



Title	Endothelin Type B Receptor–Induced Sustained Ca ²⁺ Influx Involves Gq/11/Phospholipase C–Independent, p38 Mitogen-Activated Protein Kinase–Dependent Activation of Na ⁺ /H ⁺ Exchanger
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Short communication

Endothelin type B receptor-induced sustained Ca^{2+} influx involves $G_{q/11}$ /PLC-independent, p38 MAPK-dependent activation of Na^+/H^+ exchanger.

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Running title: Mechanism for ET_B R-induced Ca^{2+} influx.

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Abstract

The mechanism for sustained Ca^{2+} influx activated by G protein-coupled receptors was examined. In Chinese hamster ovary cells expressing recombinant human endothelin type B receptor ($\text{ET}_\text{B}\text{R}$) and endogenous P2Y receptor (P2Y-R), endothelin-1 elicited a sustained Ca^{2+} influx depending on $\text{G}_{\text{q}/11}$ protein, phospholipase C (PLC), Na^+/H^+ exchanger (NHE) and p38 mitogen-activated protein kinase (p38MAPK), whereas P2Y-R-induced sustained Ca^{2+} influx was negligible. Functional study showed that NHE activation by $\text{ET}_\text{B}\text{R}$ was mediated via p38MAPK but not $\text{G}_{\text{q}/11}/\text{PLC}$, while that by P2Y-R involves only $\text{G}_{\text{q}/11}/\text{PLC}/\text{p38MAPK}$. These results suggest that $\text{G}_{\text{q}/11}/\text{PLC}$ -independent NHE activation via p38MAPK plays an important role in $\text{ET}_\text{B}\text{R}$ -mediated sustained Ca^{2+} influx.

Keywords:

- (1) endothelin type B receptor
- (2) Na^+/H^+ exchanger
- (3) p38 mitogen-activated protein kinase

G protein-coupled receptors (GPCRs) including endothelin type A receptor (ET_AR) and ET_BR transduce the binding of their agonists into activation of G protein-regulated effectors and changes in levels of corresponding second messengers. It is well-known that stimulation of G_q protein-coupled receptors induces formation of the second messengers such as inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) via phospholipase C (PLC). Binding of IP₃ to its receptor on endoplasmic reticulum (ER) triggers Ca²⁺ release from ER, resulting in a transient increase in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) (1). According to the classical theory, this is followed by a sustained increase in [Ca²⁺]_i, resulting from Ca²⁺ influx through several types of voltage-independent Ca²⁺-permeable cation channels such as store-operated Ca²⁺ channels (SOCCs) (2). On the other hand, DAG activates directly receptor-operated Ca²⁺ channels (ROCCs), leading to sustained Ca²⁺ influx. Thus, the sustained increase in [Ca²⁺]_i via SOCCs and ROCCs results from activation of G_{q/11}/PLC pathway.

Recently, we have reported that in addition to Ca²⁺ influx through SOCC and ROCC activated via G_{q/11}/PLC (3, 4), ET_AR induced G_{q/11}/PLC-independent activation of sustained Ca²⁺ influx which is not mediated through either SOCC or ROCC: the Ca²⁺ influx is mediated by the reverse mode of Na⁺/Ca²⁺ exchanger functionally coupled with Na⁺/H⁺ exchanger (NHE) via Na⁺ transport (5). Furthermore, NHE activation in response to ET_AR stimulation is mediated via p38 mitogen-activated protein kinase (p38MAPK) which is activated through G₁₂ protein (6). Because G_{q/11}-coupled receptors can couple to other G proteins, these findings imply that mechanisms for sustained Ca²⁺ influx might be different depending on the type of GPCRs to be activated.

In the present study, in order to expand the functional significance of G_{q/11}/PLC-independent, p38MAPK-dependent NHE activation in sustained Ca²⁺ influx to other GPCRs coupled with G_{q/11}/PLC and G_{12/13}, we examined whether this mechanism is involved in the Ca²⁺ influx induced by recombinant ET_BR and endogenous P2Y receptor (P2Y-R) expressed in CHO cells (7).

To generate CHO cells stably expressing ET_BR (ET_BR-CHO), the gene of human ET_BR

fused with yellow fluorescence protein at the C terminus was introduced into CHO cells by retroviral gene transfer as previously described (5, 6). $[Ca^{2+}]_i$ was monitored by using fluorescent Ca^{2+} indicators, fura-2/acetoxymethyl ester (fura-2/AM), and fluo-3/AM (5, 6). To determine molecular mechanisms for NHE activation upon stimulation of ET_B R and P2Y-R with ET-1 and adenosine triphosphate (ATP), respectively, the change in extracellular acidification rate (ECAR) was measured by the eight-channel CytosensorTM microphysiometer (Molecular Devices Corp., California, U.S.A.) (5, 6). To estimate the degree of p38MAPK activation after stimulation of ET_B R and P2Y-R, the phosphorylation levels of p38MAPK were estimated by Western blot analysis (6).

The concentration-response curves for ET-1 and ATP were constructed to evaluate its EC_{50} value (M) using GraphPad PRISMTM (version 3.00, GraphPad Software Inc., San Diego, CA, U.S.A.). The EC_{50} values were converted to negative logarithmic values (pEC_{50}) for analysis. Results of CytosensorTM microphysiometer studies are expressed as percentages of the basal ECAR prior to exposure to vehicle or inhibitors. All data were presented as means \pm S.E.M. where n refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad PRISMTM by Student's paired t -test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A P value less than 0.05 was considered to indicate significant differences.

In ET_B R-CHO, stimulation of ET_B R with 1 nM ET-1 elicited a biphasic increase in $[Ca^{2+}]_i$ consisting of an initial transient peak (304.9 ± 31.6 nM, $n = 5$) and a subsequent sustained increase (199.8 ± 16.0 nM, $n = 5$). The pEC_{50} values for ET-1-induced increase in $[Ca^{2+}]_i$ were 10.09 ± 0.13 for the transient phase and 9.72 ± 0.18 for the sustained phase (Fig. 1A, 1B). ATP also induced a transient increase in $[Ca^{2+}]_i$ with a pEC_{50} value of 5.86 ± 0.08 and the maximum increase in $[Ca^{2+}]_i$ of 386.4 ± 39.5 nM ($n = 5$). ATP can activate two families of P2 receptors, the ligand-gated ion channels (P2X-R) (8) and the G protein-coupled P2Y-R (7), which are endogenously expressed in CHO cells. ATP-induced increase in $[Ca^{2+}]_i$ seems to be mediated via P2Y-R but not P2X-R, since the Ca^{2+} response was completely inhibited by 1 μ M YM-254890, a $G_{q/11}$ inhibitor (data not shown). Notably, the

sustained increase in $[Ca^{2+}]_i$ were very small throughout the tested concentrations of ATP (Fig. 1B). Interestingly, although the transient $[Ca^{2+}]_i$ increases induced by 1 nM ET-1 and by 10 μ M ATP in ET_BR-CHO are comparable with each other (Fig. 1A), there is a marked difference in the amplitude of the sustained Ca^{2+} responses. These results suggest that store depletion does not necessarily activate SOCCs, and hence that the ET_BR-induced sustained Ca^{2+} influx is not due to SOCCs activated by the emptying of intracellular Ca^{2+} store.

To determine the signaling molecules involving sustained Ca^{2+} influx induced by ET-1, a specific inhibitor was added during the sustained phase of $[Ca^{2+}]_i$ increase. Maximally effective concentrations of a $G_{q/11}$ inhibitor (1 μ M YM-254890), a PLC inhibitor (3 μ M U-73122), an NHE inhibitor (10 μ M 5-(N-ethyl-N-isopropyl)amiloride; EIPA), and a p38MAPK inhibitor (50 μ M SB203580) inhibited the ET-1-induced sustained increases, indicating that the sustained Ca^{2+} responses to ET-1 are mediated via $G_{q/11}$, PLC, NHE, and p38MAPK. This result in turn implies that like ET_AR (6), NHE/p38MAPK-dependent mechanism is involved in the ET_BR-induced sustained Ca^{2+} influx.

Recently, we have shown that $G_{q/11}$ /PLC-independent, p38MAPK-dependent pathway regulates NHE activity after ET_AR stimulation (6). To clarify intracellular mechanisms responsible for NHE activation by ET_BR, we used the CytosensorTM microphysiometer which is a valuable tool for evaluation of NHE function in living cells (5, 6). Functional study with this instrument showed that 1 nM ET-1 and 10 μ M ATP evoked an increase in ECAR. The increases by ATP were far smaller than those by ET-1 (e.g., 124.9% for ATP vs. 162.2% for ET-1 at 6 min) (Fig. 2). The responses to ET-1 and ATP were markedly inhibited by 10 μ M EIPA, indicating the involvement of NHE. To identify upstream signaling molecules in NHE activation by ET_BR and P2Y-R, the effects of YM-254890, U-73122, and SB203580 on the ECAR response were examined. In contrast to Ca^{2+} response, the ECAR response was resistant to both YM-254890 and U-73122, but sensitive to SB203580 (Fig. 2A). On the other hand, the ATP-induced increase in ECAR was sensitive to all of these inhibitors (Fig. 2B). These findings suggest that activation of NHE is mediated via $G_{q/11}$ /PLC-independent, p38MAPK-dependent pathway for ET_BR and via $G_{q/11}$ /PLC/p38MAPK-dependent pathway

for P2Y-R.

Increasing evidence indicates that p38MAPK plays an important role in both activation of NHE by GPCRs (9, 10) and sustained Ca^{2+} response induced by ET_AR (5, 6). In addition, activation of p38MAPK by ET_AR is reported to be independent of $\text{G}_{q/11}/\text{PLC}$ pathway (6). To examine a role of p38MAPK activation in the signaling for ET_BR and P2Y-R, p38MAPK phosphorylation was measured by Western blot analysis. The p38MAPK phosphorylation by 1 nM ET-1 was exceedingly strong and persistent, whereas that by 10 μM ATP was very weak and transient (Fig. 3A). At 15 min following stimulation, ET-1 induced a concentration-dependent phosphorylation of p38MAPK with a pEC_{50} value of 10.28 ± 0.12 and the maximum level of $879.9 \pm 52.2\%$ ($n = 6$, Fig. 3B), but ATP (1 μM - 30 μM) induced no significant increase. To determine upstream regulatory molecules for p38MAPK, the effects of inhibitors for $\text{G}_{q/11}$ and PLC on the ET-1-induced p38MAPK phosphorylation were examined. Like p38MAPK activation via ET_AR (6), YM-254890 (1 μM) and U-73122 (10 μM) had little effect on p38MAPK phosphorylation by 1 nM ET-1 (data not shown). Taken together with the above-mentioned CytosensorTM microphysiometer study, these data provide further evidence for the involvement of $\text{G}_{q/11}/\text{PLC}$ -independent pathway in ET_BR -induced p38MAPK activation. We were unable to determine the upstream molecules for ATP-induced p38MAPK phosphorylation, since its phosphorylation level was too weak to analyze.

Like ET_AR , both ET_BR and P2Y-R can couple with a member of G_{12} family (G_{12} and G_{13} proteins) in addition to $\text{G}_{q/11}$ protein (11-13), and activation of G_{12} protein is involved in ET_AR -induced NHE activation via p38MAPK, causing a sustained increase in $[\text{Ca}^{2+}]_i$ (6). Interestingly, G_{13} protein is a potential candidate responsible for NHE activation mediated via ET_BR (13), despite stimulation of GPCRs coupled to G_q (10) and $\text{G}_{12/13}$ (14) can activate NHE. In analogy with the case of ET_AR (6), EIPA-sensitive part of the ET_BR -induced sustained increase in $[\text{Ca}^{2+}]_i$ is mediated by NHE, that is in turn activated via p38MAPK: the remaining part of the $[\text{Ca}^{2+}]_i$ increase which is resistant to EIPA is considered to be mediated through ROCCs and/or SOCCs, whose activation requires both $\text{G}_{q/11}/\text{PLC}$ and p38MAPK. An

activator of p38MAPK may be $G_{12/13}$ protein. In contrast, the minimum level of the sustained increase in $[Ca^{2+}]_i$ following stimulation of P2Y-R seems to be due to a low level of p38MAPK activation, causing weak activation of NHE and also ROCCs/SOCCs. The low level of p38MAPK activation might be weak coupling of P2Y-R with $G_{12/13}$ protein. Further studies will be required to confirm the possible involvement of G_{12} and/or G_{13} proteins in ET_B R-mediated p38MAPK activation. In addition, the reasons for these differences in signaling cascade and sustained Ca^{2+} response between ET_B R and P2Y-R are not known, but such phenomena may result from the difference in receptor expression level that affects receptor-G protein coupling in recombinant expression systems (9, 15). Recently, we showed that a difference in expression level of human ET_A R results in a multiplicity of receptor signaling as follows: the ECAR response to ET-1 in low-expressor clone is mediated via the $G_{q/11}$ /PLC/p38MAPK/NHE pathway, while the response in high-expressor clone via either $G_{q/11}$ /PLC/NHE or non- $G_{q/11}(G_{12})$ /p38MAPK/NHE cascades (6). The signaling cascades for low- and high-expressor are consistent with those utilized by P2Y-R and ET_B R, respectively. Therefore, there is the possibility that the difference in intracellular signaling mechanism between ET_B R and P2Y-R are due to differences in their expression levels and/or the difference of receptor type.

In summary, the present study demonstrated that $G_{q/11}$ /PLC-independent activation of NHE via p38MAPK are involved in a sustained increase in $[Ca^{2+}]_i$ triggered by ET_B R but not P2Y-R. Taken together with our previous reports indicating the participation of G_{12} /p38MAPK/NHE cascade in the ET_A R-mediated sustained $[Ca^{2+}]_i$ increase (6), these results imply that $G_{q/11}$ /PLC-independent, p38MAPK-dependent activation of NHE plays an important role in the sustained Ca^{2+} response to stimulation of ET_B R in addition to ET_A R.

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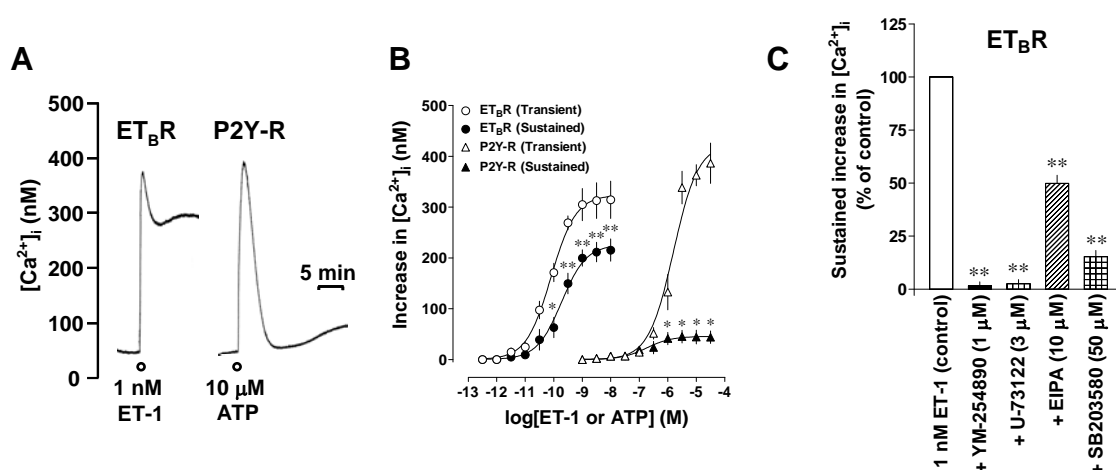
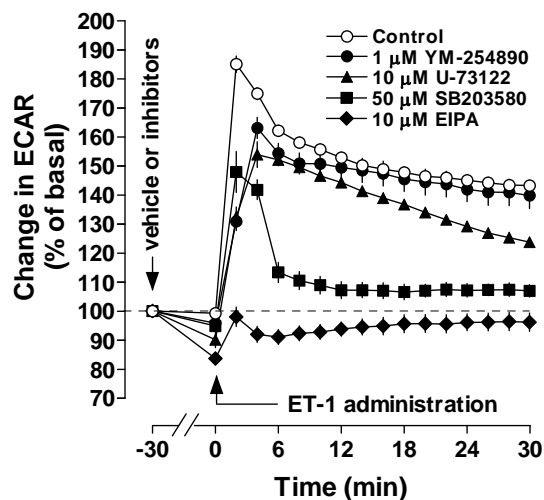


Fig. 1.

Characterization of the increases in $[Ca^{2+}]_i$ induced by ET-1 and ATP in ET_BR-CHO. (A) representative traces showing the $[Ca^{2+}]_i$ increases induced by stimulation of ET_BR with ET-1 and of P2Y-R with ATP at indicated concentrations. (B) concentration-response curves for the transient and sustained $[Ca^{2+}]_i$ increases triggered by ET-1 and ATP. The sustained $[Ca^{2+}]_i$ increases were measured 10 min and 20 min after addition of ET-1 and ATP, respectively. * $P < 0.05$, ** $P < 0.01$, sustained $[Ca^{2+}]_i$ increases induced by these agonists versus basal Ca^{2+} level. (C) effects of YM-254890, U-73122, EIPA, and SB203580 on the 1 nM ET-1-induced sustained increases in $[Ca^{2+}]_i$. Data are presented as means \pm S.E.M of the results obtained from 5 experiments. ** $P < 0.01$, versus its control (1 nM ET-1 alone, open column).

A. ET_BR



B. P2Y-R

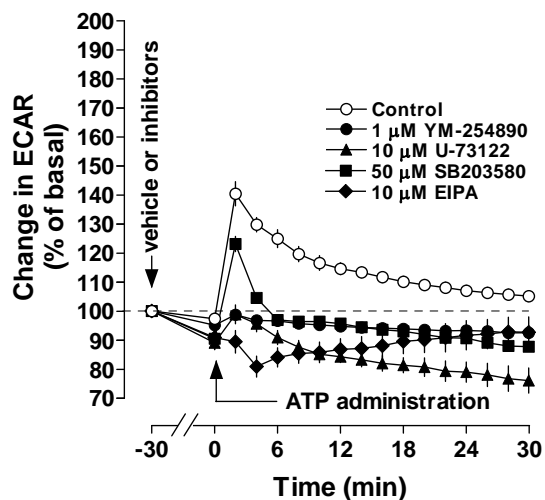
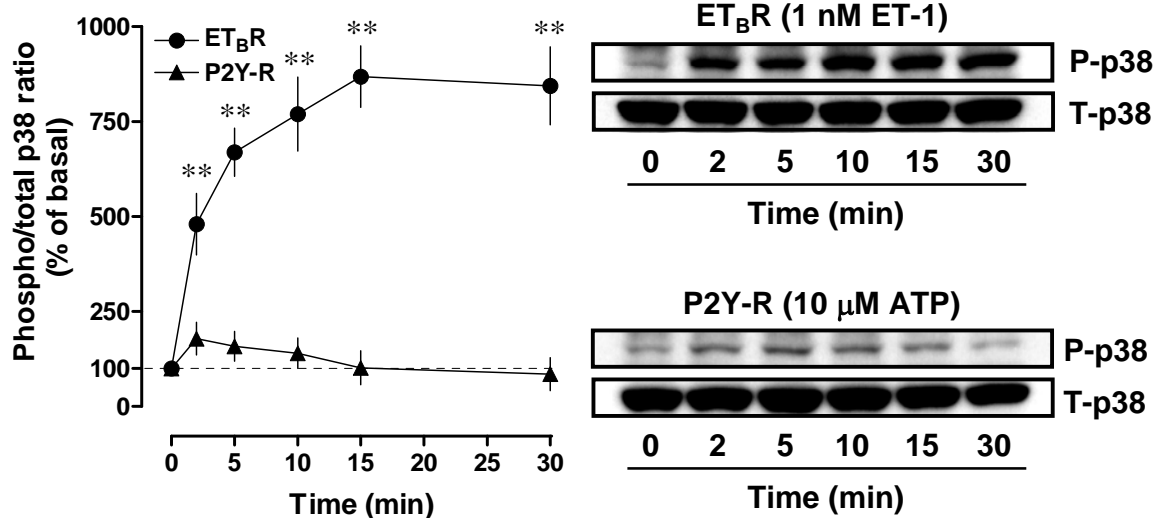


Fig. 2.

Characterization of changes in ECAR induced by 1 nM ET-1 (A) and 10 μ M ATP (B) in ET_BR-CHO. The change in ECAR was measured by the CytosensorTM microphysiometer every 2 min. The cells were treated with either vehicle (0.2 % dimethylsulfoxide) or inhibitors for 30 min before stimulation with ET-1 (ET-1 administration) or ATP (ATP administration) for 30 min. Data are presented as means \pm S.E.M of the results obtained from 5 experiments.

A. Time course



B. Concentration-dependency

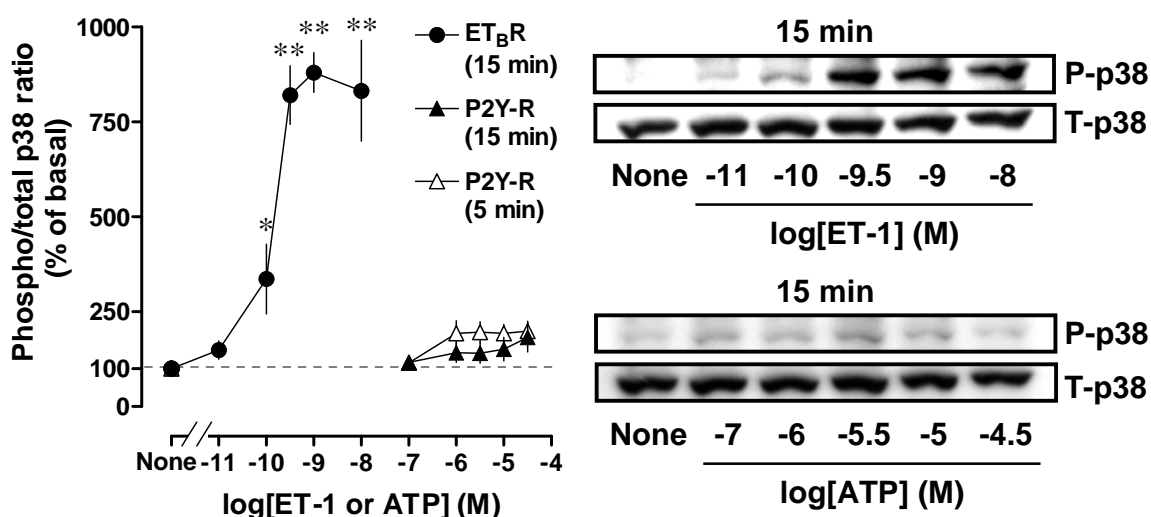


Fig. 3.

Characterization of p38MAPK phosphorylation in response to ET-1 and ATP in ET_B-R-CHO. (A) the time course of p38MAPK phosphorylation induced by 1 nM ET-1 and 10 μM ATP with, at the right, representative immunoblots (P-p38, phosphorylated p38MAPK; T-p38, total p38MAPK). (B) concentration-response curves for p38MAPK phosphorylation in response to 15 min exposure to ET-1 and 5 or 15 min exposure to ATP, with, at the right, representative immunoblots. Data are presented as means ± S.E.M of the results obtained from 6 experiments. **P* < 0.05, ***P* < 0.01, p38MAPK phosphorylation induced by these agonists versus basal p38MAPK phosphorylation level.