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Insight Box

Insight: Canonically, GPCR signaling is viewed to be initiated at the cell membrane, and evidence for the presence of an intracellular signaling wave is sparse in literature. Herein we demonstrated that the endogenous β_2-adrenergic receptor (β_2-AR) mediates an intracellular signaling wave.

Innovation: Label-free biosensor antagonist reverse assays were developed.

Integration: The biosensor permits the kinetic measure of receptor signaling, showing that the β_2-AR signaling results in a sustained cellular response. The biosensor offers non-invasive pharmacological profiling under different conditions, revealing that diverse β_2-AR ligands are clustered into different classes. The biosensor under microfluidics permits the precise control of ligand exposure, enabling the deconvolution of β_2-AR signaling pathways and waves.

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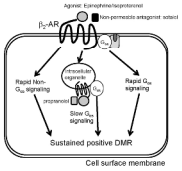
Label-free biosensor with microfluidics discovers an intracellular signaling wave mediated through the β_2-adrenergic receptors in A431.



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Paper

Label-free optical biosensor with microfluidics identifies an intracellular signalling wave mediated through the β_2-adrerengic receptor

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The canonical model of G protein-coupled receptor (GPCR) signalling states that it is solely initiated at the cell surface. In the recent years, a handful of evidences start emerging from high-resolution molecular assays that the internalized receptors can mediate the third wave of signalling, beside G protein- and β-arrestin-mediated signalling both initiating at the cell surface. However, little is known about the functional consequences of distinct waves of GPCR signalling, in particular, at the whole cell system level. We here report the development of label-free biosensor antagonist reverse assays and their use to differentiate the signalling waves of endogenous β_2-adrenergic receptor (β_2-AR) in A431 cells. Results showed that the persistent agonist treatment activated the β_2-ARs, leading to a long-term sustained dynamic mass redistribution (DMR) signal, a whole cell phenotypic response. Under the persistent treatment scheme in microplate, a panel of known β-blockers all dose-dependently and completely reversed the DMR signal of epinephrine at a relatively low dose (10 nM), except for sotalol which partially reversed the DMR. Under the perfusion condition with microfluidics, the subsequent perfusion with sotalol only reversed the DMR induced by epinephrine or isoprotenerol at 10 nM, but not at 10 μM. Furthermore, the degree of the DMR reversion by sotalol was found to be in an opposite relation with the duration of the initial agonist treatment. Together, these results suggest that the hydrophilic antagonist sotalol is constrained outside the cells throughout the assays, and the early signalling wave initiated at the cell surface dominates the DMR induced by epinephrine or isoproterenol at relatively low doses, while a secondary and late signalling wave is initiated once the receptors are internalized and contributes partially to the long-term sustainability of the DMR of epinephrine or isoproterenol at high doses.

Introduction

G-protein-coupled receptors (GPCRs) are the largest family of cell surface receptors that participate in ubiquitous transmembrane signal transduction processes primarily mediated through their coupled G proteins.[1] GPCR signalling is encoded by the spatial and temporal fluxes of downstream signalling networks.[2] The consensus model concerning GPCR signalling assumes that receptor signalling is initiated by the binding of an agonist, which stabilizes the active form of the receptor and promotes its coupling to heterotrimeric G proteins.[3,4] After the GDP–GTP exchange on the G_α subunit, the activated G protein is dissociated into GTP-bound G_α (G_α-GTP) and $G_{\beta\gamma}$ dimers from the receptor, both of which then modulates the activity of several intracellular enzymes, triggering a myriad of cell signalling. The agonist bound receptors then become phosphorylated, followed by the recruitment of arrestins to terminate receptor signalling by impairing receptor–G-protein coupling and promote receptor internalization into intracellular endosome vesicles.[5,6] The internalized receptors are further subject to degradation or recycling back to the cell plasma membrane.[7] Beside the negative regulation roles in GPCR signalling, β-arrestins were later found to be able to trigger their own signalling wave downstream the receptor activation.[8] Both G protein- and β-arrestin-mediated waves are believed to be initiated at the cell membrane.[9-11]

In the past couple of years, a handful of evidence emerging from high-resolution molecular assays suggest that instead of being functionally inactive the internalized receptors in intracellular organelles such as endosomes or Golgi apparatus may mediate a third wave of signalling.[12-19] To date, such an intracellular signalling has been reported for a small group of GPCRs including parathyroid hormone receptor[13,16], thyroid-stimulating hormone receptor[14,18], sphingosine-1-phosphate $S1P_1$ receptor[15], dopamine D_1 receptor[17], and β_2-adrenergic receptor (β_2-AR)[19]. Furthermore, the internalized receptor mediated signalling is primarily inferred from the G protein activity such as alterations in cyclic AMP (cAMP). Little is known about the functional consequences of distinct waves of GPCR signalling, in particular, at the whole cell system level.

Here we report the identification of an intracellular signalling wave after the activation of endogenous β_2-ARs in A431 using label-free resonant waveguide grating (RWG) biosensor under different stimulation schemes including microfluidics. The RWG biosensor employs a surface bound evanescent wave to translate the dynamic redistribution of cellular matters within the bottom portion of cells arising from a drug-receptor interaction into a

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real-time dynamic mass redistribution (DMR) signature.[20,21] The DMR is often recorded as a time-series of the shifts (in picometer, pm) of the biosensor resonance wavelength, a direct function of the local refractive index near the sensor surface.[21] The DMR assay permits a holistic and kinetic representation of drug action and receptor signalling in native cells.[22-24] Owing to its non-invasiveness, DMR assay can be performed under different stimulation schemes, including commonly used agonism, competitive antagonism, antagonism/desensitization, and pathway deconvolution assays (**Fig.1**).[24-26] Microfluidics-integrated biosensor systems further allow the precise control of drug-target interactions temporally[27] and spatially[28], and create new revenue for elucidating receptor signalling and drug pharmacology.[27-31] Taking advantage of the long-term sustainability of the DMR signals of certain receptors including the β_2-AR, we developed an antagonist reverse assay, wherein the receptor is first activated, followed by challenging with its antagonists (**Fig.1**). Combining the antagonist reverse assay with microfluidics showed that the activation of the β_2-ARs in A431 cells mediates distinct waves of signalling.

Materials and methods

Materials

Cholera toxin (CTx), clenbuterol, fenoterol, isoetharine, labetalol, (R)-(+)-atenolol, ritodrine, and terbutaline were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bopindolol was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). All other chemicals were obtained from Tocris Chemical Co. (St. Louis, MO, USA). The ligand library was made by dissolving ligands in dimethyl sulfoxide (DMSO) and stocking them at 10 mM at -80 ^{o}C. All ligands were freshly diluted using the assay buffer (HBSS; 1x Hanks' balanced salt buffer, 10mM Hepes-KOH, pH 7.1). Polydimethylsiloxane (PDMS) was obtained from Dow Chemical (Midland, MI, USA). Epic® 384-well biosensor cell culture compatible microplates were obtained from Corning Incorporated (Corning, NY, USA). Human epidermoid carcinoma A431 cell line was obtained from American Type Cell Culture (Manassas, VA, USA).

Cell culture

A431 cells were cultured using the complete medium (that is, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/liter glucose, 2 mM glutamine, and antibiotics) in a humidified 37°C/5% CO_2 incubator. For DMR assays in microplate, the cells were seeded at a density of 25,000 cells per well in Epic® biosensor microplates. After ~20hrs culturing in the complete medium at 37°C/5% CO_2, the cells were further starved about ~20hrs in the serum free medium to reach a quiescent state.

For DMR assays under microfluidics the cells were cultured in a PDMS microfluidic biosensor device, as previously described.[27,29] Briefly, a PDMS microfluidic device was made by casting the PDMS pre-polymer solution containing a mixture of PDMS oligomers and a reticular agent with 10:1 mass ratio (Sylgard 184 Kit, Dow Corning Corp., Midland, MI, USA) onto patterned silicon wafers, followed by curing at room temperature for about 24hrs to minimize shrinkage. After being punched to generate inlet and outlet holes, the PDMS replicas were then aligned and reversibly bonded onto the top of the biosensor inserts. The microfluidic device has a 3 x 4 array of functional RWG biosensors, each having a dimension of 2 mm x 2 mm, and a 3 x 4 array of microfluidic chambers. Each chamber has three inlets and one outlet. Each inlet was connected to an independently operated syringes using Tygon Ò tubing (Tygon S-54-HL, Saint-Gobain Performance Plastics, Akron, OH, USA), so that continuous perfusion of cells with three different solutions, one at a time, is possible without introducing any abrupt changes of shear stress or laminar flow perturbation inside the microfluidic chamber. The distance from an inlet to the outlet is 9 mm, while the central width of the chamber is 5 mm, and the

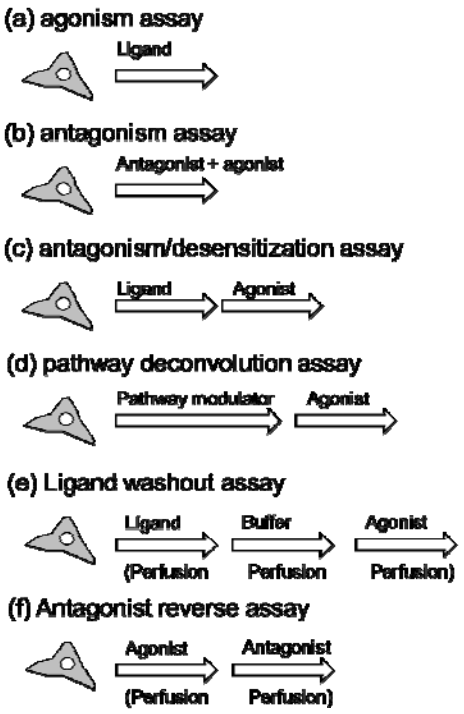

Fig. 1 Distinct DMR assay formats. (a) Agonism assay wherein the cells are directly stimulated with a ligand. (b) Competitive antagonism assay wherein the cells are stimulated with an agonist together with a known antagonist for the same receptor. (c) Antagonism/desensitization assay wherein a ligand is introduced before stimulation with an agonist, so an agonist ligand for the same receptor desensitizes the cells responding to the subsequent agonist stimulation, but an antagonist ligand blocks the agonist response. (d) Pathway deconvolution assay wherein the cells are first treated with a pathway modulator (e.g., G protein toxin), followed by the stimulation with an agonist. (e) Ligand washout assay wherein the cells are first treated with a ligand, followed by the removal of the ligand through washing or perfusion with a microfluidic device, and finally the stimulation with an agonist. (f) Antagonist reverse assay wherein the cells are first stimulated with an agonist for a receptor, followed by the treatment with an antagonist for the same receptor. A washout or perfusion step can also be applied between the two treatments. Except for perfusion which is performed using active pump-equipped microfluidic biosensor devices, all other assays are done in biosensor microplates.

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height of the microchannel is 200 μm. The total volume required to fill up a single chamber is 6 μl. The assembled device, tubing and syringes (500 ml, gas tight 1700 series, Hamilton, Reno, NV, USA) were sanitized with 70% ethanol and dried using nitrogen flow. Afterwards, 4×10^4 cells suspended in 6 μl of the complete medium were injected into each chamber. After 30 min incubation at room temperature, tubing was plugged into the microchamber inlets and was connected to syringes connecting to a syringe pump (Model: SP230IW; World Precision Instruments, Sarasota, FL, USA). The cells were finally cultured at 37°C/5% CO_2 with a continuous perfusion with the complete media for 20hrs, followed by perfusion with the serum free medium for 22hrs, both at a flow rate of 5 μl hr^{-1}. The cell confluency was ~95% at the time of assaying for all conditions.

DMR assays

DMR assays in microplate were performed using Epic® system, a bulky standalone wavelength interrogation reader system tailored for high throughput screening.[25] This system consists of a temperature-control unit (26°C), an optical detection unit, and an on-board liquid handling unit operated by robotics. The detection unit is centred on integrated fibre optics which scans across the plate with a temporal resolution of 15 sec. For persistent DMR assays in microplate,[30] the cultured cells were first washed with the assay buffer HBSS, and incubated inside the Epic® system for ~1hr. After a steady baseline was established, the ligand solutions were introduced using the on-board liquid handling device, and the cell responses were then recorded over time. For the ligand washout assay in microplate, an offline washing step consisting of a five time buffer washing (~5min in total) using a plate washer (Bio-Tek Microplate Washers ELx405™, Bio-Tek, Winooski, VT) and an additional two-hour incubation with the buffer was introduced between the two stimulation steps. For CTx treatment, the cells were pre-treated with 400 ng ml^{-1} CTx in the complete medium for 3 hours at 37°C/5% CO_2.

DMR assays under microfluidics were performed using a high resolution Epic® BT system, a small-footprint swept wavelength interrogation-based imaging system.[32] This system has a spatial resolution of 12 μm and a temporal resolution of 3 sec. This system uses a light beam from a swept tunable light source to illuminate the 3x4 microfluidic biosensor device, and a high speed complementary metal-oxide semiconductor (CMOS) digital camera to record the escaped and reflected resonant lights. The tunable light source sweeps the wavelength range from 825 to 840 nm in a stepwise fashion. Total 150 spectral images were acquired within a single sweeping cycle (3 sec), and were then processed into sensor resonance wavelength or DMR image in real time. For perfusion DMR assay under microfluidics the cells were first perfused with the assay buffer for ~1 hr, followed by perfusion sequentially with two different ligands with a flow rate of 1 μl/min to minimize the effect of shear stress on cellular status.[27]

All real-time DMR signals were reported as a 2min baseline right before stimulation with a ligand. All DMR signals were background corrected. Owing to the heterogeneity of cell signalling at the single cell level[32], all DMR signals were reported as an averaged response from a population of cells. For DMR assays in microplate, an averaged DMR response was obtained from the cells within the scanning path.[33] For DMR assay under microfluidics an averaged response from the cells located within the central area (0.3×1.0 mm) of a biosensor.[27] All studies were carried out with at least three replicates unless specifically mentioned.

Data analysis

All dose-dependent responses were analysed by using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The EC_{50} and IC_{50} values were obtained by fitting the DMR dose response curves with nonlinear regression. For cluster analysis, we performed five distinct assays to separate these ligands into different groups (see below), and extracted the real responses at the six distinct time points (3, 5, 9, 15, 30, 45min) for each DMR to form a numerical description of its characteristics.[34-36] All the six time points refer to the stimulation duration after renormalizing the responses starting from the time when the compound was added. The real responses at these discrete time points were colour coded to illustrate relative differences in the direction and strength of a DMR signal. The red colour indicates a positive value, while the green colour refers to a negative value, the black a value at or near zero. Differences in colour intensity illustrate differences in signal strength. In the ligand heatmap matrix (**Fig. 2**) each column represents one DMR value at a specific time in a specific assay condition, and each row represents one ligand. The Ward hierarchical clustering algorithm and Euclidean distance metrics (http://www.eisenlab.org/eisen/) were used for similarity analysis, and every row and column carries equal weight. DMSO in the assay buffer at a concentration that equals to those for all ligands was also included as a negative control. Each DMR represents an average of four replicates. To assist with direct visualization of DMR characteristics of each ligand in an assay, we did not carry out similarity analysis among distinct columns, so that each assay was arranged in six consecutive columns to form a column group for clear understanding the key characteristics of a DMR.

Results and Discussion

Label-free profiling of β_2-AR ligands

To identify probe molecules for elucidating the receptor signalling waves, we first profiled a small library of β_2-AR ligands, all at 10 μM, in A431 cells using five different DMR assays in microplate. A431 cell line has been used as a model cell line to study the signalling of the β_2-AR.[37,38] The negative controls were also included; these were the buffer solution containing equal amount DMSO, the α_2-AR-selective antagonist rauwoscine, and α_2-AR-selective agonists including UK14,304,

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oxymetazoline, rilmenidine, and tizanidine. As expected, all α_2-AR ligands behaved identically to the DMSO buffer control under all conditions, while the remaining ligands were active at least in one of the five assay conditions (**Fig.2**), suggesting that A431 indeed expresses functional β_2-ARs.

The five different assays include DMR agonism assay, antagonism/desensitization assay, ligand reverse assay, and two ligand washout assays. For the ligand washout assays, the cells were first treated with a ligand for either one-hour or twenty-two hours in the serum-free medium at 37°C/5% CO_2, followed by the ligand washout and finally stimulation with 10 nM epinephrine, a non-selective natural agonist for adrenergic receptors. The DMR agonism assay is used to separate different ligands based on their agonistic activities. The antagonism/desensitization assay is used to further separate different ligands based on their specificity, potency, and modes of action (agonist *versus* antagonist). The ligand washout assays are used to separate different ligands mostly based on their binding kinetics, in particular drug residence time (the reciprocal of K_{off}), related functional consequence (short or long acting antagonists *versus* short or long acting agonist).[27,29,30] The ligand reverse assay is used to examine the ability of an antagonist or partial agonist to reverse the sustained DMR of epinephrine. Except for the DMR agonism assay, all other assays used 10nM epinephrine before (the ligand reverse assay) or after the ligand treatment (the antagonism/desensitization and ligand washout assays). The DMR signals arising from the last stimulation were used to perform cluster analysis. Results showed that the similarity analysis led to a heatmap segregating thirty-nine ligands into six clusters (**Fig.2**).

The first cluster consists of the negative buffer control (DMSO), R-(+)-atenolol, and all five α_2-AR-selective ligands including UK14,304 and rauwolscine. R-(+)-atenolol, a less active enantiomer than S-(-)-atenolol at the β_2-AR, only gave rise to a small activity in the ligand reverse assay (**Figs.2** and **3**). All others were inactive in the DMR agonism assay (**Fig.3a**), and had little effect on the DMR of 10nM epinephrine, regardless of the assay conditions (**Fig.3b,c**). These results suggest that ligands in this cluster had little activity at the β_2-AR.

The second cluster consists of a group of β-blockers including betaxolol, bisoprolol, metoprolol, sotalol, S-(-)-atenolol and nadolol. These ligands were inactive in the DMR agonism assay, but blocked the DMR of 10nM epinephrine (**Fig.3d**). All these ligands reversed the DMR of 10nM epinephrine (**Fig.3e**), but had little impact on the DMR of 10nM epinephrine after the ligand removal (**Fig.3f**). These results suggest that these ligands are reversible β_2-AR neutral antagonists with short-acting activity.

The third cluster consists of dopamine, epinephrine, norepinephrine, phenylephrine, dobutamine, ritodrine, isoetharine and terbutaline. Ligands in this cluster all resulted in a robust DMR sharing characteristics similar to epinephrine, and desensitized the cells responding to the subsequent stimulation with 10nM epinephrine (**Fig.3g**). All these agonists triggered little response after the 10nM epinephrine treatment (**Fig.3h**). However, the behaviour of these ligands was divergent in the ligand washout assays – the cells responded more robustly to the subsequent stimulation with 10nM epinephrine after the removal of epinephrine or dopamine, compared to other agonists (**Fig.3i**).

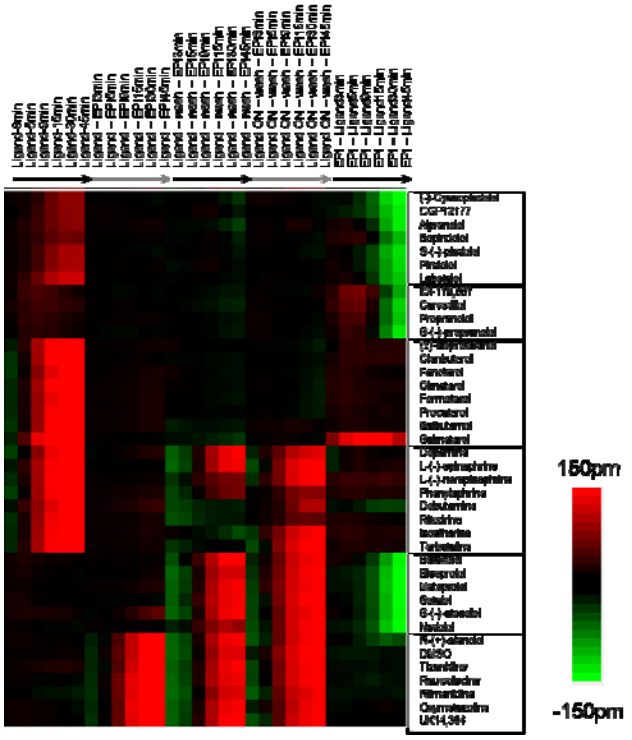

Fig. 2 DMR heat map of adrenergic receptor ligands in A431. This heat map was obtained using similarity analysis the Ward hierarchical clustering algorithm and Euclidean distance metrics of the DMR profiles of the ligands under five conditions. The five DMR signals for each ligand were: (1) a ligand-induced DMR in the cells without any pre-treatment (Ligand); (2) the DMR of 10nM epinephrine one-hour after the pre-treatment with a ligand, wherein the ligand is presented throughout the assay (Ligand-EPI); (3) the DMR of 10nM epinephrine one-hour after the treatment with the ligand, followed by five times wash with the assay buffer (Ligand-Wash-EPI); (4) the DMR of 10nM epinephrine after the overnight pre-treatment with a ligand, followed by five times wash with the assay buffer (Ligand ON-Wash-EPI); and (5) the DMR of a ligand one-hour after the pre-treatment with 10nM epinephrine, wherein epinephrine is presented throughout the assays (EPI – Ligand). For (4, 5), there are two-hour incubation between the wash and the last stimulation. The real responses at six discrete time points post-stimulation (3, 5, 9, 15, 30, 45min) for each DMR signal were used for the cluster analysis.

These results suggest that ligands in this cluster are β_2-AR agonists with short-acting agonist activity.

The fourth cluster consists of isoproterenol, clenbuterol, fenoterol, cimaterol, formoterol, procaterol, salbutamol, and salmeterol. All these ligands triggered a DMR similar to epinephrine and caused complete desensitization to the repeated stimulation with 10nM epinephrine (**Fig.3g**). All these ligands had little effect on the DMR of 10nM epinephrine, regardless of the assay conditions (**Fig.3h,i**). These results suggest that these ligands are β_2-AR agonists with moderately long or long-acting agonist activity.

The fifth cluster consists of ICI118,551, carvedilol, (±)propranolol and S-(-)-propranolol, a group of known inverse

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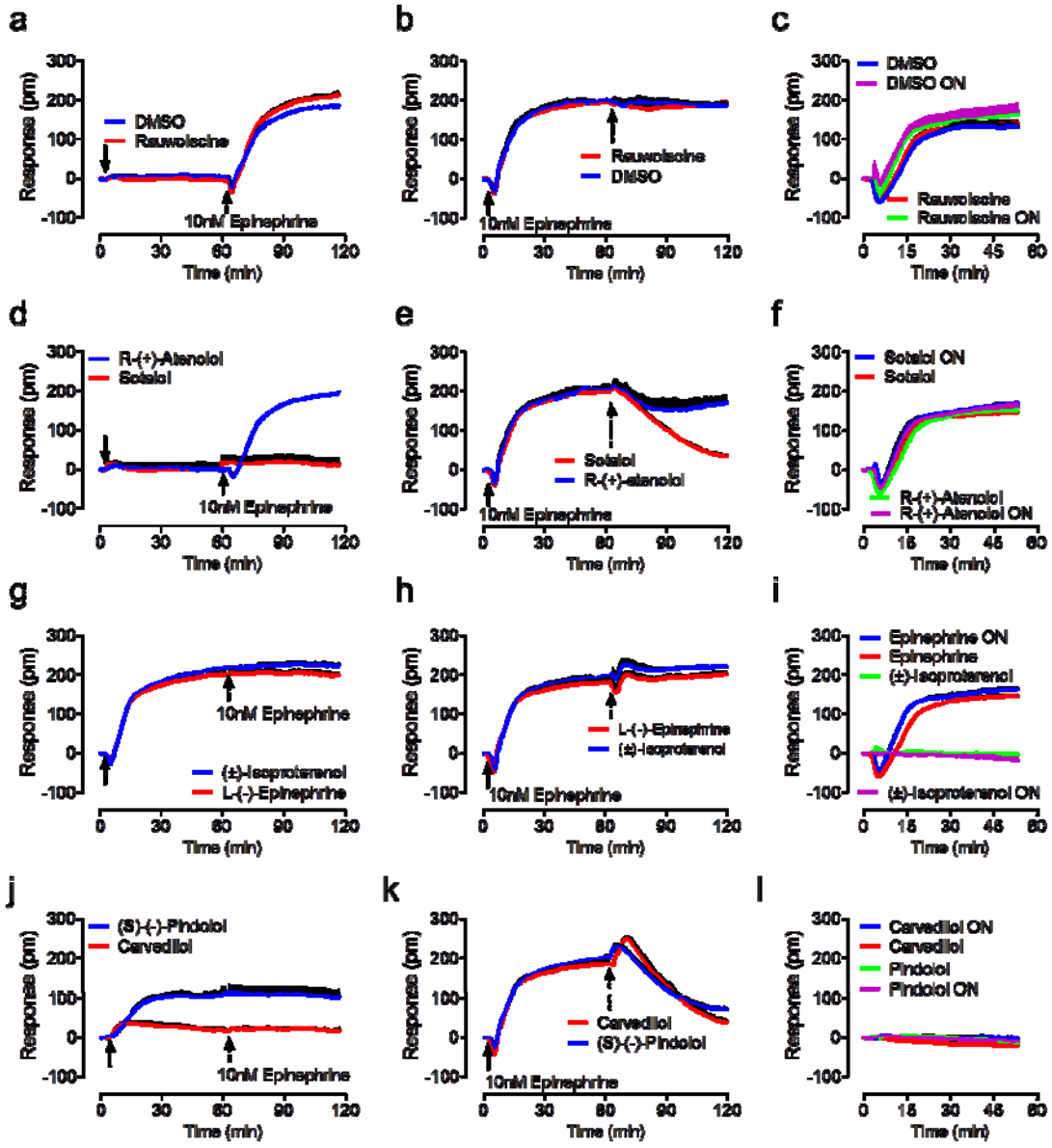

Fig. 3 DMR characteristics of different ligands under different assay conditions. (**a, d, g, j**) The real time DMR signals of cells responding to stimulation with distinct ligands, each at 10 μM, and subsequent stimulation with 10 nM epinephrine. (**b, e, h, k**) The real time DMR signals of cells responding to 10 nM epinephrine, followed by stimulation with distinct ligands, each at 10 μM. (**c, f, I, l**) The DMR signals of 10 nM epinephrine after the pre-treatment with distinct ligands, each at 10 μM, for one-hour (Ligand), or overnight (Ligand ON), followed by ligand washout and 2 hrs incubation with the assay buffer. Data represents mean±s.d. (n = 4 for all).

agonists. They all triggered a small DMR with distinct characteristics, and blocked the DMR of epinephrine under the persistent stimulation scheme (**Fig.3j**), or the ligand washout conditions (**Fig.3l**). Interestingly, all these ligands displayed a distinct reverse pattern of the epinephrine DMR – they caused an initial increase followed by a decrease after the epinephrine stimulation (**Fig.3k**). These results suggest that these ligands are β_2-AR biased agonists with relatively long-acting antagonist activity.

The last cluster consists of cyanopindolol, CGP12177, alprenolol, bopindolol, S-(-)-pindolol, pindolol, and labetalol. They all gave rise to a DMR without the initial negative phase of the epinephrine DMR, and blocked the epinephrine DMR without the ligand removal (**Fig.3j**), or with the ligand removal (**Fig.3l**).

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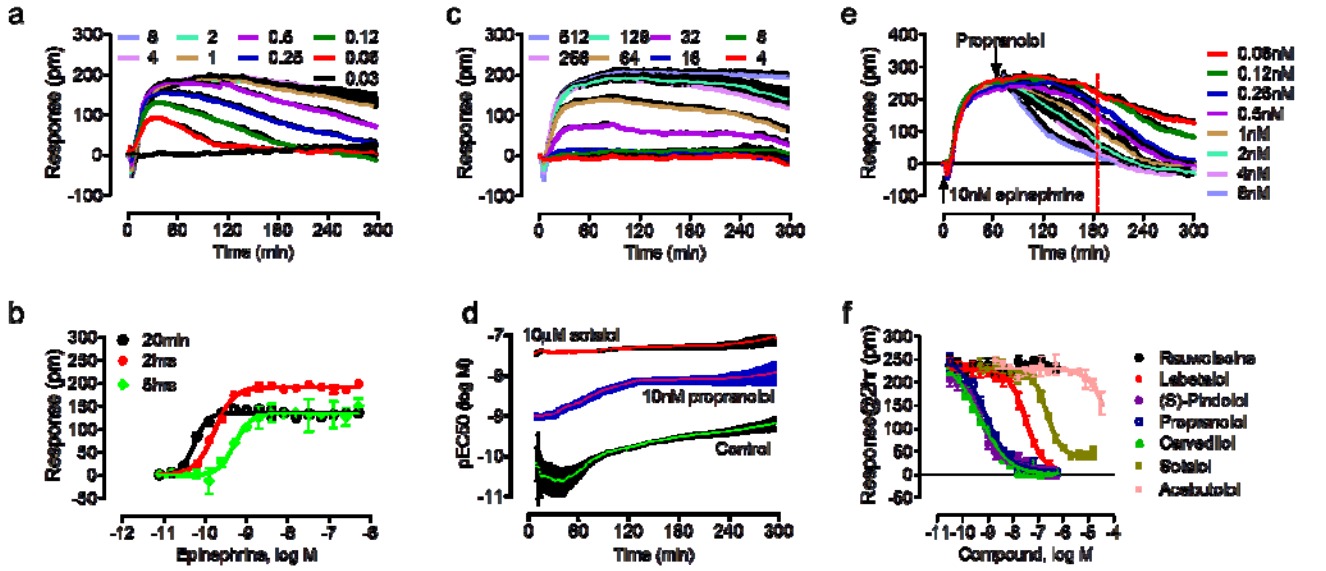

Fig. 4 The long-term DMR responses of epinephrine under different conditions in A431 cells. (a) The real-time DMR dose responses of epinephrine (in nM). (b) The real values at three discrete time points post simulation (20min, 2 hrs, 5 hrs) as a function of epinephrine doses. (c) The real-time DMR dose responses of epinephrine (in nM) in the presence of 10 μM sotalol. (d) The pEC_{50} values of epinephrine in the absence (Control), or presence of 10 nM propranolol or 10 μM sotalol as a function of the time points post stimulation. (e) The real-time DMR of 10nM epinephrine followed by the treatment with propranolol at different doses. (f) The real values of the epinephrine DMR at 2 hrs after the treatment with an antagonist (as indicated by the red dotted line) as a function of antagonist doses. Data represents mean±s.d. (n = 8 for a and b; n = 4 for c and d; n =2 for e and f).

All these ligands reversed the epinephrine DMR (**Fig.3k**). These results suggest that these ligands are β_2-AR partial agonists with relatively long-acting antagonist activity.

Collectively, these results suggest that ligands active at the β_2-AR display rich behaviours toward the receptor. Compared to our previous findings obtained using label-free integrative pharmacology on target (iPOT) analysis[34-36] and DMR multi-parameter analysis[39], there are a couple of novel findings. First, the receptor resensitization is relatively rapid for the cells pre-treated with dopamine, epinephrine or norepinephrine, but moderate for phenylephrine, dobutamine, ritodrine, isoetharine or terbutaline, and much slower for all other agonists, as revealed by the ligand washout assays. The second one is that all the β-blockers tested can reverse the DMR of 10nM epinephrine, as revealed by the ligand reverse assay. The inverse agonists including carvedilol and propranolol at 10μM initially increased and then decreased the DMR of epinephrine at 10nM.

Distinct signalling waves of the β_2-AR

β-Blockers are known to have a wide range of lipophilic property.[40,41] Sotalol, S-(-)-atenolol and nadolol are highly hydrophilic, while propranolol, pindolol, timolol, and metoprolol are highly lipophilic, and carvedilol, bisoprolol, betaxolol, labetalol, and acebutolol are moderately lipophilic. Hydrophilic drugs such as sotalol tend to have much lower permeability in cultured cells than lipophilic drugs.[42] We hypothesized that compared to lipophilic drugs, hydrophilic drugs may be much less efficient to reverse the DMR arising from the activation of the β_2-AR, if there is a late intracellular signalling wave initiated from the internalized receptors. Therefore, it would be possible to use appropriate receptor probe molecules, together with DMR assays, in particular ligand reverse assay under microfluidics, to elucidate distinct signalling waves of the β_2-AR.

First, we examined the long-term epinephrine dose responses. Results showed that epinephrine triggered a dose-dependent response (**Fig.4a**). At low doses (≤1nM) epinephrine triggered a tri-phasic DMR signal consisting of a rapid decreasing event, a slow increasing event and a much slower decaying event in a sequential manner. The DMR of epinephrine at a dose ≥1nM showed clear sustainability over 5 hrs at 26 °C. The pEC_{50} of epinephrine was found to be stimulation duration dependent, and was -10.26±0.03, -9.77±0.02, and -9.30±0.04 (n=4) at 20min, 2hrs and 5hrs post stimulation (**Fig.4b**). Given that epinephrine is an agonist binding to the β_2-AR with a moderate affinity (K_i of 741.3 nM),[43] the low EC_{50} values observed suggest that epinephrine has high efficacy to activate the DMR in A431[27]. Furthermore, the reducing EC_{50} values over stimulation time suggest that distinct pathways may govern the early response *versus* the long-term sustainability of the epinephrine DMR.

Second, we examined the long-term epinephrine dose responses in the presence of two β-blockers, each at a fixed dose. Propranolol is a lipophilic β-blocker with a high affinity binding

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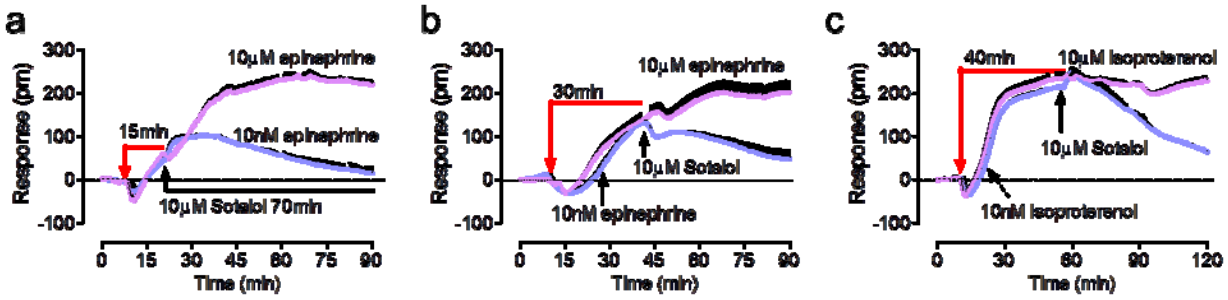

Fig. 5 The agonist exposure time-dependent DMR in A431 and its reverse pattern by sotalol. (a) The DMR of cells responding to the sequential perfusion with epinephrine (10nM and 10μM) for 15 min and 10 μM sotalol for ~1hr. (b) The DMR of cells responding to the sequential perfusion with epinephrine (10nM and 10μM) for 30 min and 10 μM sotalol for ~1hr. (c) The DMR of cells responding to the sequential perfusion with isoproterenol (10nM and 10μM) for 40 min and 10 μM sotalol for ~1hr. All were performed under perfusion condition with a flow rate of 1μl/min. Data represents mean±s.d. (n = 4).

to the β_2-AR (K_i of 0.8 nM), while sotalol is a hydrophilic β-blocker with a moderate affinity (K_i of 141.3 nM).[44] Given that propranolol only at relatively high doses (>1 μM) triggered a noticeable DMR (**Fig.2**; *ref*. 39), we decided to use 10 nM propranolol and 10 μM sotalol for the co-stimulation studies. Results showed that the presence of both antagonists shifted the potency of epinephrine to the right, suggesting that both antagonists are competitive against epinephrine (**Fig.4c** and **d**). Interestingly, the presence of the two antagonists altered the pattern of the time-dependent potency of epinephrine, wherein the potency was determined by non-linear regression analysis of the epinephrine dose-dependent real values at discrete time points. Given the epinephrine DMR characteristics, we limited such an analysis for time points ≥10 min post stimulation. Results showed that the presence of sotalol led to a potency of epinephrine that is insensitive to the stimulation time, while the presence of propranolol to a large degree caused a parallel shift of the potency of epinephrine to the right over the assay duration (**Fig.4d**). Given the binding of β-blockers to the β_2-AR at the cell membrane is believed to be through a well-defined pathway starting from the extracellular side of the receptor,[45] it is highly possible that the hydrophilic sotalol is less permeability to the cells and selectively blocks the signalling initiated from the receptors at the cell plasma membrane, but not the internalized receptors, compared to propranolol that is effective to block both signalling waves.

Third, we performed the antagonist reverse assays in microplate, wherein the cells were first stimulated with epinephrine of 10 nM for about 1hr, followed by the treatment with different antagonists in the presence of epinephrine. Results showed that all β-blockers tested dose-dependently reversed the sustained DMR, but the α_2-AR-selective antagonist rauwolscine did not and the weak β_2-AR antagonist acebutolol up to 10μM only reversed it partially (**Fig.4e**). The pIC_{50} to reverse the epinephrine DMR was found to be -9.32±0.10, -9.22±0.07, -9.10±0.07, -7.49±0.05, and -6.67±0.03 (n=2) for carvedilol, S-(-)-pindolol, S-(-)-propranolol, labetalol, and sotalol, respectively (**Fig.4f**). The rank order in pIC_{50} obtained was in good agreement with that based on their known binding affinities.[43] Notably, the four lipophilic drugs completely reversed the epinephrine DMR, but sotalol only partially reversed it (**Fig.4f**). One possibility is that the hydrophilic blocker sotalol selectively reverses the persistent signalling initiated from the cell surface receptors.

Fourth, we examined the ability of sotalol to reverse the epinephrine DMR under microfluidic perfusion condition. Perfusion is an effective means to replace a solution with a different solution.[27,29,32] Previously, we had used perfusion with microfluidics to investigate the effect of the agonist exposure duration on the receptor signalling, showing that the activated β_2-ARs can continuously mediate signalling after the agonist removal; as short as 1 min pulse stimulation with epinephrine is effective to initiate receptor signalling that is persistent for hours; and its increasing DMR event is split into two distinct phases when the pulse stimulation is ≤5 min.[27] To elucidate intracellular signalling waves, we examined and found that the agonist stimulation duration ≥10min is sufficient to ensure the minimal impact of the agonist removal by perfusion with the assay buffer on the epinephrine DMR. Thus, we used the agonist exposure duration ≥15min for the antagonist reverse assays under microfluidics. Given that for GPCRs the β-arrestin mediated signalling often requires high receptor occupancy, while the G_α pathway occurs at low receptor occupancy state,[27,46,47] both epinephrine and isoproterenol were assayed at two different doses, 10 nM and 10 μM. Results showed that perfusion with 10μM sotalol completely reversed the DMR of epinephrine at 10nM, but not at 10μM, when the sotalol solution was perfused 15min after the epinephrine stimulation (**Fig.5a**). Similar trends were observed when the sotalol solution was perfused 30min or 40min after the stimulation with epinephrine or isoproterenol, respectively (**Fig.5b** and **c**). Furthermore, the degree of the DMR reversion by sotalol was found to be in an opposite relation with the duration of the initial agonist treatment. Given that the

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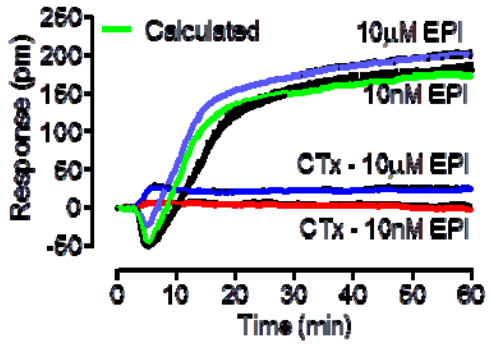

Fig. 6 The sensitivity of the epinephrine DMR in A431 cells to the cholera toxin pre-treatment. The cells were pre-treated with 400ng/ml chorea toxin for 3 hrs. Epinephrine was assayed at both 10nM and 10μM. Subtracting the DMR of 10μM epinephrine in the native cells with that in the toxin treated cells resulted in a DMR (Calculated), which is similar to that of 10nM epinephrine. Data represents mean±s.d. (n = 8).

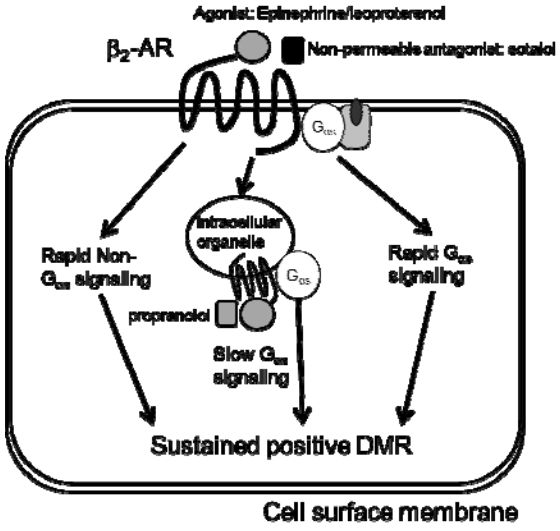

Fig. 7 The activation of the β_2-ARs in A431 triggers multiple signalling waves. The rapid $G_{\alpha s}$-dependent wave initiated at the cell surface is activated by epinephrine at low doses (e.g., 10nM), and be reversed by the hydrophilic antagonist sotalol. The rapid $G_{\alpha s}$-independent wave initiated at the cell surface is activated by epinephrine at high doses (e.g., 10μM). The slow $G_{\alpha s}$-dependent intracellular wave is also activated by epinephrine at high doses, and cannot be reversed by sotalol. All three signalling waves contribute to the long-term sustainability of the epinephrine DMR response, among which the $G_{\alpha s}$-dependent waves dominate. The cell membrane non-permeable antagonists such as sotalol selectively block the signalling waves initiated at the cell surface, while the cell membrane permeable antagonists such as propranolol block the signalling waves initiated both at the cell surface and inside the cell.

perfusion with sotalol effectively replaces the initial agonist solution as well as the rapid dissociation rates of both agonists, these results suggest that both agonists at high doses (e.g., 10μM) activate an intracellular signalling wave that cannot be blocked by the hydrophilic blocker sotalol; but, both agonists at low doses (e.g., 10nM) primarily activates signalling wave(s) initiated at the cell surface that can be blocked by sotalol.

Lastly, we examined the signalling pathways using the pathway deconvolution approaches.[26] Given that the β_2-AR in A431 is a prototypic $G_{\alpha s}$-coupled receptor, we studied the effect of chorea toxin (CTx) on the DMR of epinephrine at two different doses, 10nM and 10μM. Binding of CTx to the $G_{\alpha s}$ is known to result in permanent activation of the $G_{\alpha s}$ by ADP ribosylation of an arginine residue and cAMP production.[48] Results showed that the pre-treatment with CTx completely blocked the DMR of 10nM epinephrine, suggesting that its DMR is mostly mediated through the $G_{\alpha s}$ pathway (**Fig.6**). In contrast, the CTx pre-treatment significantly but not completely blocked the DMR of 10μM epinephrine. In addition, subtracting the DMR of 10μM epinephrine in the native cells with the toxin treated cells led to a DMR similar, but not identical to that of 10nM epinephrine. These results suggest that epinephrine at 10nM triggers a DMR primarily via the $G_{\alpha s}$ pathway, while at 10μM it activates both $G_{\alpha s}$-dependent and independent pathways, both contributing to its DMR. These results also suggest that the long-term sustainability of the DMR of epinephrine is mostly due to the $G_{\alpha s}$ pathway.

Conclusions

We provide evidence supporting that the activation of the β_2-ARs in A431 triggers multiple signalling waves, including $G_{\alpha s}$-dependent and independent rapid waves initiated at the cell surface, and an intracellular $G_{\alpha s}$-dependent and relatively slow wave (**Fig.7**). Both $G_{\alpha s}$-dependent waves are primarily accounted for the long-term sustainability of the epinephrine DMR response. The antagonist reverse assays in microplate as well as under microfluidics can provide new insights for receptor signalling, and open a new possibility to elucidate receptor pharmacology. Of note, further elucidation of the nature of signalling pathways, in particular how the internalized receptors contribute to the receptor signalling and DMR responses are warranted.

Notes and references

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Scheme
Label-free biosensor with microfluidics discovers an intracellular signaling wave mediated through the β_2-adrenergic receptors in A431.