The β₂-adrenoceptor activates a positive cAMP-calcium feedforward loop to drive breast cancer cell invasion

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Abstract

Activation of the sympathetic nervous system by stress increases breast cancer metastasis in vivo. Preclinical studies suggest that stress activates β-adrenoceptors (BARs) to enhance metastasis from primary tumors and that β-blockers may be protective in breast cancer. However, the subtype of BAR that mediates this effect, as well as the signaling mechanisms underlying increased tumor cell dissemination, remain unclear. We show that the β₂AR is the only functionally relevant BAR subtype in the highly metastatic human breast cancer cell line MDA-MB-231HM. β₂AR activation results in elevated cAMP (formoterol pEC50 9.86 ± 0.32), increased intracellular Ca²⁺ (formoterol pEC50 8.20 ± 0.33) and reduced phosphorylated ERK (pERK; formoterol pEC50 11.62 ± 0.31). We demonstrate that a highly amplified positive feedforward loop between the cAMP and Ca²⁺ pathways is responsible for efficient inhibition of basal pERK. Importantly, activation of the β₂AR increased invasion (formoterol area under the curve [AUC] relative to vehicle: 1.82 ± 0.36), which was dependent on the cAMP/ Ca²⁺ loop (formoterol AUC in the presence of 2′,5′-dideoxyadenosine 0.64 ± 0.03, or BAPTA-AM 0.45 ± 0.23) but independent of inhibition of basal pERK1/2 (vehicle AUC with U0126 0.60 ± 0.30). Specifically targeting the positive feedforward cAMP/Ca²⁺ loop may be beneficial for the development of therapeutics to slow disease progression in patients with breast cancer.—Pon, C. K., Lane, J. R., Sloan, E. K., Halls, M. L. The β₂-adrenoceptor activates a positive cAMP-calcium feedforward loop to drive breast cancer cell invasion. FASEB J. 30, 1144–1154 (2016).

Key Words: adrenergic receptor • GPCR • metastasis • stress

Stress causes activation of the sympathetic nervous system and the release of the catecholamines epinephrine and norepinephrine, the endogenous agonists of adrenoceptors. Preclinical studies have shown that stress or treatment with β-adrenoceptor (BAR) agonists can accelerate cancer progression and the development of metastasis in distant organs (1–4). Furthermore, an association has been found between psychosocial factors such as chronic stress and depression and accelerated progression of cancer (5, 6). The effects of stress on breast cancer metastasis in vivo may be blocked by BAR antagonists (β-blockers), suggesting that BAR signaling is necessary for stress to enhance metastasis from primary tumors and that β-blockers may play a protective role in slowing breast cancer progression (2). Studies since have found an association between β-blocker use and improved breast cancer outcome (7–9). In particular, β-blocker use had a more favorable outcome in patients with triple-negative breast cancer (TNBC), which lack expression of estrogen, progesterone, and HER2 (human epidermal growth factor receptor 2) receptors and are a subset of more aggressive breast cancers (7, 9). TNBC patients prescribed β-blockers had improved relapse-free survival as well as reduced breast cancer–related recurrence, distant metastasis, and cancer-related death. Findings from these studies provide evidence that β-blockers may have potential as adjuvant therapy for patients with TNBC.

BARs are prototypical GPCRs that signal through G proteins to regulate various cellular events that are important for cancer progression, including proliferation, invasion, and activation of immune response (10). BAR expression has been reported in both tumor and stromal cells in the local tumor microenvironment (2, 11), suggesting that BARs in multiple cell types may be activated by stress. Consistent with this, BAR activation by stress drives recruitment of immune cells to primary mammary tumors (2). However, far less is known about whether stress can also directly activate BARs on tumor cells. Breast cancer cells express functional BARs, as seen by increased production of intracellular cAMP in response to BAR agonists (2, 12) and inhibition of basal phosphorylated ERK (pERK) in some breast cancer cells (13, 14). In these studies, activation of the βAR led to an inhibition of cell proliferation and decreased growth of primary tumors in vivo (13, 14).

Abbreviations: 8-Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphate; AC, adenylyl cyclase; AUC, area under the curve; βAR, β-adrenoceptor; BSA, bovine serum albumin; db-cAMP, N⁶,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate; dDA, 2′,5′-dideoxyadenosine; Epac, exchange protein directly activated by cAMP; FBS, fetal bovine serum;

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However, stress or βAR activation do not consistently increase primary tumor growth despite accelerating metastasis (2, 4). As there was a more favorable association reported between β-blocker use and outcome in TNBC, this may suggest that βARs play a unique role in the progression of this highly aggressive subset of breast cancer.

Given the increasing evidence that activation of βARs may promote tumor metastasis in TNBC, it is important to decipher which βAR subtype is activated in response to stress or βAR agonist treatment, to discover if there is a direct effect of βAR activation on the tumor cells, and to identify the signaling pathways involved in mediating these effects. In this present study, we examined the subtype of βAR activated in response to βAR agonist treatment in the highly metastatic variant human TNBC cell line MDA-MB-231HM. We identified the signaling pathways downstream of βAR activation and investigated the signaling mediators that control breast cancer cell invasion.

**MATERIALS AND METHODS**

**Reagents**

The following compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA): (-)-propranolol, (-)-epinephrine, (-)-norepinephrine, 2′,5′-dideoxyadenosine (ddA), KT5720, ESL09, 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP), N6-O-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP), forskolin, and ionomycin. Salbutamol hemisulfate, formoterol hemifumarate, salmeterol xinafoate, ICYP, [125I]-(+)-cyanopindolol; IP3, inositol trisphosphate; KT5720, 8-Br-cyclic monophosphate (8-Br-cAMP), forskolin, and ionomycin. Saturation binding experiments were performed at room temperature in binding buffer (50 mM Tris, 5 mM MgCl2, 1 nM EDTA, pH 7.4), then homogenized with a Dounce homogenizer, and centrifuged (800 g, 10 min, 4°C). The supernatant was collected and the cell pellet subjected to another round of homogenization and centrifugation. Supernatants were pooled and centrifuged (40,000 g, 10 min, 4°C), and the pellet was resuspended in membrane buffer (50 mM Tris, 5 mM MgCl2, 1 mM EDTA, 200 mM sucrose, pH 7.2) and stored at -80°C.

**Gene expression**

RNA was extracted using the RNeasy Kit (Qiagen, Limburg, The Netherlands), and quantitative real-time PCR (qRT-PCR) was performed in triplicate from 100 ng RNA using the iScript One-Step RT-PCR Kit (Bio-Rad, Hercules, CA, USA) and CFX96 Real Time System (Bio-Rad). TaqMan probes were from Applied Biosystems (Foster City, CA, USA): ADRB1 (Hs02350048_s1), ADRB2 (Hs00240532_s1), ADRB3 (Hs00609046_m1), and ACTB (Hs99999903_m1). Data were analyzed using the 2^(-ΔΔCt) method and are expressed relative to ACTB (16).

**Radioligand binding**

Membranes were prepared from cells grown to 90% confluence. Cells were rinsed and scraped in homogenization buffer (5 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 5 mM EDTA, pH 7.4), then homogenized with a Dounce homogenizer (10 strokes per pestle) and centrifuged (800 g, 10 min, 4°C). The supernatant was collected and the cell pellet subjected to another round of homogenization and centrifugation. Supernatants were pooled and centrifuged (40,000 g, 10 min, 4°C), and the pellet was resuspended in membrane buffer (50 mM Tris, 5 mM MgCl2, 1 mM EDTA, 200 mM sucrose, pH 7.2) and stored at -80°C.

**Saturation binding experiments** were performed at room temperature in binding buffer (50 mM Tris, 5 mM MgCl2, 1 mM EDTA, pH 7.2) in 96-well plates. Membranes (5 μg) were incubated with 1 pM to 100 pM ICPY for 1 h with and without antagonists: propranolol (700 nM, 100 μM for β2AR), CGP-20712A (200 nM, 100 × Kd for β2AR or ICI-118551 (50 nM, 100 × Kd for β2AR) (17). Reactions were terminated by filtration through pre-soaked GF/C filters (1% v/v polyethyleneimine, 30 min) using a Packard Cell Harvester (PerkinElmer). Filters were washed 4 times with 50 mM Tris (pH 7.4, 4°C), and radioactivity was measured using a Packard Top Count device (PerkinElmer). Results were corrected for nonspecific binding, determined by 700 nM propranolol.

**cAMP accumulation**

Cells were treated with antagonists or inhibitors in stimulation buffer (140 mM NaCl, 5 mM KCl, 800 mM MgSO4, 200 mM Na2HPO4, 440 mM K2HPO4, 5 mM HEPES, 1.3 mM CaCl2, 5.6 mM glucose, 0.1% w/v bovine serum albumin [BSA], 500 μM 3-isobutyl-1-methylxanthine, pH 7.4) for 30 min at 37°C. Agonists were diluted in stimulation buffer, and cells were stimulated for 10 min at 37°C. Cells were lysed in 50 μl ice-cold ethanol, evaporated, and reconstituted in 50 μl detection buffer (5 mM HEPES, 0.3% v/v Tween-20, 0.1% w/v BSA, pH 7.4). Cell lysates (5 μl) were transferred to a 96-well OptiPlate (PerkinElmer) and cAMP detected using the cAMP AlphaScreen Kit (PerkinElmer). Data are expressed as pmol/well or basal subtracted and expressed relative to the 100 μM forskolin response, as stated.

**ERK1/2 phosphorylation**

Changes in ERK1/2 phosphorylation (pERK) were detected using the AlphaScreen SureFire pERK1/2 Kit (PerkinElmer). Cells were treated with antagonists or inhibitors diluted in serum-free medium for 30 min at 37°C, followed by agonists for 15 min. Medium was aspirated, cells were lysed in 100 μl lysis buffer, and cell lysates (4 μl) were transferred to 96-well ProxPlates (PerkinElmer) for detection. Data are expressed relative to basal.

**Calcium mobilization**

Cells were washed twice with assay buffer (150 mM NaCl, 2.6 mM KCl, 1.8 mM MgCl2, 10 mM d-glucose, 10 mM HEPES, 2.2 mM
CaCl₂, 0.5% w/v BSA, 4 mM probenecid, pH 7.4) and incubated with 1 μM Fluoro4-AM (Life Technologies) for 1 h at 37°C. Cells were washed and treated with antagonists or inhibitors for 30 min at 37°C. Fluorescence was detected at 485 nm excitation and 525 nm emission for 30 s, agonists were added, and the fluorescence was measured every 1.5 s for 4 min using a FlexStation 3 (Molecular Devices, Sunnyvale, CA, USA). Data are expressed as the vehicle-subtracted area under the curve (AUC) or as the baseline-subtracted increase in fluorescence, as stated.

Cell proliferation

Cells were treated with vehicle or formoterol in 1% v/v FBS medium for 48 h, and proliferation was assessed using the CellTiter 96 Aqueous One Proliferation Assay (Promega, Madison, WI, USA). Data are expressed relative to vehicle-treated cells.

Cell invasion

Cell invasion assays were performed by xCELLigence Real-Time Cell Analysis (RTCA) using CIM-16 plates (ACEA Biosciences, San Diego, CA, USA) (18) coated with 30 μl of 0.4 mg/ml Matrigel, and preincubated at 37°C for 4 h. A total of 180 μl of 10% v/v FBS medium containing ligand with or without inhibitors was added to the lower chambers, and 20 μl of serum-free medium with ligand and/or inhibitors was added to the upper chambers. The plate was incubated in the RTCA DP chamber (ACEA Biosciences) at 37°C and 5% CO₂ for 1 h, and a background measurement was taken. Cells were trypsinized and washed with serum-free medium; 3 × 10⁵ cells were seeded in the upper chambers in serum-free medium for 48 h, and proliferation was assessed using the CellTiter 96 Aqueous One Proliferation Assay (Promega, Madison, WI, USA). Data are expressed relative to vehicle-treated cells.

In vivo chronic stress metastasis model

All procedures involving mice were performed under protocols approved by the Institutional Animal Ethics Committee and in accordance with the animal ethics guidelines of the National Health and Medical Research Council of Australia. MDA-MB-231HM cells in 60 cm² cells were injected into the fourth left mammary fat pad of Balb/c nu/nu or Balb/c mice, respectively, and stress was induced as previously described (2, 4). Bioluminescence imaging (IVIS Lumina II; PerkinElmer) was used to track metastatic progression (2, 4). Data are expressed relative to nonstressed animals.

Statistical analysis

Data are expressed as means ± SEM from at least 3 independent experiments performed in at least duplicate. GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis. Data were analyzed by 1-way ANOVA using Dunnett’s or Tukey’s multiple comparison test, or Fisher’s least significant difference test.

RESULTS

The β₂AR is highly expressed in MDA-MB-231HM cells

We used qRT-PCR to identify the βAR subtypes that are expressed in MDA-MB-231HM cells. These cells express both ADRB1 and ADRB2; however, ADRB3 mRNA levels were undetectable (Fig. 1A). Radioligand binding was used to quantitate the relative protein expression of β₁AR and β₂AR on cell membranes. The total number of βAR binding sites was 71 ± 14.3 fmol/mg protein (Fig. 1B). There was no significant effect on radioligand binding after the addition of a β₂AR-selective antagonist, CGP-20712A (200 nM) (P = 0.086), which is 500-fold more selective for the β₁AR than the β₂AR (17). Conversely, addition of ICI-118551 (50 nM), a β₂AR-selective antagonist that is 550-fold more selective for the β₂AR than the β₁AR (17), resulted in a 98% reduction in radioligand binding. Therefore, the β₁AR is the predominant subtype expressed in MDA-MB-231HM cells. This is consistent with other studies showing higher expression of the β₂AR relative to the β₁AR in TNBC cell lines (12, 19).

The β₂AR is the only functionally relevant subtype in MDA-MB-231HM cells: activation increases cAMP and Ca²⁺ mobilization but inhibits pERK

Because βARs preferentially couple to Gαs to activate adenylyl cyclase (AC) and increase cAMP, we first examined whether nonselective and βAR subtype-selective agonists could affect cAMP production in MDA-MB-231HM cells. Endogenous nonselective agonists (epinephrine and norepinephrine) and β₂AR-selective agonists (salbutamol, salmeterol, and formoterol) all induced a concentration-dependent increase in cAMP (Fig. 1E). Although these cells expressed low levels of the β₁AR at the RNA level, xamoterol, a β₁AR-selective agonist, had no effect (data not shown). This suggests that the β₂AR is the only functionally relevant subtype. Activation of the β₂AR can also stimulate Ca²⁺ signaling (20), and Ca²⁺ signaling has been linked to tumor progression (21). Treatment of cells with formoterol, epinephrine, and norepinephrine increased Ca²⁺ mobilization; however, salbutamol and salmeterol (β₂AR-selective partial agonists) and xamoterol (data not shown) had no effect (Fig. 1F). Previous studies in HEK293S cells overexpressing the β₂AR also showed a very weak Ca²⁺ response to the partial agonists salbutamol and salmeterol compared to isoproterenol and epinephrine (20). The inability of the partial agonists to increase Ca²⁺ mobilization suggests that the β₂AR may be less efficiently coupled to this pathway in MDA-MB-231HM cells. Finally, β₂ARs can inhibit phosphorylation of ERK1/2 (pERK) in breast cancer cells (13, 14). Consistent with this, all βAR agonists except xamoterol (data not shown) caused a concentration-dependent inhibition of pERK in MDA-MB-231HM cells (Fig. 1G). Interestingly, all agonists were 100-fold more potent for pERK inhibition compared to activation of cAMP and Ca²⁺, suggesting that the β₂AR is efficiently coupled to inhibition of pERK in these cells (Table 1). There was no effect of the β₁AR agonists on the activation of p38, JNK, Akt, and STAT3 measured using AlphaScreen assays (data not shown).
antagonist for its receptor, thereby allowing identification of the functionally relevant receptor subtype within a mixed receptor population. Both propranolol and ICI-118551 caused surmountable competitive antagonism of epinephrine and formoterol, as indicated by the rightward shift in the concentration–response curves in both cAMP and pERK signaling assays (Supplemental Figs. 1 and 2). In contrast, CGP-20712A had no effect. Propranolol, CGP-20712A, and ICI-118551 alone did not affect levels of cAMP, pERK1/2, or Ca2+ (data not shown). The pA2 values obtained (Table 2) were similar to the reported affinity (KD) of propranolol, CGP-20712A, and ICI-118551 for the β2AR (17). This indicates that βAR agonists modulate signaling via the β2AR, but not the β1AR, in MDA-MB-231HM cells. To confirm this, ADRB1 and ADRB2 expression was knocked down by at least 50% using targeted siRNA (Fig. 1C). In cells transfected with control siRNA, IC50 concentrations of epinephrine (10 nM) and norepinephrine (100 nM) inhibited pERK (Fig. 1D). There was no effect of ADRB1 siRNA; however, ADRB2 siRNA abolished this inhibition of pERK. Together, these results confirm that the β2AR is the only functionally relevant βAR subtype expressed in MDA-MB-231HM cells.

A positive feedforward loop links cAMP and Ca2+ signaling in MDA-MB-231HM cells

We next investigated the signaling hierarchy in MDA-MB-231HM cells after β2AR stimulation using a panel of inhibitors. To confirm that the β2AR stimulated cAMP production in a Goα-dependent manner, cells were pretreated

### Table 1. pEC50 and pIC50 values of βAR agonists for cAMP, Ca2+, and pERK

<table>
<thead>
<tr>
<th>Agonist</th>
<th>cAMP</th>
<th>Ca2+</th>
<th>pERK</th>
</tr>
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<tbody>
<tr>
<td>Formoterol</td>
<td>9.86 ± 0.32 (4)</td>
<td>8.20 ± 0.33 (5)</td>
<td>11.62 ± 0.31 (6)**</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>8.84 ± 0.14 (3)</td>
<td>ND</td>
<td>11.11 ± 0.16 (3)**</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>7.32 ± 0.21 (6)</td>
<td>ND</td>
<td>9.69 ± 0.18 (4)***</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>7.43 ± 0.33 (4)</td>
<td>6.57 ± 0.12 (4)</td>
<td>10.05 ± 0.11 (3)***</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>6.01 ± 0.01 (4)</td>
<td>5.59 ± 0.15 (4)</td>
<td>8.24 ± 0.28 (3)***</td>
</tr>
</tbody>
</table>

Agonist potencies expressed as mean ± SEM pEC50 or pIC50 values for (n) experiments. ND, not detected. ***P < 0.001 compared to cAMP/Ca2+, 1-way ANOVA with Tukey’s posttest.
with a Goi inhibitor, NF449 (23). Blockade of Goi-inhibited formoterol-induced cAMP, suggesting that the β2AR activates Goi, to increase cAMP (Fig. 2A). βARs can also couple to Goi/Go0 proteins (24, 25); however, NF023, a Goi inhibitor (26), had no effect on formoterol-induced cAMP in these cells (P = 0.938). In addition to the Goi subunit, Gβγ subunits can also activate or inhibit different AC subtypes (27) to affect cAMP; however, treatment of cells with gallein (28) had no effect on cAMP after β2AR activation (P = 0.415).

Finally, to determine if activation of AC was required for the increase in cAMP (rather than inhibition of the phosphodiesterases that degrade cAMP), cells were treated with ddA, an AC inhibitor (29). Preincubation of cells with ddA decreased formoterol-induced cAMP production (Fig. 2B). Therefore, the β2AR activates a Goi-AC pathway to increase cAMP in MDA-MB-231HM cells.

Next we investigated how activation of the β2AR induces Ca2+ mobilization. As observed for activation of cAMP signaling, increased Ca2+ mobilization was dependent on Goi, but independent of Goi/Go0, as the Ca2+ response to formoterol was decreased after pretreatment of cells with NF449 but unaffected by NF023 (P = 0.543) (Fig. 3A, B). In contrast to the cAMP signaling pathway, increased Ca2+ mobilization was also dependent on Gβγ subunits, as preincubation of cells with gallein inhibited the formoterol-induced increase in intracellular Ca2+ (Fig. 3A, B).

Table 2. pA2 values for βAR antagonists from Schild analyses

<table>
<thead>
<tr>
<th>Signaling pathway</th>
<th>Agonist</th>
<th>Propranolol</th>
<th>CGP-20712A</th>
<th>ICI-118551</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>Formoterol</td>
<td>10.31 ± 0.75 (3)</td>
<td>6.14 ± 0.54 (3)</td>
<td>9.45 ± 0.04 (3)</td>
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<tr>
<td></td>
<td>Epinephrine</td>
<td>9.51 ± 0.38 (3)</td>
<td>6.37 ± 0.35 (3)</td>
<td>9.41 ± 0.13 (3)</td>
</tr>
<tr>
<td>pERK</td>
<td>Formoterol</td>
<td>10.25 ± 0.15 (5)</td>
<td>5.91 ± 0.71 (5)</td>
<td>10.11 ± 0.07 (5)</td>
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<tr>
<td></td>
<td>Epinephrine</td>
<td>9.99 ± 0.11 (4)</td>
<td>6.32 ± 0.26 (3)</td>
<td>10.06 ± 0.08 (4)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM for (n) experiments.

Figure 2. β2AR activation increases intracellular cAMP in MDA-MB-231HM cells through Goi-AC and Ca2+ mobilization. A) Inhibition of Goi (10 μM NF449), but not Goi/Go0 (10 μM NF023) or Gβγ subunits (50 μM gallein), decreased 1 nM formoterol-induced cAMP (n = 3–9). B, C) Formoterol (1 nM)-induced cAMP was also blocked after (B) inhibition of AC (100 μM ddA; n = 3–9) or (C) chelation of Ca2+ (20 μM BAPTA-AM; n = 4–9). Stimulation of Ca2+ influx by ionomycin (1 μM) further increased formoterol-induced cAMP (n = 5–9). D) Ionomycin alone increased basal cAMP production (n = 5–9). Bars represent means, error bars SEM. *P < 0.05. **P < 0.01. ***P < 0.001, 2-way ANOVA with Sidak’s (B) or Dunnett’s (A, C) multiple comparison test or unpaired Student’s t test (D).
β2AR with formoterol stimulates a Gox-AC pathway to increase cAMP; cAMP then activates PKA and Epac to increase Ca2+ mobilization, which is also influenced directly by Gβγ subunits; increases in intracellular Ca2+ then further increase cAMP.

The positive feedforward cAMP/Ca2+ loop leads to efficient inhibition of pERK

We then determined the signaling pathway that contributes to inhibition of pERK upon β2AR activation. There was no effect of inhibition of Gox, or Gox/o using NF449 (P = 0.608) or NF023 (P = 0.069), respectively (Fig. 4A). However, inhibition of AC (ddA) partially reversed the formoterol-induced inhibition of pERK (Fig. 4B), suggesting that this response is dependent on cAMP. cAMP can inhibit ERK phosphorylation via a PKA-Src-Rap1 pathway, which blocks Ras activation, to prevent activation of the Ras-Raf1-ERK1/2 pathway (34–36). However, inhibition of PKA (KT5720) had no effect on the formoterol-induced inhibition of pERK (P = 0.668) (Fig. 4B). We could not determine any involvement of Epac, as preincubation of MDA-MB-231HM cells with ESI-09 significantly decreased basal levels of pERK such that no further inhibition was induced by formoterol (data not shown). However, inhibition of Ca2+ signaling (Gβγ subunits using gallein, or Ca2+ chelation with BAPTA-AM) reversed the formoterol-induced inhibition of pERK (Fig. 4C). These findings suggest that both cAMP and Ca2+ signaling contribute to the inhibition of pERK in response to formoterol.

The lack of effect of Gox and PKA blockade on the formoterol-induced inhibition of pERK may be due to the very efficient coupling of the β2AR to this pathway, as suggested by the increased potency of β2AR agonists for pERK compared to both cAMP and Ca2+ (Table 1). Therefore, an alternative approach was used to confirm that activation of the cAMP/Ca2+ feedforward loop by formoterol leads to inhibition of basal pERK. Cells were treated with either forskolin, which activates AC to increase cAMP, or the synthetic cAMP analogs 8-Br-cAMP or db-cAMP (Fig. 4D). All compounds mimicked formoterol inhibition of pERK, reducing basal pERK levels by 90%, 53%, and 46%, respectively. Treatment of cells with ionomycin, a Ca2+ ionophore, also reduced basal pERK levels by 72%. This suggests that activation of the cAMP/Ca2+ feedforward loop can inhibit basal pERK.

Only the feedforward cAMP/Ca2+ loop, and not pERK inhibition, contributes to increased invasion

To determine the functional consequence of β2AR signaling in TNBC cells, we measured proliferation and invasion, 2 key steps in stress-induced metastasis. The MAPK signaling cascade has a major role in regulating cell growth and proliferation (37). Because activation of β2AR signaling in MDA-MB-231HM cells inhibited pERK, we examined whether proliferation was altered in these cancer cells. However, increasing concentrations of formoterol had no effect on cell proliferation (Fig. 5A). We then assessed tumor cell invasion in real time using xCELLigence (18). An EMax concentration of formoterol (0.5 μM) significantly increased the invasion of MDA-MB-231HM cells over 5 d compared to vehicle-treated controls (Fig. 5B, C), and this was abolished in the presence of ICI-118551, suggesting that increased invasion is due to activation of
Figure 4. β2AR-mediated inhibition of pERK is dependent on the positive feedforward cAMP/Ca^{2+} loop. A) Formoterol (10 pM)-induced inhibition of pERK was unaffected after inhibition of Gαs (10 µM NF449) or Gαi/o (10 µM NF023) (n = 3–11). B) Blockade of AC (100 µM ddA) reversed inhibition of pERK by 10 pM formoterol, but inhibition of PKA (1 µM KT5720) had no effect (n = 5–11). C) Inhibition of Gβγ subunits (50 µM gallein) or Ca^{2+} chelation (20 µM BAPTA-AM) reversed 10 pM formoterol-induced inhibition of pERK (n = 3–11). D) Inhibition of pERK by 10 pM formoterol was mimicked by 10 µM forskolin, 100 µM db-cAMP, 100 µM 8-Br-cAMP, or 1 µM ionomycin (n = 5–11). Bars represent means, error bars represent SEM. *P < 0.05, ***P < 0.001, 1-way ANOVA with Dunnett’s multiple comparison test (D) or 2-way ANOVA with Dunnett’s multiple comparison test (C, B).

The β2AR. To determine which signaling pathways were responsible for the β2AR-dependent increase in MDA-MB-231HM cell invasion, we used a panel of inhibitors. Treatment of cells with U0126, a MEK inhibitor, had no effect on the basal level of cell invasion (Fig. 5D, E). This suggests that inhibition of pERK after β2AR activation is not directly associated with increased cell invasion. Next we assessed whether formoterol-induced breast cancer cell invasion required activation of the cAMP/Ca^{2+} feedforward loop. Inhibition of Gαs and AC with NF449 and ddA, respectively, completely reversed formoterol-induced invasion (Fig. 5F, G). Moreover, blockade of Gβγ subunits with gallein and Ca^{2+} chelation with BAPTA-AM also abrogated cell invasion induced by formoterol (Fig. 5H, I). The inhibitors alone had no significant effect on basal invasion. These results indicate that formoterol inducers breast cancer cell invasion through the activation of the β2AR-dependent positive feedback cAMP/Ca^{2+} loop.

To determine whether the positive feedforward cAMP/Ca^{2+} loop was a common feature in other breast cancer cells, we used the less aggressive parental MDA-MB-231 cells and a 66 cl4 mammary adenocarcinoma cell line that metastasizes in response to stress in vivo (2). The 66 cl4 cells showed a significantly delayed onset of metastasis in response to stress compared to MDA-MB-231 cells (Supplemental Fig. 3A). Parental MDA-MB-231 cells expressed both ADRB1 and ADRB2, whereas the 66 cl4 cells only expressed aDR2 at the mRNA level (Supplemental Fig. 3B, C). Interestingly, in both cell lines, β2AR agonists stimulated a much smaller increase in cAMP production compared to MDA-MB-231HM cells (Supplemental Fig. 3D–I). Moreover, there was no effect of the β2AR agonists on Ca^{2+} mobilization, and β2AR activation had varying effects on pERK (Supplemental Fig. 3G–L). It is possible that the lack of a Ca^{2+} signal, and therefore the absence of the feedforward cAMP/Ca^{2+} loop in these cells, may explain the low levels of cAMP production and the slower development of metastasis in response to stress.

**DISCUSSION**

We have previously reported that activation of the βAR by chronic stress increases metastasis from primary tumors in mouse orthotopic models of cancer (2, 4). Retrospective studies have also demonstrated a clinical association between β-blocker usage and reduced distant metastasis, cancer recurrence, and mortality (7–9, 11). However, the subtype of βAR that mediates this effect, whether the increased sympathetic activity acts directly on the tumor and/or via the tumor microenvironment, and the cellular mechanisms underlying increased metastatic dissemination, remain unclear. The results presented here uncover molecular mechanisms that may underlie the capacity of stress to promote cancer metastasis at the level of the tumor cell. We show that the β2AR is highly expressed and is the only functionally relevant βAR subtype in MDA-MB-231HM breast cancer cells. β2AR activation results in elevated cAMP, increased intracellular Ca^{2+}, and reduced pERK levels. We reveal a highly amplified positive feedforward loop between the cAMP and Ca^{2+} pathways, which is responsible for efficient inhibition of basal pERK (Fig. 6). Importantly, we link the positive cAMP/Ca^{2+} loop, but not inhibition of pERK, to increased invasion of MDA-MB-231HM cells.

The β2AR, a predominantly Gαs-coupled receptor, increased cAMP production in response to endogenous and β2AR-selective agonists, consistent with other studies in breast cancer cells (2, 12, 19). We show that this increase in cAMP is due to Gαs activation of AC and was not affected by
Ga\textsubscript{i/o}. Activation of the \(\beta_2\)AR increased intracellular Ca\textsuperscript{2+} by 2 pathways: directly via G\(\beta\gamma\) subunits and indirectly via a pathway involving the cAMP-dependent PKA and Epac. Interestingly, this increase in Ca\textsuperscript{2+} signaling also activated the cAMP pathway. This suggests a novel feedforward mechanism activated by \(\beta_2\)AR signaling in MDA-MB-231HM cells in which initial G protein coupling increases both cAMP and Ca\textsuperscript{2+} mobilization. The cAMP effectors PKA and Epac further increase intracellular Ca\textsuperscript{2+} levels, and elevated Ca\textsuperscript{2+} leads to further stimulation of AC and additional increases in cAMP to amplify the signal (Fig. 6). A synergistic relationship between cAMP and Ca\textsuperscript{2+} signaling has long been observed (27, 38, 39). cAMP itself, in addition to its effectors PKA and Epac, can have direct effects on numerous aspects of Ca\textsuperscript{2+} signaling, including activation of hyperpolarization-activated cyclic nucleotide-gated channels, voltage-gated Ca\textsuperscript{2+} channels, and inositol trisphosphate (IP\textsubscript{3}) receptors (27). Indeed, after \(\beta_2\)AR activation, cAMP-dependent PKA can either directly phosphorylate L-type voltage-gated Ca\textsuperscript{2+} channels to stimulate Ca\textsuperscript{2+} influx (40) or activate the ryanodine receptor (41) to mobilize Ca\textsuperscript{2+} from IP\textsubscript{3}-gated intracellular stores (20). Epac can also directly increase intracellular Ca\textsuperscript{2+} by activating a Rap GTPase–phospholipase C-IP\textsubscript{3} receptor pathway in HEK293 cells (42, 43). Conversely, it is also well established that Ca\textsuperscript{2+} signaling can regulate ACs and thereby modulate intracellular cAMP levels, either directly by Ca\textsuperscript{2+} and/or calmodulin binding or via effectors including calmodulin kinase, PKC, or calcineurin (27). In MDA-MB-231HM cells, activation of this positive feedforward loop mediates 2

Figure 5. Activation of \(\beta_2\)AR by formoterol increases breast cancer cell invasion. A) Increasing concentrations of formoterol had no effect on proliferation of MDA-MB-231HM cells (\(n = 3\)). B, C) Formoterol (0.5 \(\mu\)M) induced invasion of MDA-MB-231HM cells over 5 d, and this was blocked by 100 nM ICI-118551 (\(n = 3–10\)). D, E) Inhibition of MEK (10 \(\mu\)M U0126) had no effect on invasion (\(n = 3\)). F–I) The effect of formoterol on invasion was blocked after inhibition of Ga\textsubscript{s} (10 \(\mu\)M NF449) or AC (100 \(\mu\)M ddA) (F, G) or G\(\beta\gamma\) subunits (50 \(\mu\)M gallein) or Ca\textsuperscript{2+} chelation (20 \(\mu\)M BAPTA-AM) (H, I) (\(n = 3–10\)). Symbols/bars represent means, error bars SEM. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), 1-way ANOVA with Fisher’s least significant difference test.
distinct and independent events: highly efficient inhibition of pERK, demonstrated by significantly increased potency of all ligands in pERK assays compared to cAMP and Ca\textsuperscript{2+} assays (Table 1), and increased cell invasion.

The 2 cAMP effectors, PKA and Epac, have been linked to increased tumor cell invasion. PKA can activate Src, a kinase involved in the regulation of cell survival, motility, and invasion (44). Indeed, hyperactivation of PKA signaling and therefore elevated Src phosphorylation (Ser17 and Tyr419) are associated with accelerated mammary tumorigenesis (45). Moreover, previous studies have linked βAR-dependent PKA activation to the stimulation of Src and increased cancer cell invasion. In ovarian cancer cells, stimulation of βAR leads to cAMP-dependent PKA phosphorylation of Src at Ser17 and Tyr419, which was required for tumor cell invasion and stress-induced tumor growth (46). Similarly, activation of βARs in MDA-MB-231 cells caused PKA activation of Src, which was essential for invasion (47). There is also evidence for an involvement of cAMP-Epac pathways in tumor cell invasion. Activation of Epac is important for pancreatic cancer cell migration and invasion (51), and in fibrosarcoma cells, the Go\textsubscript{s}-cAMP-Epac pathway was linked to the formation of invadopodia (48), plasma membrane–localized complexes that are important for invasion. Interestingly, there is also evidence that the Go\textsubscript{s}-cAMP-Epac pathway can regulate Src kinase activity (49). As such, we favor the hypothesis that Src links the feedforward cAMP/Ca\textsuperscript{2+} loop to enhanced MDA-MB-231HM cell invasion.

The link between increased cell invasion and the positive feedforward cAMP/Ca\textsuperscript{2+} loop has important ramifications for TNBC, as the main cause of mortality in breast cancer patients is metastatic dissemination of the primary tumor (50). Tumor cells are exposed to multiple growth factors and cytokines in the local tumor microenvironment, which critically influences cancer progression to metastasis (51). The transformation of tumor cells into a metastatic phenotype in response to the tumor microenvironment requires epithelial to mesenchymal transition. In invasive breast cancer, this involves significant reorganization of plasma membrane domains and the underlying cytoskeleton (52, 53). In fact, the remodeling of lipid-rich plasma membrane domains is highly associated with the transition to more aggressive breast carcinomas (52, 54). Interestingly, Ca\textsuperscript{2+}/calmodulin activation of AC requires direct interactions with A kinase anchoring protein, AKAP79, which in turn scaffolds the Ca\textsuperscript{2+} channel components STIM1 and Orai1 in proximity to the AC in lipid-rich domains (27, 55, 56). In addition to interacting with AC/STIM1/Orai1, AKAP79 can also interact with the β\textsubscript{2}AR to form large signaling complexes, often dependent on lipid-rich plasma membrane domains (57–60). It may be that the plasma membrane organization within these highly invasive cells facilitates the positive feedforward loop between cAMP and Ca\textsuperscript{2+} signaling pathways, thus promoting increased invasion.

Retrospective clinical studies have reported a variable strength of association between β-blocker use and cancer outcomes. Although some studies have reported that breast cancer patients receiving β-blockers have a more favorable outcome, defined by reduced distant metastasis, reduced recurrence, and reduced mortality (7–9, 11, 61), others found no association between β-blocker use and outcome (62–65). Interestingly, β-blocker use in TNBC patients was reported to have a more favorable outcome compared to estrogen receptor–positive breast cancer patients (7, 9). It is plausible that in addition to β\textsubscript{2}AR signaling within the tumor microenvironment (2), the positive feedforward cAMP/Ca\textsuperscript{2+} loop identified here may occur in a tumor cell–type dependent manner that could impact β-blocker efficacy in vivo. Indeed, previous studies have reported that β\textsubscript{2}AR-dependent inhibition of pERK leads to reduced cell proliferation and/or tumor growth in the breast cancer cell lines MDA-MB-231, IBH-4, and IBH-6 and in the normal mammary epithelial cell line MCF10A (13, 14, 60). The authors suggested that β\textsubscript{2}AR agonists may be useful adjuvant treatments for breast cancer. This directly contrasts with our finding that β-blockers would be useful treatments for TNBC, as they prevented invasion in the highly metastatic variant of the MDA-MB-231 cell line.
used in this study (15). These important differences in the outcome of β2AR activation in distinct tumor cell types could be influenced by factors including the organization and expression levels of proteins required for efficient coordination of cAMP and Ca²⁺ signaling. In support of this, the feedforward cAMP/Ca²⁺ loop was absent in parental MDA-MB-231 and 66cl4 cells, and this correlated with lower β2AR expression in triple-negative breast cancer postmenopausal women. Breast Cancer Res Treat. 140, 567–575

In summary, we have identified the β2AR as the only functionally relevant βAR subtype in MDA-MB-231HM cells. Activation of the β2AR in these breast cancer cells results in a positive feedforward loop between cAMP and Ca²⁺ signaling that causes increased invasion. The identification of a tightly coupled signaling loop that is activated by β2ARs may be beneficial for future development of therapeutics to slow cancer progression in patients with aggressive TNBC.

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