

Label-free optical biosensor for ligand-directed functional selectivity acting on β_2 adrenoceptor in living cells

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Abstract Recent realization of ligand-directed functional selectivity demands high-resolution tools for studying receptor biology and ligand pharmacology. Here we use label-free optical biosensor to examine the dynamic mass redistribution of human epidermoid A431 cells in response to diverse β_2 -adrenoceptor ligands. Multi-parameter analysis reveals distinct patterns in activation and signaling of the receptor induced by different agonists. Sequential and co-stimulation assays categorize various ligands for their ability to modulate signaling induced by catechol, a structural component of catecholamines. This study documents multiple ligand-specific states of the β_2 -adrenoceptor and highlights the power of the biosensor assays for screening pathway-biased ligands.

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Keywords: Optical biosensor; G protein-coupled receptor; β_2 -adrenoceptor; Ligand-directed functional selectivity

1. Introduction

G protein-coupled receptors (GPCRs) are the largest family of cell membrane receptors and the richest class of drug targets in the human genome. Classical receptor-occupancy theories define the efficacy of ligands as their ability to alter the equilibrium between inactive and active states of the receptor, assuming that all GPCR activities are correlated [1]. However, the recent data have challenged this simple kinetic model. Amassing evidence suggests that GPCR signaling is sophisticated – a receptor may couple simultaneously to more than one G protein subtype and interact with other signaling molecules such as arrestins [2]. In many cases the activation of a receptor can mediate both G protein-dependent and independent signaling, often in a ligand-dependent manner [3–5]. As a result, GPCRs display rich behaviors in cells, and many ligands can induce operative bias to favor specific portions of the cell machinery and exhibit pathway-biased efficacies [6,7].

Given the significance of ligand-directed functional selectivity in drug development [8], the possibility of a ligand having

multiple efficacies demands high-resolution pharmacological assays for screening pathway-biased ligands. It is obviously difficult in practice for conventional cell assays, which are mostly pathway-biased and assay only a single signaling event [9], to systematically represent the signaling potentials of GPCR ligands [10]. Label-free optical biosensors including surface plasmon resonance (SPR), resonant-waveguide grating (RWG), and plasmon-waveguide resonance (PWR) are routinely used for biomolecular interaction analysis [11,12]. Recently, we had applied label-free optical biosensors for whole cell sensing, and found that these biosensors are capable of monitoring endogenous receptor activation, leading to high-information and physiologically relevant measures of a receptor–ligand pair [13–16]. These assays do not require prior knowledge of cell signaling, and are pathway-unbiased [16]. The optical responses recorded are pathway-sensitive, and do reflect the complexity of receptor signaling [13]. Thus, we hypothesized that the biosensor-based cellular assay is amenable to studying ligand-selective signaling. Here we examined the functional selectivity of various ligands for endogenous β_2 -adrenoceptor (β_2 AR) in A431 cells using the biosensor. Results showed that there is a strong correlation between the structures of ligands and the characteristics of their optical signals.

2. Methods

2.1. Materials

Alprenolol, cell preamble dynamin inhibitory peptide (DIPC), CGP12177, dopamine, epinephrine, forskolin, ICI 118551, isoproterenol, labetalol, norepinephrine, pindolol, S(–)pindolol, propranolol, salbutamol, salmeterol, timolol, and xamoterol were obtained from Tocris (St. Louis, MO). Catechol, halostachine, tyramine, and phenylethylamine were purchased from Sigma (St. Louis, MO). Cell culture compatible Epic[®] 384 well RWG biosensor microplates were obtained from Corning Inc. (Corning, NY).

2.2. Cell culture

Human epidermoid carcinoma A431 cells (American Type Cell Culture) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/l glucose, 2 mM glutamine, and antibiotics. $\sim 1.8 \times 10^4$ cells at passage 3–15 suspended in 50 μ l the medium containing 10% FBS were placed in each well of a 384 well microplate, and were cultured at 37 °C under air/5% CO₂ for ~ 1 day, followed by ~ 20 h starvation through continuously culture in the serum-free DMEM.

2.3. Optical biosensor system and cell assays

Corning[®] Epic[®] wavelength interrogation system was used. This system consists of a temperature-control unit, an optical detection unit, and an on-board liquid handling unit with robotics. The detection unit

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Abbreviations: GPCR, G Protein-coupled receptor; β_2 AR, β_2 -Adrenoceptor; DMR, dynamic mass redistribution; RWG, resonant-waveguide grating; cAMP, cyclic adenosine monophosphate; DIPC, dynamin inhibitory peptide; SPR, surface plasmon resonance; PWR, plasmon-waveguide resonance

is centered on integrated fiber optics, and enables kinetic measures of cellular responses with a time interval of ~7 or 15 s.

The RWG biosensor exploits its evanescent wave, created by the total internal reflection of light at a solution–surface interface, to measure ligand-induced dynamic mass redistribution (DMR) signals in cells. The evanescent wave extends into the cells and exponentially decays over distance, leading to a characteristic sensing volume of ~150 nm [15], implying that any optical response mediated through the receptor activation only represents an average over the portion of the cell that the evanescent wave is sampling. Such sampling with the biosensor is sufficient to differentiate the signaling of distinct classes of GPCRs in living cells, and offers a simplified representation of GPCR signaling [13,16,17].

Like SPR, the RWG biosensor is sensitive to refractive index – an intrinsic property of biomolecules. Since the refractive index of a given volume within a cell is largely determined by the concentrations of biomolecules such as proteins [18], we found, based on a three-layer waveguide grating theory, that a ligand-induced optical response is largely associated with DMR [15]. The relocation of cellular targets towards the sensor surface (e.g., relocation of intracellular targets to the activated receptors at the basal membrane surface) makes a positive contribution to the DMR; conversely, the movement of cellular targets away from the sensor surface (e.g., receptor internalization) is a negative contributor to the DMR. The aggregation of these events determines the kinetics and amplitudes of a ligand-induced DMR. However, recent studies, using PWR technology and *in vitro* reconstituted GPCRs immobilized onto the sensor surface, showed that a ligand-induced optical response of the receptor–lipid membrane system consists of two components – changes in mass density and changes in

structure [12,19]. Since the RWG biosensor used here is unable to differentiate the contributions of these components, ligand-induced changes in organization of biomolecules in living cells may also contribute to the overall response measured.

For biosensor cellular assays, a 2-min baseline was first established, compound solutions were then transferred into the sensor plate having cells maintained in Hanks balanced salt solution (20 mM Hepes, pH 7.1), and the cell responses were recorded continuously. All studies were carried out at controlled temperature (28 °C) and with three replicates for each measurement, unless specifically mentioned. The assay coefficient of variation was found to be <10%. All dose-dependent responses were analyzed using non-linear regression method with the Prism software (Graph Pad).

3. Results

3.1. Ligand-specific DMR signals

We chose the endogenous β_2 AR in A431 as a model system for assaying ligand-directed functional selectivity with the biosensor, because of its well-established signaling, and the availability of a rich source of structurally similar ligands having a wide spectrum of efficacies.

Fig. 1 graphically depicts the chemical structures and DMR signals in quiescent cells of distinct ligands, whereas Table 1 summarizes their DMR characteristics. The β_2 AR ligand-

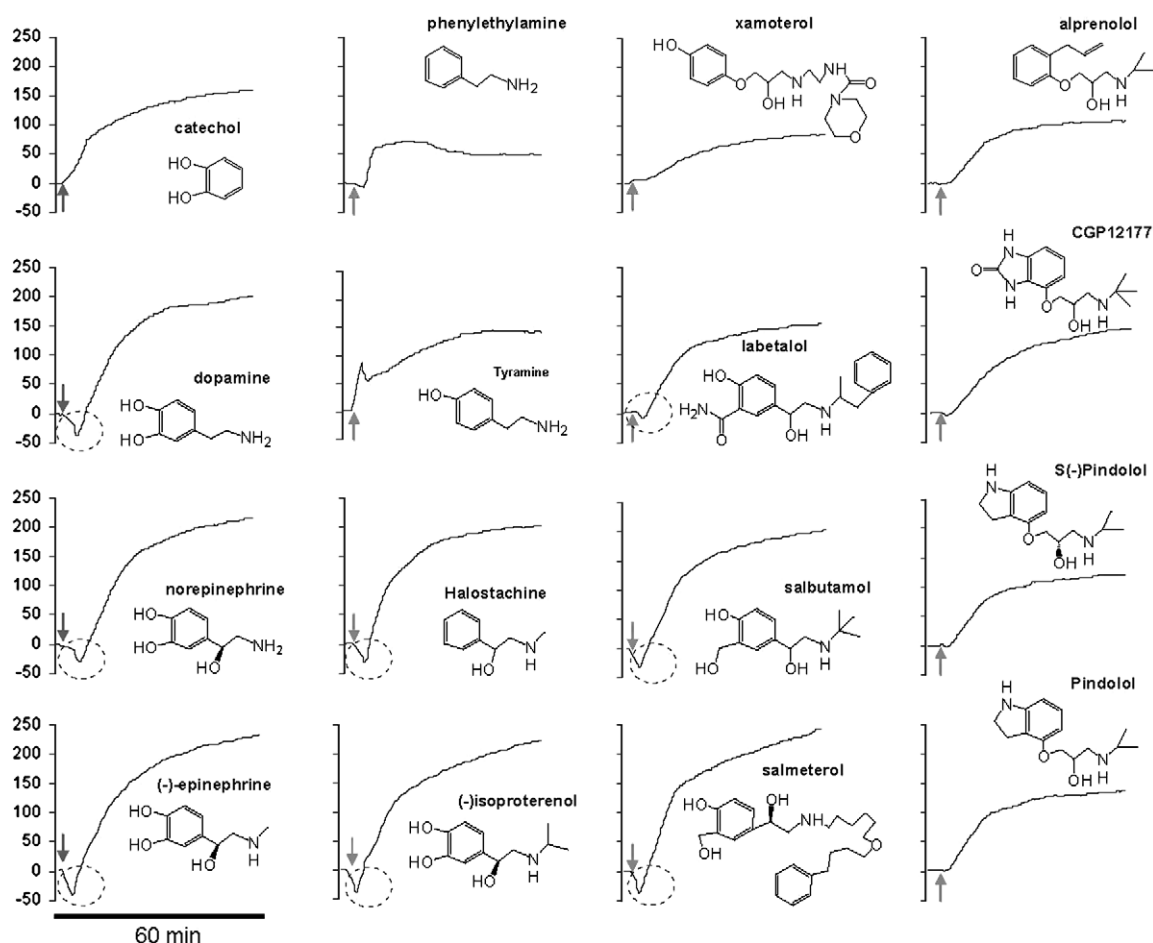


Fig. 1. The structures of β_2 AR ligands and their DMR in quiescent A431 cells. The ligands included (–)epinephrine (8 nM), (–)isoproterenol (10 nM), norepinephrine (100 nM), dopamine (32 μ M), halostachine (500 μ M), catechol (500 μ M), tyramine (125 μ M), phenylethylamine (500 μ M), salmeterol (8000 nM), salbutamol (164 nM), labetalol (2 μ M), xamoterol (1 μ M), pindolol (8 μ M), S(–)pindolol (8 μ M), CGP12177 (100 nM), and alprenolol (4 μ M). The grey arrows indicated the time when the agonist was introduced.

Table 1
The characteristics of β_2 AR ligands

Ligand	$-\log K_i$	$-\log EC_{50} \pm S.E.$	P-DMR (pm)	N-DMR (pm)	τ (s)	$t_{1/2}$ (s)
Isoproterenol	6.97	11.07 ± 0.07	232 ± 15	37 ± 5	130 ± 20	534 ± 32
Epinephrine	7.16	10.13 ± 0.06	232 ± 12	37 ± 7	132 ± 20	521 ± 25
Norepinephrine	5.40	7.99 ± 0.07	209 ± 16	29 ± 5	180 ± 17	559 ± 15
Dopamine	4.35	5.96 ± 0.06	214 ± 32	31 ± 4	252 ± 15	480 ± 23
Catechol	3.80	3.30 ± 0.07	152 ± 13	0 ± 3	130 ± 15	289 ± 42
Halostachine	5.08	4.63 ± 0.05	208 ± 21	20 ± 7	200 ± 32	349 ± 41
Salmeterol	8.89	9.68 ± 0.07	160 ± 14	0 ± 4	290 ± 17	370 ± 45
		6.90 ± 0.10	244 ± 23	40 ± 11	152 ± 20	n.a.
Salbutamol	5.35	9.07 ± 0.04	209 ± 13	32 ± 3	132 ± 20	539 ± 32
Labetalol	7.97	8.05 ± 0.05	149 ± 17	8 ± 4	250 ± 15	544 ± 46
CGP12177	9.20	9.95 ± 0.07	123 ± 14	5 ± 5	238 ± 26	582 ± 32
Alprenolol	9.49	10.23 ± 0.09	124 ± 16	0 ± 4	233 ± 15	540 ± 26
S(-)-pindolol	10.16	10.85 ± 0.06	137 ± 12	0 ± 3	249 ± 26	580 ± 56
Pindolol	9.15	9.97 ± 0.04	142 ± 19	0 ± 4	250 ± 15	516 ± 47
Xamoterol	6.06	7.32 ± 0.08	75 ± 18	0 ± 5	260 ± 26	974 ± 150

The binding affinities were obtained from the GPCR ligand database (<http://www.gpcr.org/7tm/ligand/ligand.html>). The EC_{50} was calculated based on the DMR response at the time point of 50 min. The $t_{1/2}$ was calculated based on the fitting of the P-DMR with a one-phase exponential association non-linear regression.

induced DMR can be classified into five categories, based on their dynamics. The first group included isoproterenol, epinephrine, norepinephrine, dopamine, halostachine, labetalol, and salbutamol, each of which mediated a biphasic DMR response. Following a small decrease in signal (termed as negative-DMR, N-DMR) with a short duration was an increased signal (termed as positive-DMR, P-DMR) to an elevated level with ligand-dependent maximal amplitudes and kinetics. The second group included catechol, xamoterol, alprenolol, CGP12177, S(-)-pindolol, and pindolol, each of which mediated an initial steady phase with a short duration, and a succeeding P-DMR. The third group included salmeterol. Salmeterol induced a biphasic dose-dependent DMR (Fig. 2). Salmeterol at low doses (<50 nM) induced a CGP12177-like DMR, but at high doses an epinephrine-like DMR. The fourth group included phenylethylamine and tyramine, both of which induced a complicated DMR signal, possibly due to crosstalks

with another receptor(s) (see [Supplementary material](#)). Thus both were excluded from further analysis. The fifth group included ICI 118551, propranolol, timolol, satolol, betaxolol, and atenolol and CGP20712, none of which caused any significant DMR (data not shown). Given the sensitivity of the DMR signals to signaling pathway involved [13,16], these results suggest that these ligands differ greatly in their ability to activate the β_2 AR and direct its signaling.

3.2. Ligand-induced DMR are specific to the β_2 AR

Since A431 also naturally expresses other receptors [16], we were interested whether the DMRs obtained are specific to the β_2 AR. Results showed that the β -blocker propranolol dose-dependently attenuated the DMR induced by all agonists at their corresponding EC_{100} , yielding similar IC_{50} s (see [Supplementary material](#)). Interestingly, dopamine of high doses (>100 μ M) led to a DMR that can only be partially inhibited by 1 μ M propranolol (see [Supplementary material](#)), suggesting that the DMR of dopamine only at low doses is specific to the β_2 AR. Thus, the following discussions were limited to dopamine <100 μ M. Nonetheless, these results suggest that the ligand-induced DMRs are β_2 AR-specific.

3.3. Ligand-specific efficacy

Since these agonists exhibit different capacities in activating the β_2 AR, we were interested in their efficacies based on DMR measures. The maximal amplitudes of the P-DMR, normalized to the epinephrine response, were in the following order: isoproterenol (105%) $\sim$ epinephrine (100%) $>$ norepinephrine (91%) $\sim$ dopamine (92%) $\sim$ salbutamol (91%) $\sim$ halostachine (90%) $>$ catechol (66%) $\sim$ alprenolol (65%) $\sim$ labetalol (62%) $\sim$ pindolol (61%) $\sim$ S(-)-pindolol (60%) $>$ CGP12177 (54%) $\sim$ alprenolol (54%) $>$ xamoterol (33%) ($n = 16$) (Table 1). A similar but more pronounced trend was observed for the N-DMR event. These results suggest that both isoproterenol and epinephrine fully or nearly fully activate the β_2 AR, while the other ligands partially activate the receptor.

3.4. Ligand-specific shift of apparent potency relative to affinity

Many studies have shown that, similar to the efficacy, the ligand potency is also signal output-dependent; and the shifts

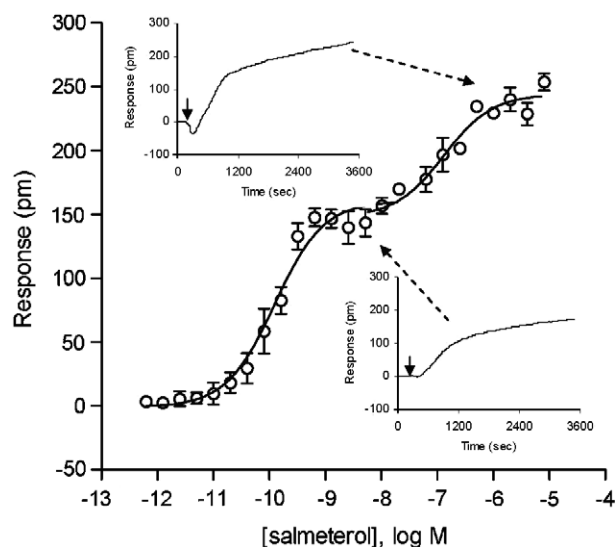


Fig. 2. The salmeterol-induced DMR. The inset showed two representative DMRs induced by salmeterol of 10 nM and 1000 nM, respectively.

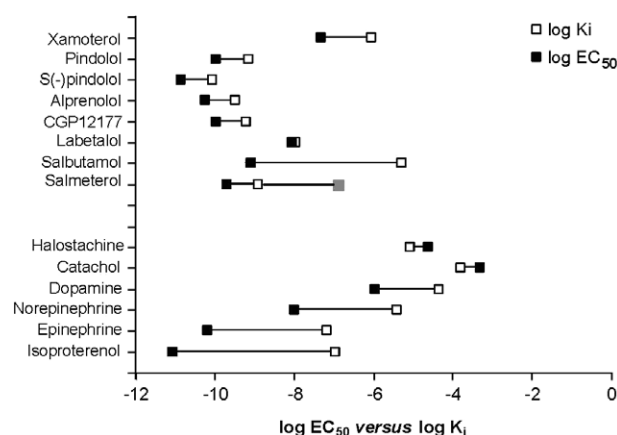


Fig. 3. The shifts in apparent DMR EC_{50} relative to binding affinity of the β_2AR agonists.

in apparent potency relative to affinity have been attributed to the differential coupling efficiencies of ligands. Results showed that all β_2AR ligands induced a dose-dependent and saturable response, yielding a single EC_{50} (see [Supplementary material](#)), except for salmeterol which exhibited two well-separated EC_{50} values (0.12 ± 0.05 nM and 130 ± 17 nM) (Fig. 2). Because of its biphasic dose responses, the potency of salmeterol shifted in both directions (Fig. 3). In comparison, the EC_{50} of the two full agonists, isoproterenol and epinephrine, significantly shifted towards the left. Similar shifts, but to lesser extent, were observed for the three strong partial agonists, norepinephrine, dopamine and salbutamol. Conversely, little shift was observed for the other weak partial agonists that poorly activate G_s . These results suggest that the weaker the agonist is, the closer to k_d the EC_{50} is; and the greater shift in EC_{50} indicates that the ligand is more effective in causing cyclic adenosine monophosphate (cAMP) accumulation.

3.5. Ligand-specific DMR kinetics

Since the DMR is a real-time kinetic cellular response, we examined the kinetic characteristics of these β_2AR ligand-induced DMRs, including the transition time τ for the P-DMR event to occur. Ligands that caused a rapid transition ($\tau \sim 140$ s) included isoproterenol, epinephrine, salmeterol of high doses, catechol and salbutamol. In comparison, all other partial agonists including salmeterol of 10 nM resulted in a slow transition ($\tau \sim 200$ –300 s).

Except for salmeterol at high doses, the P-DMR events induced by all agonists seem to fit well with a one-phase exponential association, leading to a characteristic $t_{1/2}$ (see examples in [Supplementary material](#); Table 1). Ligands that are known to be ineffective or less effective in causing receptor internalization [20] resulted in a rapid P-DMR. These ligands were dopamine, catechol, halostachine, and salmeterol of low doses. Ligands that are known to be effective to cause receptor internalization resulted in a slow P-DMR with a $t_{1/2}$ of ~ 540 s; these ligands were isoproterenol, epinephrine, norepinephrine, and salbutamol. Interestingly, xamoterol induced the slowest P-DMR.

The receptor internalization involves the relocation of the activated receptor/effector complexes away from the basal cell membrane and is a negative contributor to the overall response [15]. Thus, we speculated that the differentiated kinetics in the

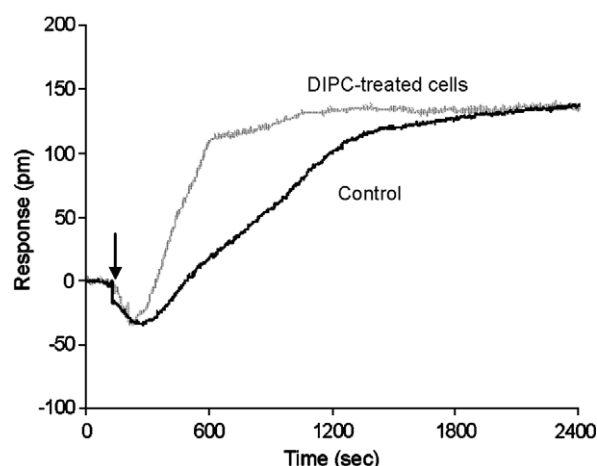


Fig. 4. The DMR signals of A431 cells induced by 2 nM epinephrine without (control) and with the pretreatment with 25 μ M dynamin inhibitory peptide (DIPC-treated cells).

P-DMR may be related to internalization in part. Since dynamin plays an important role in the ligand-induced β_2AR internalization [21], we examined the effect of inhibiting dynamin activity on the ligand responses. Results showed that the pretreatment of cells with DIPC significantly accelerated the kinetics of the epinephrine P-DMR, leading to a $t_{1/2}$ of ~ 200 s (Fig. 4), suggesting that the receptor internalization has negative impact on the kinetics of the epinephrine P-DMR. Conversely, the DIPC pretreatment had little effect on the P-DMR induced by catechol or halostachine (data not shown). Together, these results suggest that at least for catecholamine agonists, the difference in P-DMR kinetics seems to be an indicator for the ability of ligands to cause receptor internalization.

3.6. Catechol exhibits different abilities to modulate β_2AR ligand-induced DMR

Catechol seems to be an effective molecular probe to differentiate mechanistic differences between β_2AR activation by catecholamine agonists and by the structurally related non-catechol partial agonist salbutamol [22]. Thus, we examined the ability of catechol to modulate the DMR induced by other ligands. Results showed that the co-stimulation of A431 with pindolol of 10 nM and catechol of 500 μ M led to a DMR that closely resembled, but was not identical to, the sum of the two individual DMRs (Fig. 5a). The co-stimulated P-DMR exhibited faster transition time and kinetics. Moreover, pindolol in the presence of 500 μ M catechol yielded an EC_{50} almost identical to pindolol alone (Fig. 5b). Similarly, other partial agonists including alprenolol, CGP12177 and halostachine resulted in an almost identical pattern (see [Supplementary material](#); data not shown). These results suggest that these partial agonists do not directly compete with the binding of catechol to the β_2AR , and lead to signaling mostly independent of the catechol-induced signaling. Conversely, the co-stimulation of A431 with epinephrine or norepinephrine and 500 μ M catechol shifted the EC_{50} of either agonist to the right (Fig. 5b; data not shown), indicating that catechol does directly compete with both agonists; and either agonist can override the catechol response with a cost of decreased potency. Together,

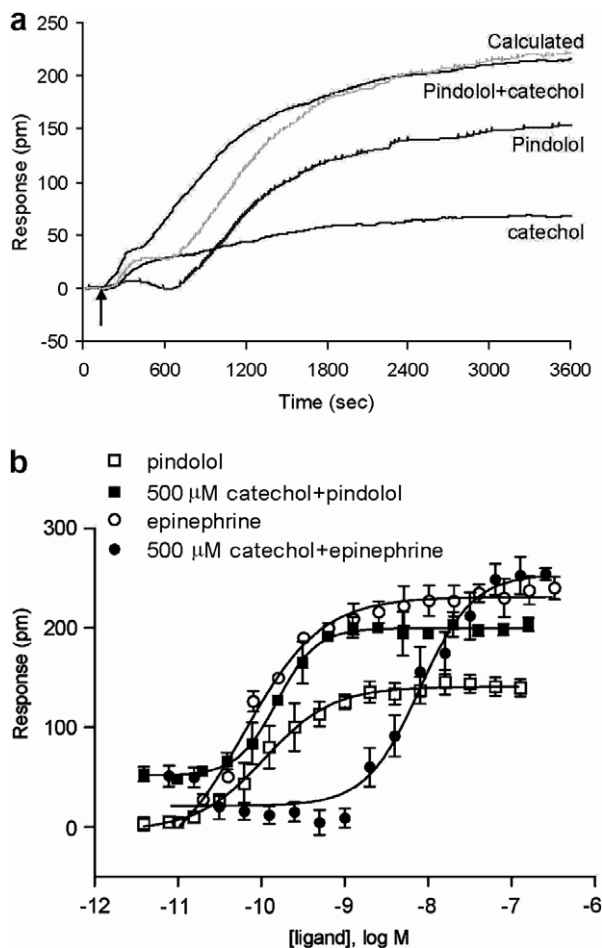


Fig. 5. The effect of catechol co-stimulation on the ligand-induced DMR. (a) The DMR signals induced by 500 μM catechol (Catechol), or 10 nM pindolol (Pindolol) were compared with that co-stimulated with 500 μM catechol and 10 nM pindolol (Pindolol + Catechol). The simple sum of both catechol and pindolol responses (Calculated) was also presented. (b) The dose-dependent responses induced by pindolol or epinephrine individually were in comparison with those induced by co-stimulation of either ligand with 500 μM catechol.

these results indicate the presence of multiple ligand-specific states of the receptor.

3.7. β -Blockers partially attenuate the catechol-induced DMR

We further examined the ability of several β -blockers to modulate the catechol-induced DMR. The blockers were inverse agonists ICI 118551 and propranolol, and antagonists timolol, sotalol, betaxolol, and atenolol. All these blockers attenuated, but were unable to completely inhibit, the catechol response (Fig. 6). Moreover, both ICI 118551 and propranolol altered the dynamics of the catechol signal from a single P-DMR to a G_q -like DMR [15,16,23]. Conversely, the other β -blockers, but not SCH23390, simply suppressed in a dose-dependent manner the amplitude and duration of the catechol signal. The apparent IC_{50} was 1.8 ± 1.5 nM, 193 ± 23 nM, 46.0 ± 11.3 nM, 415 ± 32 nM, 5.8 ± 1.3 nM, 0.07 ± 0.04 nM, and 12.7 ± 3.5 nM for propranolol, betaxolol, CGP20712, atenolol, sotalol, timolol, and ICI 118551, respectively. These results suggest that these β -blockers may not directly occupy the catechol-binding pocket, but do impact the catechol downstream signaling.

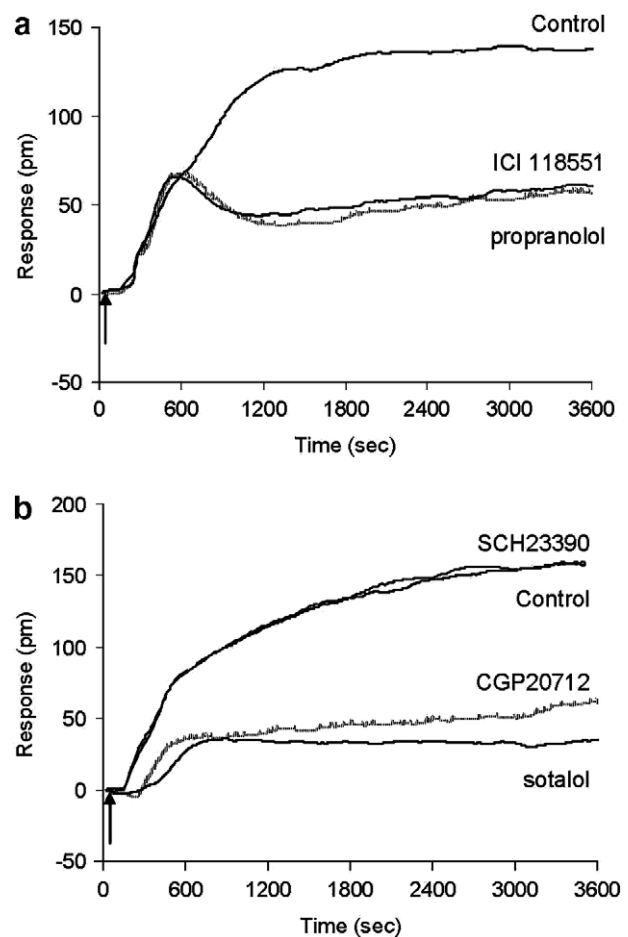


Fig. 6. The impact of β -blockers on the catechol (500 μM) response. (a) The catechol response (control) was compared with those in cells pretreated with 500 nM ICI 118551 or 10 nM propranolol. (b) The catechol response (control) was compared with those in cells pretreated with 1 μM SCH 23390, 500 nM CGP20712, or 250 nM sotalol.

4. Discussion

The β_2 AR signaling involves a series of orderly spatial and temporal events, many of which contribute to a ligand-induced DMR. Thus, it is difficult to link specific cellular events to the DMR. Here we primarily used multi-parameter-based pattern analysis, and discussed the functional selectivity of various ligands in the context of known characteristics of β_2 AR signaling. The agonists examined differ greatly in their abilities to cause cAMP accumulation, receptor internalization and/or interaction with β -arrestin. Their functional selectivity is clearly evident in DMR characteristics.

Epinephrine, isoproterenol, norepinephrine and dopamine are structurally similar catecholamine agonists, all of which led to a similar type of DMR, characterized by the occurrence of the N-DMR (Fig. 1). All four ligands are known to produce comparable cAMP through the β_2 AR, but differ greatly in causing receptor internalization [20,24]. Conversely, catechol was unable to produce an epinephrine-like DMR – there is no N-DMR event in the catechol response. Catechol is a very weak partial agonist to activate G_s , leading to very small increase in cAMP but not receptor internalization [22]. These results suggest that the occurrence of the N-DMR is

an indicator for the ability of the agonists to elevate intracellular cAMP level.

For the four catecholamine agonists, their P-DMR exhibited clearly different kinetics. The inhibition of dynamin activity significantly accelerated the kinetics of the epinephrine P-DMR, but not the catechol response (Fig. 4). Furthermore, both dopamine and catechol that are not effective in causing receptor internalization induced a faster P-DMR, compared to the other three catecholamines. These results suggest that the receptor internalization contributes negatively in part to the P-DMR, and is hidden behind other cellular events that occur simultaneously but cause increase in local mass density (possibly an increase in cell adhesion; data not shown). The delicate balance between them governs the apparent kinetics of the P-DMR. Thus the P-DMR kinetics indicates the ability of ligands to induce receptor internalization.

Halostachine also induced an epinephrine-like DMR (Fig. 1). Halostachine was shown to cause a maximal increase in cAMP at the whole cell level that was ~20% of that induced by epinephrine [20,22]. Since the cAMP produced due to the β_2 AR activation is much restricted initially in some microdomains in cells [25], the halostachine response suggests that the threshold of localized cAMP resulting in the N-DMR occurrence is relatively small, and/or halostachine is effective to cause localized cAMP production, but not at the whole cell level. Interestingly, consistent with the poor ability of halostachine to cause receptor internalization is that its P-DMR is also rapid.

Salbutamol, salmeterol and labetalol are three structurally related non-catechol ligands. These ligands exhibited great difference in mediating DMR signals. Salbutamol, the strong partial agonist in activating G_s , induced an epinephrine-like DMR. Labetalol induced a DMR in-between the catechol and epinephrine responses, with a small but noticeable N-DMR. Interestingly, salmeterol at low doses led to a labetalol-like DMR, while salmeterol of >100 nM caused a salbutamol-like DMR. Salmeterol is a long acting β -agonist with low intrinsic activity, and exhibits dual efficacies – a weak partial agonist for producing an effective interaction between the receptor and β -arrestin 2, and a full agonist for causing cAMP accumulation [26,27].

The four structurally related β -blockers alprenolol, CGP12177, S(-)pindolol and pindolol all led to a catechol-like DMR, but with distinct kinetics. CGP12177 is a partial agonist of the β_2 AR, and is capable of stimulating G_s and causing receptor internalization [28]. The co-stimulation with pindolol and catechol induced a DMR being close to the sum of the two DMRs obtained independently, and catechol did not alter the EC_{50} of pindolol. A similar behavior was also observed for the other three ligands. In contrast, the presence of catechol shifted the EC_{50} of epinephrine or norepinephrine to the right. These results suggest that these partial agonists do not compete with the binding of catechol, and mediate signaling mainly independent of catechol.

The other structurally diverse β -blockers led to no or little DMR. However, these β -blockers were able to attenuate the catechol response, with a maximal inhibition of ~55%. The catechol responses in the propranolol- and ICI 118551-pre-treated cells were similar, but were different from the cells pre-treated with the other β -blockers (Fig. 6). These results were surprising. A recent *in vitro* biophysical study showed that ICI 118551 has little effect on the catechol-induced fluores-

cence changes of a fluorescently engineered β_2 AR, suggesting that ICI 118551 is unable to inhibit the catechol-induced receptor activation [22]. However, these studies were based on the changes between only two points within an engineered receptor, and their functional consequences were largely limited to certain cellular events. Nonetheless, the present study suggests that these β -blockers may not directly compete with catechol, but do impact the catechol signaling through unknown mechanism(s).

In summary, we have used non-invasive optical biosensor to study the functional selectivity of diverse β_2 AR ligands. Multi-parameter analysis uncovers distinct patterns linking the structure to the ability of these ligands to mediate distinct receptor signaling. The data presented here clearly demonstrates biased agonisms of various β_2 AR ligands, and illustrates the power of optical biosensors for probing ligand-directed functional selectivity. However, there are still many unanswered questions – which pathways are involved in the DMR and how these pathways are regulated in a ligand-specific manner, and whether these pathways proceed collectively or independently. Furthermore, contributions from many cellular responses mediated through a receptor that make the biosensor cellular assays so valuable, however, also render the optical signal obtained “non-specific” relative to conventional cellular assays. Further functional genomics and cell biology studies are in progress to depict the nature of DMR signals mediated through the activation of GPCRs, including the β_2 AR.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2008.01.021](https://doi.org/10.1016/j.febslet.2008.01.021).

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