G_i-Dependent Localization of β_2-Adrenergic Receptor Signaling to L-Type Ca^{2+} Channels

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ABSTRACT A plausible determinant of the specificity of receptor signaling is the cellular compartment over which the signal is broadcast. In rat heart, stimulation of β_1-adrenergic receptor (β_1-AR), coupled to G_s-protein, or β_2-AR, coupled to G_s- and G_i-proteins, both increase L-type Ca^{2+} current, causing enhanced contractile strength. But only β_1-AR stimulation increases the phosphorylation of phospholamban, troponin-I, and C-protein, causing accelerated muscle relaxation and reduced myofilament sensitivity to Ca^{2+}. β_2-AR stimulation does not affect any of these intracellular proteins. We hypothesized that β_2-AR signaling might be localized to the cell membrane. Thus we examined the spatial range and characteristics of β_1-AR and β_2-AR signaling on their common effector, L-type Ca^{2+} channels. Using the cell-attached patch-clamp technique, we show that stimulation of β_1-AR or β_2-AR in the patch membrane, by adding agonist into patch pipette, both activated the channels in the patch. But when the agonist was applied to the membrane outside the patch pipette, only β_1-AR stimulation activated the channels. Thus, β_1-AR signaling to the channels is diffusive through cytosol, whereas β_2-AR signaling is localized to the cell membrane. Furthermore, activation of G_i is essential to the localization of β_2-AR signaling because in pertussis toxin-treated cells, β_2-AR signaling becomes diffusive. Our results suggest that the dual coupling of β_2-AR to both G_s- and G_i-proteins leads to a highly localized β_2-AR signaling pathway to modulate sarcolemmal L-type Ca^{2+} channels in rat ventricular myocytes.

INTRODUCTION

How myriad receptors coupling to a small number of G-proteins and sharing common second messengers can have highly specific effects is a fundamental question of cell signaling. As a distinctive example, stimulation of β_1- or β_2-adrenergic receptor subtype (β_1-AR, β_2-AR) in rat ventricular myocytes activates G_s-protein, leading to activation of adenylate cyclase, generation of cAMP, and activation of protein kinase A (PKA; Mcdonald et al., 1994; Xiao and Lakatta, 1993; Skeberdis et al., 1997a; Kuznetsov et al., 1995). The consequent PKA phosphorylation of L-type Ca^{2+} channels increases the Ca^{2+} influx during depolarization, augments the intracellular Ca^{2+} transient, leading to enhanced contractile strength. However, only β_1-AR stimulation causes substantial phosphorylation of phospholamban, which accelerates Ca^{2+} sequestration into sarcoplasmic reticulum (SR), resulting in hastened muscle relaxation and SR-generated arrhythmogenic spontaneous Ca^{2+} oscillations (Xiao and Lakatta, 1993; Xiao et al., 1994; Xiao et al., 1995; Altschuld et al., 1995); and of troponin-I and C-protein, which reduces myofilament sensitivity to Ca^{2+} (Kuschel et al., 1999a). The global effect of β_1-AR stimulation on multiple target proteins in both cell membrane and intracellular organelles is consistent with the classic notion of β-adrenergic signaling, in which the Gs-coupled receptor signaling is mediated by a diffusive cAMP/PKA pathway. In contrast to the multiple effects of β_1-AR stimulation, β_2-AR stimulation seems to specifically activate L-type Ca^{2+} channels, without affecting the aforementioned intracellular proteins. Two immediate questions arise from the differential effect of β_2-AR versus β_1-AR. What causes β_2-AR stimulation to specifically affect L-type Ca^{2+} channels? Does β_2-AR stimulation affect the channel in the same manner as β_1-AR stimulation? This paper is focused on answering these two questions.

We hypothesized that β_2-AR signaling might be localized to the cell membrane compartment, and hence affect only the Ca^{2+} channels in the membrane, but not intracellular proteins distant to the membrane. Localized signal propagation may serve as an important mechanism for targeting receptor signaling to specific subcellular domains. However, little is known about the subcellular localization of signaling because it is difficult to quantify the spatial range of signal propagation. In this study, we intend to decipher the specific effect of β_2-AR by comparing the spatial ranges of β_1-AR and β_2-AR signal propagation.

We used cell-attached patch-clamp technique to isolate a small patch of cell membrane ($\sim 1\ \mu m^2$) from the rest of the cell membrane to create two isolated membrane compartments: the patch membrane and the surrounding membrane. We then measured the single L-type Ca^{2+} channel activity

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inside the patch and monitored the effect of stimulating the receptors either in the surrounding membrane by adding agonist into bath (remote receptor stimulation), or in the patch membrane by adding agonist into pipette (local receptor stimulation). This approach allows us to examine whether remote receptor stimulation in the surrounding membrane can affect the channels in the patch membrane and how does the effect of remote receptor stimulation compare to that of local receptor stimulation. (Soejima and Noma, 1984) This approach also allows us to study, in detail, how the single channel gating kinetics are modulated by β_2-AR or β_1-AR stimulation and whether the two receptor subtypes affect the channels in a similar manner. The results of our study clearly indicate that β_2-AR signaling to the channels is indeed localized to the membrane vicinity, whereas β_1-AR signaling to the channels is diffusive through the cytosol. This finding explains why β_2-AR signaling specifically activates the L-type Ca^{2+} channels without substantially affecting the intracellular proteins.

To study the signaling mechanism underlying the localized β_2-AR signal propagation, we examined the role of G_i-proteins. Previous studies show that whereas β_1-AR couples exclusively to G_s-protein, β_2-AR couples dually to G_s- and G_i-proteins (Xiao et al., 1995; Daaka et al., 1997; Xiao et al., 1999). It is known that G_i counteracts the G_s-coupled activation of adenylate cyclase, reducing the production of cAMP in some cell types (Gilman, 1987; Wong et al., 1991; Katada et al., 1987). The interplay of G_s and G_i signaling has been clearly demonstrated in the cross-talk of different receptor families. For example, stimulation of G_i-coupled muscarinic receptors counteracts the positive inotropic effect of G_s-coupled β-adrenergic stimulation (Levy et al., 1981; Gupta et al., 1994; Zhang et al., 2000). However, β_2-AR presents an interesting case in which the receptor couples to both G_s- and G_i-proteins, generating cross-talk between the two signaling pathways originating from a single receptor (Xiao et al., 1999). In this study, we examined the role of G_i in the β_2-AR signaling to L-type Ca^{2+} channels. Our data suggest that G_i activation is essential to the localization of β_2-AR signaling.

MATERIALS AND METHODS

Cell preparation

Rat ventricular myocytes were isolated from 2- to 4-month-old Wistar rats using a standard enzymatic technique (Xiao et al., 1995). Cells were dispersed in the HEPES buffer containing (in mM) NaCl 137, KCl 5, dextrose 15, $MgSO_4$ 1.3, NaH_2PO_4 1.2, HEPES 20, $CaCl_2$ 1, with pH 7.4 adjusted using NaOH. For pertussis toxin (PTX) treatment, the cells were incubated in 1.5 μg/ml PTX at 37°C for 3 h. Experiments were performed at room temperature of 20–22°C.

Single channel recording

Single L-type Ca^{2+} channel activity was recorded using the cell-attached patch clamp technique on a electrophysiology setup consisting of an AxoPatch 200B patch clamp amplifier (Axon Instruments, Inc., Foster City, CA), a Digidata 1200 analog/digital converter (Axon Instruments), and a IBM compatible personal computer. The bath solution contained (in mM) potassium aspartate 110, KCl 30, $MgCl_2$ 3.8, $CaCl_2$ 1.2, EGTA 5, HEPES 5, glucose 10, and Mg-ATP 2, with pH 7.4 adjusted using KOH. The pipette solution contained (in mM) $BaCl_2$ 100, TEACl 20, and HEPES 10, with pH 7.4 adjusted using TEAOH. The solutions containing drugs were made by adding the drug stock solution into the pipette or bath solution, at no less than 100 times dilution. CGP 20712A (CGP) was provided by Ciba-Geigy Corp. (Basel, Switzerland). ICI 118,551 (ICI) was provided by Imperial Chemical Industry (UK). Zinterol was provided by Bristol-Myers-Squibb (Stamford, CT). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

The patch membrane potential was held at −80 mV or −70 mV, depolarized to 0 mV in a step pulse for 150 ms, then repolarized to the holding potential for 850 ms before the next pulse. The current signal was filtered through a 4-pole lowpass Bessel filter at a cutoff frequency of 1 KHz, digitized at a sampling rate of 10 KHz, and recorded on the computer hard disk.

Data analysis

Single channel activities were analyzed using the pClamp software package (Axon Instruments) and a home written program for open probability calculation. No digital filtering was used. The linear leak current and capacitive transient was subtracted using averaged blank sweeps. Events were detected using the half amplitude criterion. To avoid potential bias in selecting records, we accepted all the records that were long enough (>200 sweeps) to reflect the average channel behavior, except those with noisy or drifting baselines. We calculated the channel open probability (NPo) as the ratio of open time to the total time, during 150 ms depolarization pulse in each sweep. We then calculated the average open probability from all the sweeps in an entire record. In order to compare the channel activities in different patches, we estimated the total number of channels in a patch by counting the maximum number of overlapped openings at high depolarization voltages of 30 mV. We then normalized the average open probability to per single channel (Po). The total number of channels in a patch so obtained might be underestimated in the control condition due to low Po. However, this potential error is less likely to occur under drug application, because these drugs increase Po (see results). Furthermore, this normalization is not necessary for studying the effects of drug applied in bath, for the comparison was made on the same patch. In any case, the potential error in estimating the number of channel does not affect the major conclusions regarding drug effects.

We characterized single channel gating kinetics using three gating modes: mode-0, mode-1, and mode-2 (Hess and Tsien, 1984). We did not include very short mode-0a openings (Yue et al., 1990) because they probably make little contribution to Po. The open dwell time histograms are well fitted to a sum of two exponential functions. The first exponential fitting gives a mean open time of mode-1 events about $\tau = 0.45$ ms. The second exponential fitting varies greatly from record to record, due to large statistical fluctuations in a small total number of mode-2 events (<50 in most records); hence, we calculated the arithmetic mean open time instead. To group the open events to mode-1 and mode-2, we used a transition criterion of 4 ms open dwell time, where the two exponential fitting lines intersect in the log plot of histogram. The frequency of mode-1 or mode-2 events (number of open events per sweep) were then calculated according to this grouping, and normalized to per channel in multi-channel patches. Note that we calculated the number of open events per sweep instead of closed times, because the latter is prone to the error introduced by the missing events and the number of channels in the patch. The frequency of mode-0 events (blank sweep %) is obtained from the patches containing only a single channel. The availability is then calculated as (100 − blank sweep)%.

The data for each experimental condition were averaged, and reported as mean ± standard error. Student's *t*-test was used to evaluate the statistical significance of the change in mean value. We used paired *t*-test to compare the data from the same patch, i.e., remote receptor stimulation versus control, and unpaired *t*-test to compare the data from different patches, i.e., local receptor stimulation versus control.

RESULTS

In order to study the spatial range of signal propagation, we used on-cell patch-clamp technique to create two separate membrane compartments on an intact cell: the patch membrane sealed in (20–50 GΩ) by the pipette (~1 μm^2), and the surrounding membrane outside the pipette. Because in this configuration the only route connecting the two membrane compartments is via the cytosol, if remote receptor stimulation in the surrounding membrane affects the channels in the patch membrane, the signaling molecules are probably diffusive through the cytosol. If the channels can only be affected by local receptor stimulation inside the patch membrane, it would suggest a localized signaling in the membrane vicinity.

Lack of an effect of remote β_2-AR stimulation on the channels

Previous experiments using whole-cell voltage-clamp technique have shown that the whole cell L-type Ca^{2+} current in rat ventricular myocytes is markedly increased by using zinterol to selectively stimulate β_2-AR (Xiao and Lakatta, 1993; Zhou et al., 1997). A similar effect is seen by using norepinephrine to selectively stimulate β_1-AR (Xiao and Lakatta, 1993). The sample traces of the above experiments (Fig. 1, *A* and *C*) illustrate that at whole cell level, a global stimulation of either β_2-AR or β_1-AR augments the macroscopic L-type Ca^{2+} current. Whole cell current measurements do not, however, shed light on whether β_1-AR or β_2-AR stimulation act locally or globally. To study the range of signal propagation in subcellular domains, we tested whether stimulation of remote receptors in the surrounding membrane can activate the channels in the patch via a diffusive pathway through the cytosol.

To provide a frame of reference, we first measured the basal level single channel activity in absence of receptor stimulation. Under control condition, most channels displayed sparse basal level activity, as shown in the sample traces (Fig. 1 *B*). The single channel conductance is ~25 pS with Ba^{2+} 100 mM as charge carrier, and the unitary current is ~0.84 pA at 0 mV depolarization. Fig. 1 *B* shows the history of channel activity in a plot of NPo per sweep (1 s interval between two subsequent sweeps). Because of the stochastic nature of single channel activity, the NPo per sweep fluctuates along time. Hence, we use the average Po (calculated as the arithmetic average of Po per sweep for entire record containing 200 to 900 sweeps) to assess the overall channel activity (see Methods). The L-type Ca^{2+} channels showed an average Po of 1.45 ± 0.12% (average ± standard error, 14 cells) under the control condition. More detailed analysis on single channel gating kinetics will be presented later when relevant to the argument.

Maximal stimulation of remote β_2-AR, by bath application of zinterol 10 μM (Zint) (Skeberdis et al., 1997a; Xiao et al., 1994; Zhou et al., 1997), did not cause discernable change in the channel activity (Fig. 1 *B*). In paired experiments in 5 cells, the average Po is 1.56 ± 0.19% under control condition and 1.35 ± 0.25% following remote β_2-AR stimulation. Thus, Po before and after drug application is not significantly different (*t*-test $P = 0.3$).

In contrast, remote β_1-AR stimulation by bath application of norepinephrine 10 μM and prazosin 2 μM (NE + Praz) clearly increased the channel activity (Fig. 1 *D*). The average Po increased from 1.51 ± 0.36% to 3.94 ± 0.36% following remote β_1-AR stimulation ($P = 0.02$, 4 cells).

To double-check the differential effects of remote β_2-AR and β_1-AR stimulation, we did similar experiments using isoproterenol 1 μM in combination with CGP 0.3 μM (Iso + CGP) to selectively stimulate β_2-AR, or in combination with ICI 0.1 μM (Iso + ICI) to selectively stimulate β_1-AR (Xiao and Lakatta, 1993). Consistent with the zinterol experiment, stimulation of remote β_2-AR by Iso + CGP did not significantly alter channel activity (Po of 1.48 ± 0.06% before and 1.81 ± 0.16% after drug application, *t*-test $P = 0.28$, 3 cells). Fig. 1 *E* depicts an experiment where the channel NPo did not show discernable change following remote β_2-AR stimulation by Iso + CGP; however, showed significant increase after a subsequent remote β_1-AR stimulation by Iso + ICI in the very same cell.

The results of these experiments consistently demonstrate that remote β_2-AR stimulation does not affect the channel activity, whereas remote β_1-AR stimulation activates the channels, most likely via a diffusive signaling pathway through the cytosol. The absence of an effect of remote β_2-AR stimulation on the single channel activity, in the presence of a robust effect of global β_2-AR stimulation on the whole cell L-type Ca^{2+} current, suggests that β_2-AR signaling might be localized to the membrane receptor vicinity.

Effect of local β_2-AR stimulation on the channel activity

To test whether β_2-AR signaling is membrane delimited, we compared single L-type Ca^{2+} channel activity in the absence (control condition) or presence of the agonist in the pipette. Under control condition, most channels displayed low basal level activity (Fig. 2 *A*). The average Po is 1.45 ± 0.12% (14 cells). Stimulation of local β_2-AR, by including zinterol 10 μM (the same concentration used for the remote β_2-AR stimulation experiments) in the pipette, effectively activated the channels (Fig. 2 *B*). The average Po markedly

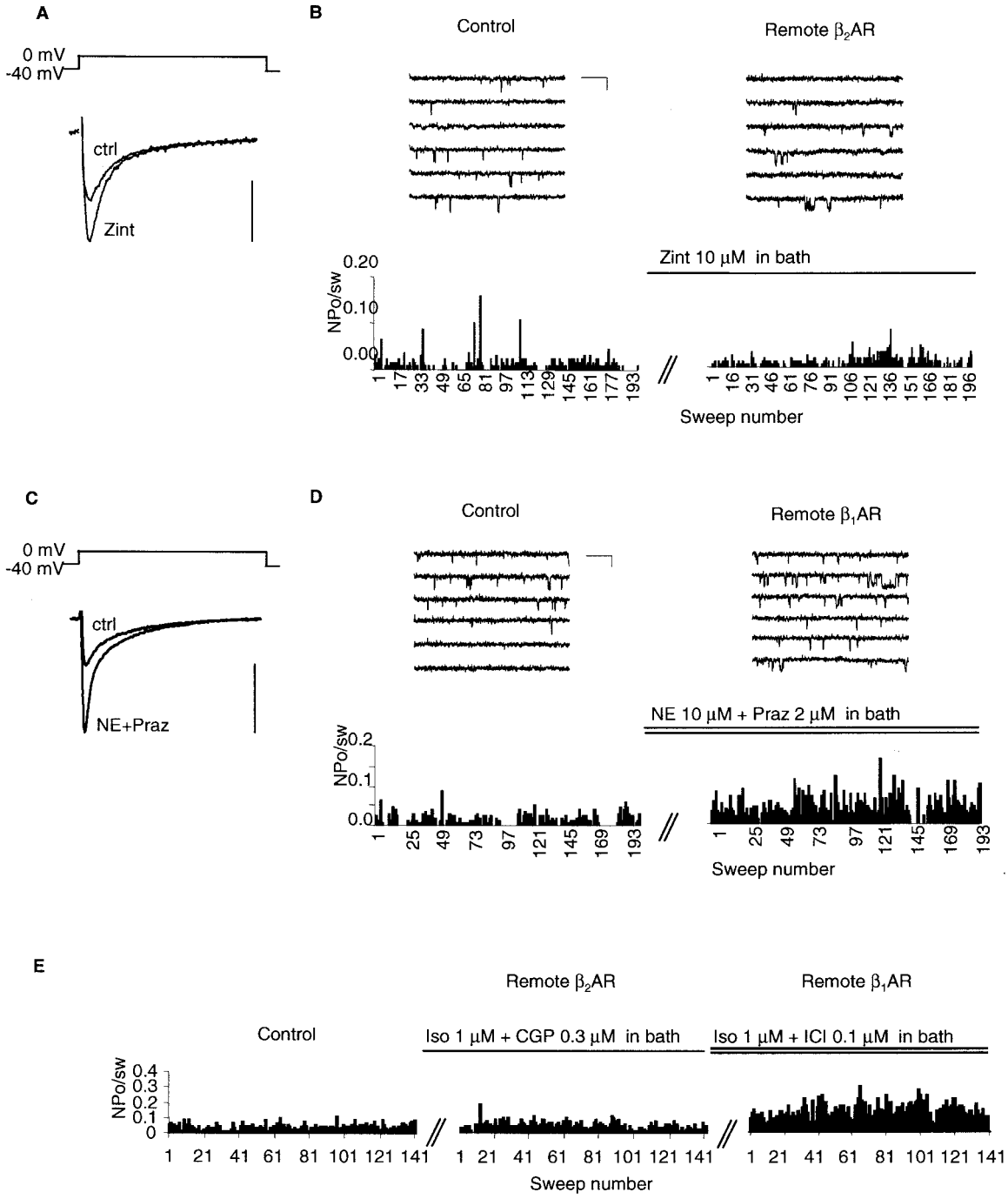

FIGURE 1 Remote β_2-AR stimulation does not affect the channel activity, whereas remote β_1-AR stimulation activates the channels. (*A*) Whole cell L-type Ca^{2+} current elicited by depolarization (step pulse of 200 ms duration) under the control condition (ctrl), and under the β_2-AR stimulation using zinterol 10 μM (Zint). Scale bar represents 0.5 nA. The bath solution is (in mM): $CaCl_2$ 1.0, NaCl 137, KCl 5.0, dextrose 15, $MgSO_4$ 1.3, NaH_2PO_4, and HEPES 20, with pH 7.4 adjusted using NaOH. The pipette solution is (in mM): CsCl 110, TEACl 20, Na_2-phosphocreatine 5.0, Na_2GTP 0.2, HEPES 10, MgATP 3, with pH 7.2 adjusted using CsOH. (*B*) Upper panels show representative traces of single L-type Ca^{2+} channel activity under the control condition, and following remote β_2AR stimulation using zinterol 10 μM in the same patch. The scale bars represent 30 ms (horizontal) and 1.0 pA (vertical). Lower panel shows the history of channel activity by open probability per sweep (NPo/sw) over time. The interval between sweeps is 1 s. The bath exchange rate in the perfusion chamber is ~95% change within 30 s. The channel activity was recorded at least 5 min after the drug application. (*C*) Whole cell L-type Ca^{2+} current elicited by depolarization (step pulse of 150 ms duration) under the control condition (ctrl), and under the β_1-AR stimulation using norepinepherin 1 μM and prazosin 0.1 μM (NE + Praz). Scale bar: 1.0 nA. (*D*) Upper panels show representative traces of single L-type Ca^{2+} channel activity under the control condition, and following remote β_1AR stimulation using norepinepherin 10 μM and prazosin 2 μM in the same patch. The scale bars represent 30 ms (horizontal) and 1.0 pA (vertical). Lower panel shows the open probability per sweep (NPo/sw) over time. (*E*) The open probability of a channel under the control condition (*left*), following remote β_2-AR stimulation using isoproterenol 1 μM and CGP20712A 0.3 μM, a β_1-AR inhibitor (*middle*), and following subsequent remote β_1-AR stimulation using isoproterenol 1 μM and ICI118,551 0.1 μM, a β_2-AR inhibitor (*right*).

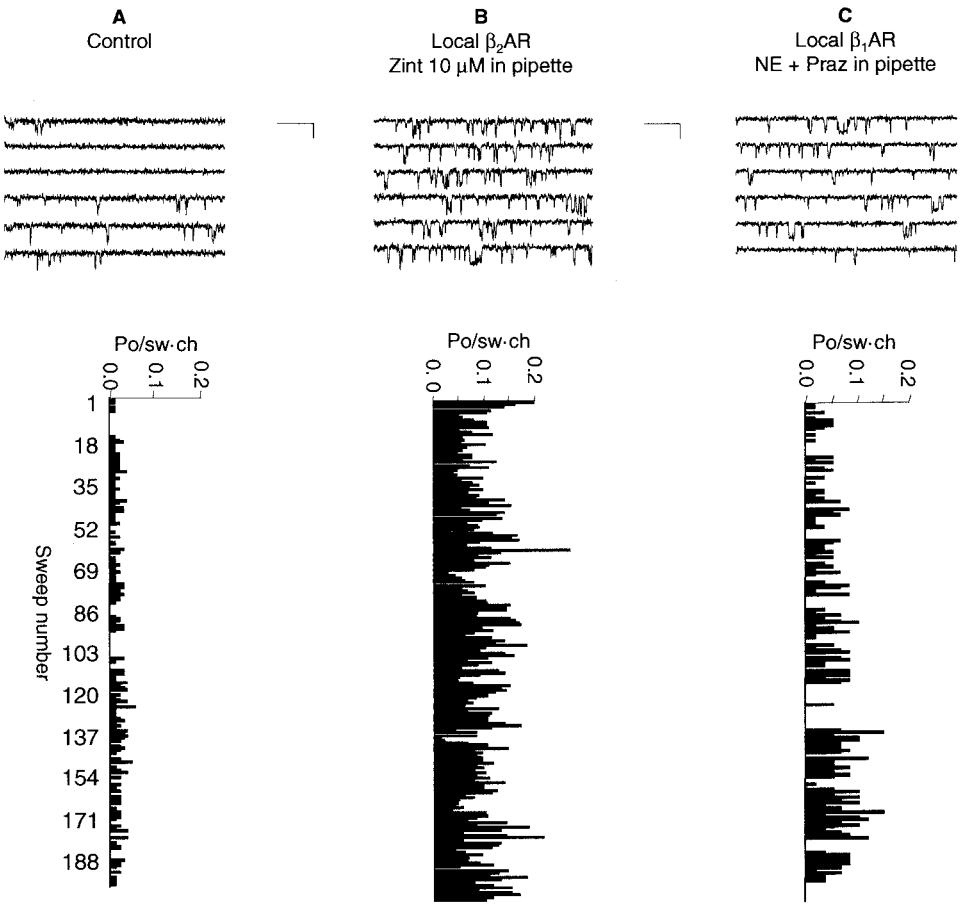

FIGURE 2 Stimulation of local β_2-AR or β_1-AR activates single L-type Ca^{2+} channels. Upper panels show representative traces of single L-type Ca^{2+} channel activity under the control condition (*A*), local β_2AR stimulation using zinterol 10 μM (*B*), and local β_1AR stimulation using norepinepherin 10 μM and prazosin 0.2 μM (*C*). The scale bars represent 30 ms (*horizontal*) and 1 pA (*vertical*). Lower panels show the channel open probability per sweep (Po/sw), normalized to per channel, over time. The interval between two subsequent sweeps is 1 s.

increased to 4.81 $\pm$ 0.94% per channel ($P < 0.05$, 6 cells), a 3.3-fold increase.

In order to study the effect of local β_2-AR stimulation on the channel activity in detail, and compare it to the effect of β_1-AR stimulation, we characterized the single channel gating kinetics using three gating modes (Hess and Tsien, 1984) (see Methods). Under control condition, the channel has an availability of 68.3%; that is, 68.3% of the sweeps had at least one opening event in a sweep (Table 1). Mode-1 open events have a mean open time of 0.42 ms and a frequency of 3.6 events per active sweep. Mode-2 open events are rare with a frequency of only 2.8 events per 100 sweeps. Because of the small total numbers, the mode-2 event is prone to large statistical fluctuations, rendering its measurement less meaningful. Therefore we will not use mode-2 statistics in the following discussion, although the measurements are listed in Table 1 for a complete analysis.

Under local β_2-AR stimulation using zinterol 10 μM, the availability of the channel increased to 94.8%. The mode-1 frequency increased to 8.7 events per active sweep (Table 1). The mean open time of mode-1 events is 0.46 ms, without significant change from the control condition. Thus, the increase of average Po under local β_2-AR stimulation is mainly attributed to the increase of availability and mode-1 open frequency, without significant change in the mode-1 mean open time.

To ensure that above changes in channel activity are due to β_2-AR stimulation, we also used lower concentration of zinterol 1 μM, or a different agonist Iso + CGP to stimulate β_2-AR. The channel open probability increased to 3.83 $\pm$ 1.37% ($P < 0.05$, 4 cells) and 3.09 $\pm$ 0.26% ($P < 0.05$, 3 cells) respectively under these two conditions. The increase of Po is mainly attributed, again, to the increase of availability and mode-1 open frequency, without significant change in mode-1 mean open time (Table-1). The potent effect of local β_2-AR stimulation on the channels, and a lack of effect of remote β_2-AR stimulation, strongly support the conclusion that β_2-AR signaling to the L-type Ca^{2+} channel is localized.

Local β_1-AR stimulation using NE + Praz also increased average Po to 4.82 $\pm$ 0.83%, a 3.3-fold increase from the control condition (Fig. 2 *c*). This increase of Po arises

mainly from an increase of availability from 68.3% to 89.0% and an increase of mode-1 frequency from 3.6 to 10.0 events per active sweep, without significant change in the mode-1 mean open time (Table 1).

To study the mechanism of β_2-AR signaling, we compared the effect of local β_2-AR stimulation on the single channel gating kinetics with that of β_1-AR. Local β_2-AR stimulation activates the channel by increasing the channel availability and mode-1 open frequency, without changing mode-1 mean open time (Table 1). The mode-2 open frequency seems also increased, although the small sample numbers prohibit meaningful statistical testing. Thus, there is no discernable difference in the effect of β_2-AR and β_1-AR signaling at the level of single channel gating kinetics.

Effect of PTX treatment on the β_2-AR signaling

To study the mechanism for the localization of β_2-AR effect on the channels, we examined the role of G_i-protein. It is known that β_2-AR couples dually to G_s- and G_i-proteins, whereas β_1-AR couples exclusively to G_s protein (Xiao et al., 1995; Daaka et al., 1997; Xiao et al., 1999; Kuschel et al., 1999b). We hypothesized that G_i activation might be responsible for the localization of β_2-AR signaling. We pretreated cells with PTX for 3 h to decouple G_i from β_2-AR stimulation (Oinuma et al., 1987), then examined the effect of remote β_2-AR stimulation on the L-type Ca^{2+} channels. In PTX-treated cells, under the control condition, the basal level channel activity is similar to that of untreated cells (Fig. 3 and Table 1). The average Po is 1.49 ± 0.14% (7 cells), availability 70.8%, mode-1 frequency 3.5 events per active sweep, and mode-1 mean open time 0.45 ms. However, unlike in untreated cells, stimulation of remote β_2-AR by bath application of zinterol 10 μM markedly activated the channels in PTX-treated cells (Fig. 3 and Table 1). The average Po increased 2.1-fold ($P < 0.05$, in the same 7 cells), attributed mainly to an increase of availability to 75.1% and an increase of mode-1 frequency to 9.1 events per active sweep, without a significant change of mode-1 mean open time.

Using PTX to decouple G_i from β_2-AR transformed the nature of β_2-AR signaling from localized to diffusive. Therefore, G_i-protein may be responsible for the localization of β_2-AR signaling in untreated cells. In the PTX-treated cells, upon removal of the G_i influence, the G_s signaling pathway alone is activated by β_2-AR, leading to a diffusive signaling from β_2-AR to the L-type Ca^{2+} channel, resembling that of G_s-coupled β_1-AR signaling.

DISCUSSION

Our main finding is that β_2-AR signaling activates the L-type Ca^{2+} channel via a highly localized pathway, whereas β_1-AR signaling can activate the channel via a diffusive pathway. The localization of β_2-AR signaling is determined by the coupling of the receptor to G_i-protein.

Diffusive signaling of β_1-AR

The diffusive nature of β_1-AR signaling to activate the L-type Ca^{2+} channels is in agreement with the classic notion of β-adrenergic signaling cascade. In this scheme, receptor stimulation causes G_s-protein activation, leading to activation of adenylate cyclase, production of cAMP, and activation of PKA. In support of this notion, our data shows that remote β_1-AR stimulation increases the availability and the open frequencies of mode-1 events, without changing the mode-1 mean open time (Table 1). These changes in the single channel gating kinetics under β_1-AR stimulation are similar to the changes caused by a direct application of cAMP (Cachelin et al., 1983; Hirano et al., 1994). Local β_1-AR stimulation also changes the single channel parameters in a similar manner, except that it is more efficacious

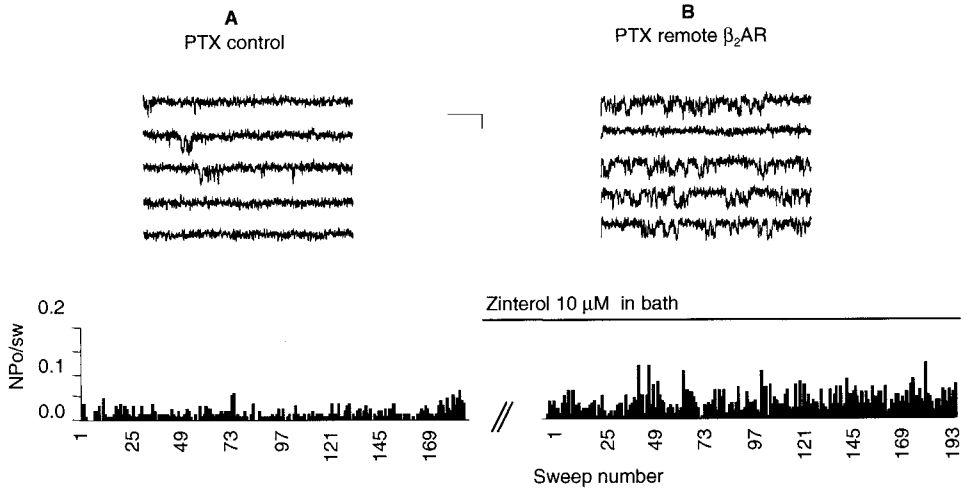

FIGURE 3 Remote β_2-AR stimulation activates L-type Ca^{2+} channels in PTX treated cells. The upper panels show representative traces of single channel activity in PTX-treated cells under control condition (*A*), and following remote β_2AR stimulation using zinterol 10 μM (*B*) in the same patch. The scale bars represent 30 ms (*horizontal*) and 1 pA (*vertical*). Lower panel shows the channel open probability per sweep (NPo/sw) over time.

TABLE 1 Summary of the single channel parameters (mean ± SE)

Experimental condition	P_o % (n)	Availability (%)	Mode-1 Event/sweep	Mode-1 τ-open (ms)	Mode-2 Event/100 sweeps	Mode-2 τ-open (ms)
Control (pooled)	1.45 ± 0.12 (14)	68.3 ± 6.3	3.6 ± 0.6	0.42 ± 0.04	2.8 ± 1.2	9.1 ± 1.5
Local β_2-AR stimulation						
Pipette-Zint 10 μM	4.81 ± 0.94* (6)	94.8 ± 1.9*	8.7 ± 1.1*	0.46 ± 0.01	11.1 ± 5.8	6.1 ± 0.4
Pipette-Zint 1 μM	3.83 ± 1.37* (4)	85.4 ± 9.5*	7.5 ± 2.2*	0.57 ± 0.04	6.2 ± 5.5	17.6 ± 7.6
Pipette-Iso 1 μM + CGP 0.3 μM	3.09 ± 0.26* (3)	90.9 ± 5.7	6.5 ± 0.9*	0.45 ± 0.05	3.3 ± 0.8	10.8 ± 1.6
Local β_1-AR stimulation						
Pipette-NE 10 μM + Praz 1 μM	4.82 ± 0.83* (6)	89.0 ± 5.7*	10.0 ± 1.5*	0.43 ± 0.06	15.4 ± 7.8	8.4 ± 2.3
Remote β_2-AR stimulation						
Control (in the same patch)	1.56 ± 0.19 (5)	62.0 ± 13.0	3.5 ± 0.3	0.42 ± 0.05	3.6 ± 2.7	10.8 ± 3.1
Bath-Zint 10 μM	1.35 ± 0.25	60.5 ± 10.5	2.7 ± 0.6	0.50 ± 0.09	2.7 ± 0.8	7.6 ± 0.9
Remote β_2-AR stimulation						
Control (in the same patch)	1.48 ± 0.06 (3)	76.5	3.8 ± 0.8	0.41 ± 0.10	0.9 ± 0.5	11.2 ± 6.8
Bath-Iso 1 μM + CGP 0.3 μM	1.81 ± 0.16	68.3	3.8 ± 0.5	0.48 ± 0.09	2.3 ± 1.2	9.5 ± 7.4
Remote β_1-AR stimulation						
Control (in the same patch)	1.51 ± 0.36 (4)	66.8 ± 3.4	3.4 ± 0.8	0.41 ± 0.16	1.1 ± 0.7	10.9 ± 3.8
Bath-NE 10 μM + Praz 1 μM	3.94 ± 0.36*	92.9 ± 0.4*	8.8 ± 1.0*	0.46 ± 0.04	7.3 ± 2.4	11.7 ± 4.3
Remote β_2-AR stimulation in PTX cells						
PTX control (in the same patch)	1.49 ± 0.14 (7)	70.8 ± 8.2	3.5 ± 0.3	0.45 ± 0.03	1.4 ± 0.6	5.7 ± 0.5
PTX Bath-Zint 10 μM	3.14 ± 0.89*	75.1 ± 8.5	9.1 ± 2.0*	0.48 ± 0.03	2.5 ± 0.7	6.2 ± 0.6

*Student's t-test $P < 0.05$, experiment versus control.

P_o, average open probability normalized to per channel; n, number of cells; Availability, percentage of active sweeps in a patch containing a single channel; Mode-1 τ-open, mean open time of mode-1 events; Mode-2 τ-open: arithmetic average of mode-2 open time.

than remote β_1-AR stimulation, increasing Po 3.3-fold instead of 2.6-fold (Fig. 4 and Table 1). A simple explanation is that stimulation of local receptors may generate stronger signals to the channels in the vicinity than stimulation of remote receptors, because diffusion of cAMP and PKA from a remote site may dilute the signals and weaken the signaling strength. Hence, the relatively potent effect of local versus remote β_1-AR stimulation supports a diffusive cAMP-PKA signaling pathway. To examine the total strength of local and remote receptor stimulation, we included β_1-AR agonist in pipette first, then added the agonist in bath. The channel open probability was 4.1% under local β_1-AR stimulation, then increased to 5.9% under global (local plus remote) stimulation (data not shown), in comparison with 1.45% under control condition.

Hence, β_1-AR signaling is mediated by a diffusive cAMP-PKA pathway, which leads to phosphorylation of multiple proteins involved in the cardiac excitation-contraction coupling, including L-type Ca^{2+} channel in sarcolemmal membrane, phospholamban on the sarcoplasmic reticulum, and troponin-I and C-proteins of the myofilament (Xiao and Lakatta, 1993; Xiao et al., 1994; Altschuld et al., 1995; Kuschel et al., 1999a,b). In addition, PKA-dependent phosphorylation and activation of phosphatase inhibitor-1 may also reduce phosphatase activity, and further enhance PKA-dependent protein phosphorylation (Kuschel et al., 1999b). Thus, the diffusive β_1-AR signaling gives rise to a global cAMP/PKA-dependent modulation of cardiac muscle contraction, including an increase of the contraction amplitude, an acceleration of the relaxation, a decrease of the myofilament sensitivity to Ca^{2+}, and arrhythmogenic spontaneous Ca^{2+} oscillations (Xiao and Lakatta, 1993).

Localized signaling of β_2-AR

β_2-AR signaling to activate the L-type Ca^{2+} channel is highly localized. Maximum stimulation of remote β_2-AR by bath application of zinterol 10 μM did not cause a discernible change in the L-type Ca^{2+} channel activity in the patch membrane. However, stimulation of local β_2-AR by pipette application of zinterol at the same concentration (10 μM) or lower (1 μM) led to marked increases in the channel activity (Fig. 4). It was reported that much higher concentration of ~50 μM zinterol increased the channel activity in the patch membrane when applied to a high Na^+ bath solution (Schroder and Herzig, 1999). However, at such a high concentration, zinterol activates not only β_2- but also β_1-AR (Minneman et al., 1979; Xiao et al., 1998); the latter could activate the channels via diffusive signaling. In our study, the fact that the channels are activated by 1 μM zinterol in the pipette, but not by 10 μM zinterol in the bath, strongly suggest that the β_2-AR signaling is highly localized to the membrane, in sharp contrast to the diffusive β_1-AR signaling. When we used another agonist Iso + CGP to selectively stimulate β_2-AR, again, local β_2-AR stimulation activated the channel, but remote β_2-AR stimulation did not significantly alter channel activity (Fig. 4). This localization of β_2-AR signaling to the channels in the receptor vicinity is consistent with a lack of β_2-AR effect on the proteins that

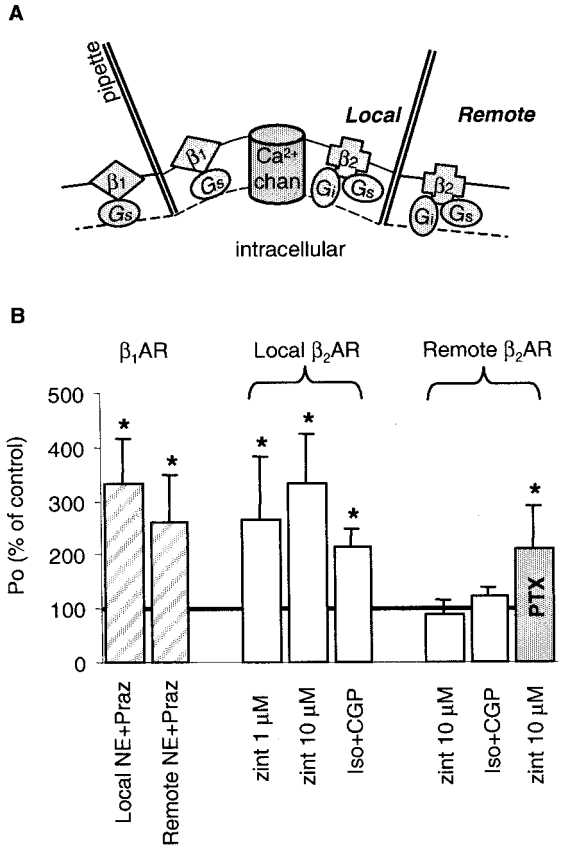

FIGURE 4 Effects of receptor stimulation on average channel open probability (Po). (*A*) Schematic of the two membrane compartments separated by the patch pipette which forms a tight seal (~20–50 GΩ) on the cell membrane, creating an isolated patch membrane compartment (~1 μm^2). Single channel activity in the patch membrane was recorded through an electrode in the pipette. The drugs were added directly into the pipette solution for local receptor stimulation, or applied into the bath for remote receptor stimulation. (*B*) The average channel open probability Po is shown as the percent of the control values (normalized to the solid horizontal line at Y = 100%). The bars from left to right show the open probability of channels under the local and remote β_1-AR stimulation using norepinephrine 10 μM plus prazosin 2 μM (NE + Praz); local β_2AR stimulation using zinterol 1 μM, zinterol 10 μM, and isoproterenol 1 μM plus CGP 0.3 μM (Iso + CGP); remote β_2-AR stimulation using zinterol 10 μM, isoproterenol 1 μM plus CGP 0.3 μM; and remote β_2-AR stimulation in PTX-treated cells using zinterol 10 μM. The difference between the mean values are deemed significantly different if *t*-test $P < 0.05$ and marked with an asterisk.

are remote from the cell membrane. It also explains the specific effect of β_2-AR stimulation on enhancing cardiac muscle contraction amplitude without changing the relaxation time (Xiao and Lakatta, 1993). This result also agrees with an earlier observation that β_2-AR effect on the macroscopic L-type Ca^{2+} current in frog ventricular myocytes was confined to the half-cell region where the receptors in the corresponding membrane area were stimulated by local application of agonist (Jurevicius and Fischmeister, 1996). The present study further reveals that β_2-AR signaling is highly localized to the membrane receptor vicinity within a submicron spatial range.

What mediates the localized β_2-AR signaling to the L-type Ca^{2+} channels? Accumulating evidences suggest that cAMP/PKA pathway mediates the β_2-AR signaling. Circumstantial evidence comes from the comparison of single channel gating kinetics under local β_2-AR stimulation to that under β_1-AR stimulation. As we have shown, the changes in the single channel gating kinetics under local β_2-AR stimulation follow a similar pattern to that under β_1-AR stimulation, or under direct application of cAMP (Cachelin et al., 1983; Hirano et al., 1994). A previous study shows that Rp-cAMP, an inhibitory cAMP analog, blocks β_2-AR effect on augmenting whole-cell L-type Ca^{2+} current and abolishes the enhancement of contractile strength (Zhou et al., 1997; Kuschel et al., 1999a). To test the Rp-cAMP effect at single channel level, we included zinterol 10 μM in the pipette solution to stimulate local β_2-AR, then applied Rp-cpt-cAMP, a membrane permeable form of Rp-cAMP, into the bath. The channel activity was high at the beginning under the local β_2-AR stimulation, then gradually decreased at about 20 min, 25 min, and 50 min after Rp-cpt-cAMP application in three cells, respectively (data not shown). However, because the decrease of channel activity occurred long after Rp-cpt-cAMP application, we can not reliably distinguish the drug effect (it could be a slow process for Rp-cpt-cAMP to permeate the cell membrane and be converted to Rp-cAMP) from "run down" of channel activity. Additional evidence supporting the role of cAMP-PKA in β_2-AR signaling comes from earlier studies showing that Rp-cAMP or a peptide PKA inhibitor blocks the effects of isoproterenol in rat ventricular myocytes (Kuznetsov et al., 1995; Minneman et al., 1979) and in frog ventricular myocytes where β_2-AR is dominantly expressed (Hartzell et al., 1991; Hartzell and Fischmeister, 1992; Skeberdis et al., 1997b).

Another proposed mechanism for localized signaling is a direct interaction between G-protein and L-type Ca^{2+} channels. Studies by Brown and his colleagues show that G_s-protein, not G_i-protein, activated the channels in excised patches (Mattera et al., 1989; Yatani et al., 1987), or the channels incorporated into lipid bilayers (Yatani et al., 1988). However, because of channel "run down", Bay K 8644 or isoproterenol was used in those experiments to maintain basal level channel activity. It remains controversial whether L-type Ca^{2+} channels are directly modulated by G_s-protein under physiological conditions in absence of an agonist or a stimulant. In the present study, we have shown that G_i-protein is responsible for the localization of β_2-AR signaling, whereas G_s-coupled β_1-AR signaling, or β_2-AR signaling in PTX-treated cells, is diffusive. Hence, the above proposed mechanism can not explain the localization of β_2-AR signaling, although it remains possible that some degree of direct interaction could exist between G_s and the channels. Taken together, the present data favor the

notion that a compartmentalized cAMP/PKA pathway mediates the localization of β_2-AR signaling to L-type Ca^{2+} channels.

Role of G_i-protein in the localization of β_2-AR signaling

β_2-AR couples dually to G_s and G_i, whereas β_1-AR couples exclusively to G_s (Xiao et al., 1995; Daaka et al., 1997; Xiao et al., 1999). Our data show that using PTX treatment to decouple G_i from β_2-AR transformed the nature of β_2-AR signaling from localized to diffusive (Fig. 4). Previous studies in our group also show that PTX treatment transformed β_2-AR signaling to cause phosphorylation of phospholamban and acceleration of cardiac muscle contraction (Xiao et al., 1995; Kuschel et al., 1999b), resembling that of G_s-coupled β_1-AR signaling.

The current understanding is that activation of G_i counteracts G_s signaling by inhibiting adenylate cyclase, thereby reducing total cAMP production (Gilman, 1987; Katada et al., 1987). In light of this scheme, a simple explanation of our results could be that activation of both G_s- and G_i-proteins by β_2-AR leads to less production of cAMP, and hence more spatially confined response, in comparison to β_1-AR stimulation. PTX treatment of cells disrupts G_i signaling, allowing β_2-AR stimulation to produce more cAMP to reach more distant target. This explanation, however, is challenged by several lines of evidence. An earlier study in rat ventricular myocytes shows that the dose-response curves of β_1-AR and β_2-AR stimulation to global cAMP production overlap each other (Xiao et al., 1994). Recent studies show that PTX treatment of cells did not alter the increase of global cAMP by β_2-AR stimulation (Zhou et al., 1997), nor did it alter the increase of total PKA activity (Kuschel et al., 1999b). Nevertheless, the global cAMP concentration or PKA activity may not reflect the activity of these molecules in localized subcellular domains. When the membrane-bound cAMP was measured instead of global cAMP, the increase of membrane-bound cAMP induced by β_2-AR stimulation was only half of that induced by β_1-AR stimulation (Xiao et al., 1994). Still, measurement of membrane-bound cAMP provides little information on the cAMP/PKA activity in highly localized domains such as sarcolemma and dyadic junction. Therefore, the above biochemical data lack sufficient resolution, and need to be interpreted with caution.

Our previous studies suggest that protein phosphatases may also be involved in the localization of β_2-AR signaling. Calyculin A, a phosphatase inhibitor, selectively enhanced β_2-AR, but not β_1-AR, mediated contractile response in rat ventricular myocytes. However, in PTX-treated cells, calyculin A cannot further enhance β_2-AR mediated contractile response, suggesting that β_2-AR-coupled G_i signaling may activate protein phosphatases, which localize and offset the G_s-mediated signaling (Kuschel et al., 1999b). Therefore, interplay between protein phosphorylation and dephosphorylation events in local domains may contribute to the localization of β_2-AR signaling.

A localized signaling could also arise from localization of signaling molecules, e.g., localization of adenylyl cyclase, phosphodiesterases, cAMP (Buxton and Brunton, 1985), PKA, phosphatases (Raymond, 1995; Sako and Kusumi, 1994), and PKA anchoring proteins (Scott, 1997; Mochly-Rosen, 1995; Coghlan et al., 1995; Gao et al., 1997). In support to this hypothesis, a close spatial association of L-type Ca^{2+} channels with adenylate cyclase and PKA has been demonstrated (Gao et al., 1997; Gray et al., 1998). Many signaling molecules including G-protein coupled receptors, G-proteins, adenylate cyclase, and the regulatory subunit of PKA have been found to localize in caveolae (Isshiki and Anderson, 1999; Schwencke et al., 1999). We speculate that localization of signaling molecules in specific microdomains may serve as a general mechanism to confer the specificity of G-protein-coupled receptor signaling.

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