic, which was typically 0.01% of total counts.

cAMP measurements. To assess alterations in cellular cAMP content, intact cells on culture plates were exposed to graded concentrations of isoproterenol at 37°C in the same medium that was used for binding experiments and contractility studies; phosphodiesterase inhibitors were not used. cAMP production was stopped at 90 s by addition of 10% TCA, preliminary experiments having shown peak cAMP content by this time. Cells and supernatant were harvested and cAMP was assayed by the RIA method of Harper and Brooker (15).

(+)PN200-110 binding experiments. For determination of equilibrium binding properties of (+)-[^{3}H] PN200-110, monolayers of cultured ventricular cells were washed twice in 37°C Hepes buffer identi-cal to that for CGP 12177 binding studies. Graded concentrations of radioligand with or without unlabeled (+)PN200-110 (1 μM) were added and the monolayer was then incubated at 37°C for 1 h. Unbound (+)-[^{3}H] PN200-110 was removed by washing the monolayer twice with Hepes buffer at 37°C; cells were removed from the substrate by treatment with 0.04% trypsin at 37°C for 30 min. Aliquots of cells suspensions were counted at 42% efficiency in a liquid scintillation counter (LKB Instruments, Gaithersburg, MD).

Equilibrium binding data were analyzed by a modification of the iterative, nonlinear least squares method of Munson and Rodbard using a VAX 780 computer as previously described (10, 11). This program uses initial estimates for agonist and antagonist K_d values, B_{max}, and nonspecific binding for each ligand to solve a series of linear equations that completely describe equilibrium binding isotherms or a competition binding curve. The sum of squares error between the actual data points and curve described by the initial parameters is calculated. The program then iteratively adjusts the parameters, solves the equations, and calculates the sum of squares error until parameters are identified that optimally describe the data. Convergence is accepted when the last iteration improves the error by < 0.01%. Binding curves are initially fit to a one-site model, then to a two-site model. Goodness of fit to a one or two affinity state model is compared by the F test. If the two-site model improves the fit at $P < 0.05$, then the single site model for the ligand is rejected. In this manner, affinities for antagonist or agonist as well as B_{max} and nonspecific binding can be directly estimated without applying simplifying assumptions.

Each point was assayed in triplicate, and each individual binding curve was replicated at least three times. All equilibrium dissociation constants are expressed as the mean±SEM.

Calcium flux measurement. The procedures for measuring calcium fluxes have been described in detail by Biedert et al. (9) and Barry and Smith (16). For determination of calcium uptake, cells were first incubated in Hepes buffer described above, along with the dihydropyridine for 5 min. Initial experiments indicated that (+)-PN200-110 and (±)-BAY k 8644 at the concentrations used reached binding equilibrium in 5 min.

The cells were then transferred to buffer containing the same concentration of the dihydropyridine and ^{45}Ca (5 μCi/ml; [Ca] 0.9 mM) for 60 s. The $t_{1/2}$ for equilibration of the rapidly exchangeable calcium pool in this experimental protocol is 11 s (9). Therefore, the 60-s incubation in ^{45}Ca labels > 95% of the rapidly exchangeable calcium pool without an appreciable contribution from the slowly exchangeable pool. Cells were washed twice for 8 s each by gently agitating the glass coverslips sequentially in two 60-ml volumes of ice-cold balanced salt solution. Cells were then dissolved in medium containing 1% SDS and 10 mM sodium borate and aliquots were assayed for radioactivity.

Materials. The (+) enantiomer of [^{3}H]PN200-110 was obtained from Amersham Corp., Arlington Heights, IL. ^{45}Ca was obtained from New England Nuclear, Boston, MA. Miles Laboratories, West Haven, CT, kindly supplied (±)-BAY k 8644, verapamil was a gift of Searle, Skokie, IL, and Sandoz Ltd., Basel, Switzerland, supplied unlabeled (+) enantiomer of PN200-110. [^{3}H]CGP12177 was from Amersham Corp. Other reagents were from Fisher Scientific, Springfield, NJ.

Dihydropyridines were dissolved in 70% ethanol at a concentration of 10^{-2} M for BAY k 8644 and 10^{-3} for PN200-110, protected from light, stored a maximum of 3 wk, and subsequently diluted in buffer before use. Control experiments confirmed that the low final ethanol concentration by itself had no measureable effect.

Results

Agonist-induced beta-adrenergic receptor regulation. [^{3}H]CGP 12177 bound to the intact cultured ventricular cells with high affinity. We have previously reported that CGP 12177 has an affinity of 0.41±0.08 nM in this intact cultured cell system at 37°C (10). Equilibrium binding isotherms were uniformly monophasic (saturable) and were best described by a single-af-

finity state for CGP 12177 (Fig. 1). Binding was typically > 70% specific at ligand concentrations near the K_d. For naive cells, the number of β-adrenergic receptors identifiable on the surface on the intact cells was 44±3 fmol/mg protein.

Exposure to 1 μM isoproterenol for 4 h produced down-regulation of cell surface β-adrenergic receptors. After 4 h incubation with isoproterenol and three washes of the intact cells at 4°C, equilibrium binding of CGP 12177 revealed no change in K_d but the B_{max} was reduced to 17±2 fmol/mg ($P < 0.05$). Thus, β receptor occupancy by agonist produces prompt down regulation of the receptors on the surface of intact cells, a finding in accordance with those of Limas and Limas (17) and Stahlen and Hertel (18).

Calcium channel antagonist binding. [^{3}H]PN200-110 equilibrium binding experiments were performed in naive cells and isoproterenol-exposed cells in randomly allocated cell plates from the same culture preparation. PN200-110 bound to intact cells with K_d = 173±24 pM and B_{max} = 113±25 fmol/mg protein. Specific binding was typically 65–80% at PN200-110 concentrations near the K_d. For cells exposed to isoproterenol for 4 h, washed free of isoproterenol and PN200-110 binding determined, there was no change in K_d (185±27pM, $P > 0.05$) but B_{max} was significantly reduced to 73±30 fmol/mg (P = 0.03, Fig. 2). Thus, exposure to a β-adrenergic agonist for 4 h produces a decline not only in cell surface β-adrenergic receptors but also in DHP binding sites in intact cultured ventricular cells studied under identical conditions.

To determine whether regulation of DHP binding sites requires β-adrenergic receptor occupancy or whether elevation of intracellular cAMP by nonreceptor mediated mechanisms is sufficient to produce DHP receptor regulation, intact cultured cells were exposed to 1 × 10^{-4} M isobutylmethylxanthine (IBMX) and forskolin (20 μM). This produced an increase in total cellular cAMP from 0.21±0.04 to 6.0±0.3 nmol/mg protein ($P < 0.01$, n = 4). Cells incubated with IBMX and forskolin for 4 h and paired control plates of cells then had DHP binding sites determined by PN200-110 binding. K_d for PN200-110 was not significantly changed by drug
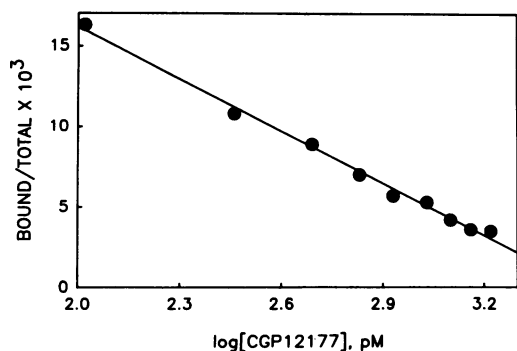
Figure 1. Equilibrium binding of [^{3}H]CGP12177. Graded concentrations of [^{3}H]CGP12177 and intact myocytes were incubated at 37°C in the presence or absence of μM ±2 propranolol to define nonspecific binding. Plotted on the abscissa is log [^{3}H-CGP12177] expressed in picomolar and on the ordinate is bound/total [^{3}H-CGP12177]. For this representative experiment, under control conditions and with each point determined in triplicate K_d = 0.3 nM and B_{max} = 39.6 fmol/mg. This experiment was replicated a total of five times for naive and for isoproterenol-exposed cells. Data were invariably well-described by a single-site model for binding.
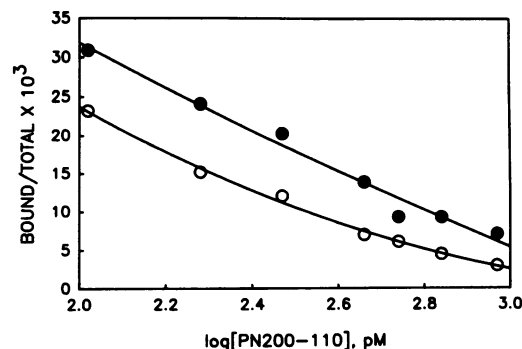
Figure 2. Equilibrium binding of (+)PN200-100. Graded concentrations of [^{3}H]PN200-110 were incubated with intact myocytes at 37°C in the presence or absence of unlabeled (+)PN200-110 (1 μM) to define nonspecific binding. Axes have the same format as in Fig. 1. Closed circles indicate binding to naive cells; open circles indicate binding to cells previously exposed to isoproterenol for 4 h. The slight curvilinear shape of the binding isotherms reflects the contribution of nonspecific binding to the nontransformed data. This component of nonspecific binding is taken into account in the model describing such data. For all experiments, goodness of fit was not significantly improved by a two-site model compared with a one-site model. Each point was determined in triplicate and this representative pair of experiments was replicated a total of eight times. For this experiment for naive cells K_d = 0.10 nM and B_{max} = 77 fmol/mg. For isoproterenol-exposed cells, K_d = 0.14 nM and B_{max} = 56 fmol/mg.

exposure. However, for this series of experiments, the number of DHP binding sites decreased from 192±26 fmol/mg protein in naive cells to 131±27 fmol/mg protein in cells with elevated cAMP content ($P < 0.01$) (Table I).

^{45}Ca *uptake.* To determine whether a decrease in DHP binding sites might be associated with altered calcium uptake kinetics in the cultured heart cells, the initial velocity of ^{45}Ca uptake was determined in naive cultured heart cells and cells that had previously been exposed to 1 μM isoproterenol for 4 h. In this preparation, $\sim$ 30% of calcium uptake into a rapidly exchangeable pool is sensitive to calcium channel antagonists and is likely to be via L-type Ca channels (16). Accordingly,

Table I. Ligand Binding in Intact Cells

	Receptors	Control	Desensitized	% of Control	P
		fmol/mg			
Beta-adrenergic CGP12177 binding					
Isoproterenol exposed	B_{max}	44±3	17±2	39	<0.05
DHP receptors- PN200-110 binding					
Isoproterenol exposed	B_{max}	113±25	73±30	65	<0.03
IBMX and forskolin	B_{max}	192±26	131±27	68	<0.01
Diltiazem exposed	B_{max}	243±21	265±54	109	0.82

Number of binding sites (B_{max}) is expressed in femtomoles/milligram protein. For conditions A and B, cells were exposed to 1 μM isoproterenol for 4 h before ligand binding for desensitization experiment. For IBMX and forskolin, cells were exposed to IBMX and forskolin for 4 h. For diltiazem exposed, cells were exposed to 1 μM diltiazem for 18–24 h. Percent control indicates percentage of ligand binding sites in drug-exposed cells compared with that in matched control cells assayed simultaneously.

small differences in total calcium uptake were sought. To augment the calcium channel–dependent calcium uptake signal, cells were exposed to either control buffer or buffer containing 0.1 μM BAYk 8644, a calcium channel agonist that specifically increases calcium channel–dependent calcium flux. The results are summarized in Table II. Although the difference in ^{45}Ca uptake velocity among the treatment groups is not numerically large, it was highly consistent. For each set of experiments, plates of cells from the same culture were used and assayed in parallel so that paired analysis was possible. For each of six sets of experiments, 0.1 μM BAYk 8644 produced a significant increase in calcium uptake compared with control cells, and for cells first treated with 1 μM isoproterenol for 4 h and then stimulated with 0.1 μM BAYk 8644, ^{45}Ca uptake velocity was less than that for cells not exposed to isoproterenol. This decrease in ^{45}Ca uptake velocity after 4 h isoproterenol exposure was not due to gross calcium overload of the cells, as the total calcium content of cells determined by 5 min uptake of ^{45}Ca was not increased after 4 h isoproterenol exposure. Indeed, the pool of intracellular calcium labelled over 5 min was less, 4,363±206 vs. 3,036±306 dpm ($P < 0.05$, $n = 7$ experiments) after isoproterenol exposure.

To determine whether ^{45}Ca uptake by mechanisms other than via the calcium channel (predominantly Na/Ca exchange) was altered by exposure to isoproterenol, initial ^{45}Ca uptake velocity was determined in the presence or absence of calcium channel blockade. Control cells were incubated in physiological buffer for 4 h, with 1 μM verapamil being present for the last 5 min. ^{45}Ca uptake was then determined for 60 s. Under these conditions, 25±5% of ^{45}Ca uptake was verapamil sensitive. For cells preincubated in isoproterenol for 4 h, 5±9% of ^{45}Ca uptake was verapamil sensitive ($P > 0.05$, $n = 7$ experiments).

Regulation of phenylalkylamine binding sites. To investigate the possibility that binding sites for phenylalkylamine calcium channel antagonists may differ from regulation of DHP binding sites, we attempted to characterize binding of a phenylalkylamine calcium channel antagonist ligand in intact cultured chick embryo ventricular cells. [^{3}H]D888 binding isotherms in intact cells were performed using unlabeled D888 (1 μM) to define nonspecific binding. Unfortunately, in the intact cell preparation, nonspecific binding for the ligand, including binding to the culture dishes, was so high that despite a wide variety of assay conditions examined and a number of maneuvers to reduce nonspecific binding, no technically satisfactory specific binding could be documented.

Homologous regulation by calcium channel effectors. To test the hypothesis that prolonged binding of a calcium channel antagonist to calcium channels in intact cultured heart cells alters the expression of DHP binding sites, we exposed cells to 1 μM diltiazem for 18–24 h and then assayed binding of the DHP ligand PN200-110. The benzothiazipine ligand, diltiazem, was used to block calcium channels so that it would not interfere with the subsequent assay of DHP binding sites. This was of concern because the dissociation of DHP calcium channel ligands from intact cells at 37°C may be very slow (unpublished observation). Exposure of cells to diltiazem for 24 h produced no alteration in expression of PN200-110 binding sites (Table I) and no alteration in K_d for PN200-110.

Contractile physiology. Exposure to 1 μM isoproterenol for 4 h produced a decrease in number of DHP binding sites and in calcium channel–dependent calcium flux. To determine whether this affects the ultimate physiological function for cardiac myocytes, which is contraction, the contractile response of control and isoproterenol-exposed cells to a calcium channel effector was examined under conditions identical to conditions for ligand binding and calcium flux experiments. Basal amplitude of contraction was determined in a medium containing 0.9 mM calcium. When the spontaneously contracting myocytes were exposed to 0.1 μM BAYk 8644, there was a robust increase in amplitude of contraction with no alteration in beating rate. When different coverslips containing cells from the same culture were exposed to 1 μM isoproterenol for 4 h and then superfused with 0.1 μM BAYk 8644, there was also a significant increase in amplitude of contraction, but the response was substantially less than that for cells not previously exposed to isoproterenol (Table III).

Discussion

A large body of evidence has made it abundantly clear that occupancy of the β-adrenergic receptor by an agonist, as well as other mechanisms of elevating intracellular [cAMP] produces phosphorylation of the calcium channel by cAMP–dependent protein kinase (1, 5). Elevation of cAMP content produces an increase in the overall open state probability of individual calcium channels, whereas unitary conductance remains unaffected (19). Dephosphorylation of the calcium channel in cardiac ventricular cells by phosphatases reverses this electrophysiological effect (20). The series of investigations

Table II. ^{45}Ca Uptake in Intact Myocytes

Treatment	^{45}Ca uptake
	nmol/mg protein
Control	3.1±0.2
BAYk 8644 (*0.1 μM*)	3.3±0.2*
Isoproterenol (*1 μM, 4 h*) then	
BAYk 8644 (*0.1 μM*)	2.9±0.3‡

^{45}Ca uptake over 60 s in intact myocytes was studied in identical, paired plates of myocytes from the same culture.
* $P = 0.006$ compared with control.
‡ $P = 0.005$ compared with Bay k 8644 (0.1 μM).

Table III. Contractile Response

Condition	Contractile amplitude
	% of basal
Basal	100
(±)BAYk 8644 (*0.1 μM*)	168±7
Isoproterenol (*1 μM, 4 h*) then	
(±)BAYk 8644 (*0.1 μM*)	134±11*

Basal condition is amplitude of contraction of spontaneously contracting myocytes in 0.9 mM Ca medium. Both BAYk 8644 and isoproterenol + BAYk 8644 produce a significant ($P < 0.01$) increase in amplitude of contraction over basal.
* Contractile amplitude for isoproterenol + BAYk 8644 is significantly less than that for BAYk 8644 ($P = 0.02$).

reported in this paper sought to determine whether tonic stimulation by the β-adrenergic receptor transmembrane signaling pathway or tonic elevation of cAMP content via other mechanisms might produce alterations in expression of DHP binding sites in intact myocardial cells and/or alterations in calcium channel function. This question is not only of fundamental importance to our understanding of Ca channel regulation in a variety of excitable and secretory tissues but is also pertinent to the possibility of calcium channel regulation in clinically important hyperadrenergic states such as congestive heart failure.

Exposure of intact cultured myocardial cells to saturating concentrations of isoproterenol produced downregulation of cell surface β-adrenergic receptors, as would be expected from previous studies from this and other laboratories (10, 12, 21, 22). A significant new finding of this study is that when DHP binding sites are assayed in intact cells after a 4-h incubation with isoproterenol, there is a decrease in the number of DHP binding sites to 65% of control with no change in the affinity of the remaining DHP binding sites for the antagonist ligand. Moreover, when cAMP content is elevated by a non–receptor-mediated mechanism (stimulation of the catalytic subunit of adenylate cyclase with forskolin and inhibition of phosphodiesterase by IBMX), there is a very similar reduction in number of PN200-110 binding sites with no change in affinity for the ligand. These results are summarized in Fig. 3.

Radioligand binding to DHP binding sites is currently the best available method for pharmacologically determining the number of calcium channels on or within an intact cell. Nevertheless, there are limitations to this approach (see below). To determine whether the change in the number of DHP binding sites, which is used as a surrogate for determination of the number of copies of the calcium channel proteins in the cell membrane has physiological importance, an index of calcium channel function was measured in control cells and cells with downregulated calcium channels under conditions identical to those for the binding studies. BAY k 8644 augmentation of ^{45}Ca uptake was used as an index of abundance of functional or recruitable calcium channels. Because only a fraction of calcium uptake in this model system is via the voltage-dependent calcium channel ($\sim$ 25–30%, reference 16), a larger signal to noise ratio could be achieved by using conditions that stimulate calcium channel–dependent flux rather than just measuring basal uptake. BAYk 8644 produced a small but highly reproducible increase in ^{45}Ca uptake velocity at 60 s in naive cells. The ^{45}Ca uptake velocity was substantially less after the cells had been exposed to isoproterenol for 4 h. Control experiments demonstrated that the decrease in BAYk 8644–stimulated ^{45}Ca uptake velocity could not be attributed to calcium overload of the cell.

To bolster further the evidence that β-adrenergic agonist-induced downregulation of the calcium channel had physiological significance, calcium channel agonist-induced increase in amplitude of contraction of the intact cells was studied under conditions identical to those of β-adrenergic receptor binding studies, calcium channel ligand binding, and ^{45}Ca uptake studies. Again, cells exposed to isoproterenol for 4 h had a smaller response to a calcium channel agonist than naive cells (Fig. 3). Moreover, under conditions where the number of DHP binding sites is decreased, and the contractile response to a calcium channel agonist is decreased, the portion of ^{45}Ca uptake sensitive to verapamil tended to decrease (25 vs. 5%). Together, this series of experiments demonstrates that when
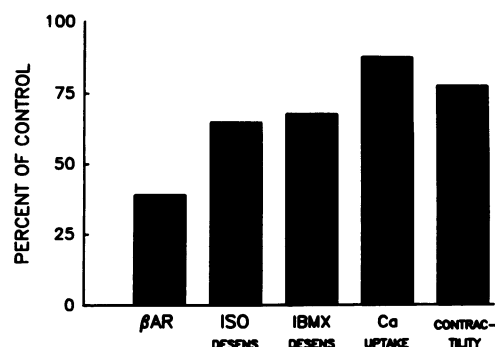

Figure 3. Summary of desensitization effects on ligand binding and physiological function. The ordinate indicates percent of control ligand binding site number or percent of physiological function. 100% is that observed in naive cells. Data show the effect of 4-h exposure to isoproterenol or IBMX. BAR, beta-adrenergic receptor number. ISO desens, number of DHP binding sites after isoproterenol exposure. IBMX desens, number of DHP binding sites after exposure to IBMX and forskolin. Ca uptake, ^{45}Ca uptake at 60 s after exposure to isoproterenol for 4 h. Contractility, contractile response to BAYk 8644 after exposure to isoproterenol for 4 h. Elevation of cAMP by beta receptor-dependent or -independent mechanisms produces a similar decline in number of DHP binding sites. ^{45}Ca uptake and contractile amplitude are determined only in part by number of Ca channels; isoproterenol exposure produces a smaller decline in these physiological parameters.

cAMP content of cultured myocardial cells is elevated over 4 h, the number of DHP binding sites decline in concert with a decline in calcium channel physiological function. Note that there is no change in affinity of the remaining DHP binding sites for the antagonist ligand after isoproterenol exposure. This argues against modulation in the conformation of the DHP receptor site as a cause for the decline in the number of binding sites.

Determination of DHP binding sites has been taken as a marker for the number of calcium channels in the intact heart cells. There are limitations to this interpretation. Alterations in membrane potential may change the apparent number of binding sites (11, 23). However, if isoproterenol were to produce depolarization of these cells, that would tend to increase the number of binding sites instead of decrease it, as was in fact observed. Moreover, the contractility experiments confirm that the cells continue to beat, which excludes major depolarization. The basal number of Ca channels (or DHP binding sites) displayed some drift during the course of these studies. Individual groups of experiments were closely grouped in time, were paired, and demonstrated acceptable variation around the mean value. However, over the course of months, as different flocks of breeder chicken were used as the source of chick embryos and different lots of serum were used for cell culture, the absolute number of DHP binding sites in control cells did vary somewhat. One possible cause for this is alteration in the lipid composition of the cell membrane for cells grown in various batches of FCS. There is now evidence that alterations in cholesterol content of the sarcolemma can regulate expression of muscarinic cholinergic receptors and β adrenergic receptors (24). The calcium channel may also be affected.

An additional finding of this study is that regulation of DHP binding sites appears to be purely heterologous; we could

find no evidence for homologous desensitization. Blockade of the calcium channel by diltiazem for 18–24 h produced no change in DHP binding site number. We have previously reported that in intact cells, unlike membrane preparations, diltiazem does not produce augmented DHP binding (8). Nevertheless, we cannot absolutely exclude the possibility that the diltiazem produced augmented DHP binding to a reduced number of calcium channels. Use of a DHP ligand to produce sustained Ca channel blockade in an attempt to produce homologous desensitization was not feasible, as there would almost certainly be retained ligand that would interfere with subsequent binding assays. Allosteric modulation of DHP binding by diltiazem has not been observed in our experimental system (8). Longer term homologous desensitization experiments will be necessary to absolutely exclude DHP receptor regulation by this mechanism over a slower (days) time course.

Calcium channel function is regulated over a time course of seconds to minutes by drugs and membrane potential (4). What previously existing evidence is there that cardiac calcium channels can be regulated over a longer time course? In heart, the evidence is limited, but there is a broader base of evidence for long-term calcium channel regulation in other systems. Previous work from this laboratory (25) has demonstrated that growth of cultured myocardial cells in excess thyroid hormone for 2–3 d causes an increase in the expression of DHP binding sites and an increase in calcium channel function. There is also evidence that during cardiac ontogeny the number of DHP binding sites increases (3). In developing skeletal muscle myotubes, calcium channel abundance is regulated by growth and long-term exposure to β-adrenergic effectors also modulates calcium channel number and function. Schmid et al. found that after prolonged isoproterenol exposure (2–4 d) cultured myotubes showed an increase in B_{max} and a decrease in K_d for a DHP antagonist (6). In a similar skeletal muscle myotube system, activation of protein kinase C has been reported to modulate DHP receptors (26) and in a smooth muscle cell line, differentiation, mitogens, and oncogenes can regulate the expression of voltage-gated calcium channels (27). Thus, it is clear that not only are calcium channel gating properties subject to rapid regulation, but over a prolonged time course, with the opportunity for alterations in gene transcription, translation, or posttranslational modification, calcium channel expression and function can also be modulated. Prolonged stimulation of the β-adrenergic receptor-adenylate cyclase transmembrane signaling pathway not only can change calcium channel gating properties but the current data demonstrate that over the time course of hours, a β-adrenergic agonist can alter the expression of DHP binding sites and indices of calcium channel function. Thus, over the course of hours changes in sympathetic tone may alter expression of cardiac Ca channels critical to excitation-contraction coupling, with important sequelae for physiological function.

Little is known about the turnover rate of the calcium channel protein in myocardial cells. The 4-h time course for regulation of DHP binding sites and Ca channel function demonstrated here is probably too rapid to be attributable to new protein synthesis. Elevation of cellular cAMP content can affect posttranslational modification of glycoproteins (28), possibly including the calcium channel. However, the observed time course may also be too rapid for that putative mechanism of channel regulation as well. Alteration in the phosphoryla-

tion state of the calcium channel is the most likely cause for loss in DHP binding sites and decline in calcium channel function. The calcium channel contains phosphorylation sites (7). One might postulate that sustained phosphorylation of the calcium channel may lead to its internalization. The most likely mechanism is prolonged activation of cAMP-dependent protein kinase (5). For another sarcolemmal structure, the β-adrenergic receptor, sustained phosphorylation by cAMP-dependent kinase and β receptor kinase has been demonstrated to uncouple the β receptor and promote its translocation into the cytosol (29–31). To confirm this hypothesis, it will be necessary to demonstrate sustained phosphorylation of cardiac calcium channels that are translocated out of the sarcolemma.

There are several areas of agreement regarding regulation of the abundance and function of cardiac calcium channels. First, it is evident that calcium channel gating properties are modulated by phosphorylation of the channel by cAMP-dependent protein kinase (5). Second, the data presented here demonstrate that elevation of cAMP in intact myocardial cells for 4 h produces a decrease in abundance of DHP binding sites and a decrease in indices of calcium channel function. Third, there is now evidence that G_s can directly modulate calcium channel function by a mechanism independent of cAMP-dependent protein kinase (32). It has also been established that over the course of days, in a process requiring protein synthesis, excess thyroid hormone can lead to increase in expression of DHP binding sites and calcium channel–dependent calcium flux (25). Unresolved issues regarding calcium channel regulation include major uncertainties about the mechanism of calcium channel recruitment and cellular mechanisms underlying the disappearance of DHP binding sites with concomitant decline in calcium channel function. It is unknown if there are silent calcium channels present in the sarcolemmal membrane that are not identifiable by DHP ligands and whether there are intracellular pools of calcium channels as well. The findings of this study do support the postulate that cardiac calcium channels are in a dynamic state and can be regulated by prolonged exposure to indirectly acting calcium channel effectors but not by exposure to a benzothiazipine calcium channel antagonist.

Acknowledgments

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