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

Keywords:

(1) endothelin type B receptor

(2) Na\textsuperscript{+}/H\textsuperscript{+} exchanger

(3) p38 mitogen-activated protein kinase
G protein-coupled receptors (GPCRs) including endothelin type A receptor (ETAR) and ETBR 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 Gq 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 Gq/11/PLC pathway.

Recently, we have reported that in addition to Ca2+ influx through SOCC and ROCC activated via Gq/11/PLC (3, 4), ETAR induced Gq/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 ETAR stimulation is mediated via p38 mitogen-activated protein kinase (p38MAPK) which is activated through G12 protein (6). Because Gq/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 Gq/11/PLC-independent, p38MAPK-dependent NHE activation in sustained Ca2+ influx to other GPCRs coupled with Gq/11/PLC and G12/13, we examined whether this mechanism is involved in the Ca2+ influx induced by recombinant ETBR and endogenous P2Y receptor (P2Y-R) expressed in CHO cells (7).

To generate CHO cells stably expressing ETBR (ETBR-CHO), the gene of human ETBR
fused with yellow fluorescence protein at the C terminus was introduced into CHO cells by retroviral gene transfer as previously described (5, 6). 

$[\text{Ca}^{2+}]_{i}$ was monitored by using fluorescent $\text{Ca}^{2+}$ indicators, fura-2/acetoxyethyl ester (fura-2/AM), and fluo-3/AM (5, 6). To determine molecular mechanisms for NHE activation upon stimulation of $\text{ET}_{B}$R and $\text{P2Y-R}$ with ET-1 and adenosine triphosphate (ATP), respectively, the change in extracellular acidification rate (ECAR) was measured by the eight-channel Cytosensor™ microphysiometer (Molecular Devices Corp., California, U.S.A.) (5, 6). To estimate the degree of p38MAPK activation after stimulation of $\text{ET}_{B}$R and $\text{P2Y-R}$, 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 PRISM™ (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 Cytosensor™ microphysiometer studies are expressed as percentages of the basal ECAR prior to exposure to vehicle or inhibitors. All data were presented as means ± S.E.M. where $n$ refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad PRISM™ 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 $\text{ET}_{B}$R-CHO, stimulation of $\text{ET}_{B}$R with 1 nM ET-1 elicited a biphasic increase in $[\text{Ca}^{2+}]_{i}$ consisting of an initial transient peak ($304.9 \pm 31.6$ nM, $n = 5$) and a subsequent sustained increase ($199.8 \pm 16.0$ nM, $n = 5$). The pEC$_{50}$ values for ET-1-induced increase in $[\text{Ca}^{2+}]_{i}$ were $10.09 \pm 0.13$ for the transient phase and $9.72 \pm 0.18$ for the sustained phase (Fig. 1A, 1B). ATP also induced a transient increase in $[\text{Ca}^{2+}]_{i}$ with a pEC$_{50}$ value of $5.86 \pm 0.08$ and the maximum increase in $[\text{Ca}^{2+}]_{i}$ of $386.4 \pm 39.5$ 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 $[\text{Ca}^{2+}]_{i}$ seems to be mediated via P2Y-R but not P2X-R, since the $\text{Ca}^{2+}$ response was completely inhibited by 1 μM YM-254890, a $\text{G}_{q/11}$ inhibitor (data not shown). Notably, the
sustained increase in \([\text{Ca}^{2+}]_i\) were very small throughout the tested concentrations of ATP (Fig. 1B). Interestingly, although the transient \([\text{Ca}^{2+}]_i\) increases induced by 1 nM ET-1 and by 10 \(\mu\text{M}\) ATP in ET\(_B\)-CHO are comparable with each other (Fig. 1A), there is a marked difference in the amplitude of the sustained \(\text{Ca}^{2+}\) responses. These results suggest that store depletion does not necessarily activate SOCCs, and hence that the ET\(_B\)-induced sustained \(\text{Ca}^{2+}\) influx is not due to SOCCs activated by the emptying of intracellular \(\text{Ca}^{2+}\) store.

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

Recently, we have shown that \(G_{q/11}/\text{PLC-independent, p38MAPK-dependent pathway regulates NHE activity after ET\(_A\)R stimulation (6). To clarify intracellular mechanisms responsible for NHE activation by ET\(_B\)R, we used the Cytosensor\textsuperscript{TM} 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 \(\mu\text{M}\) 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 \(\mu\text{M}\) EIPA, indicating the involvement of NHE. To identify upstream signaling molecules in NHE activation by ET\(_B\)R and P2Y-R, the effects of YM-254890, U-73122, and SB203580 on the ECAR response were examined. In contrast to \(\text{Ca}^{2+}\) 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}/\text{PLC-independent, p38MAPK-dependent pathway for ET\(_B\)R and via G_{q/11}/\text{PLC/p38MAPK-dependent pathway}
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\(_A\)R (5, 6). In addition, activation of p38MAPK by ET\(_A\)R is reported to be independent of G\(_{q/11}\)/PLC pathway (6). To examine a role of p38MAPK activation in the signaling for ET\(_B\)R 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 \(\mu\)M 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 ± 52.2% (\(n = 6\), Fig. 3B), but ATP (1 \(\mu\)M - 30 \(\mu\)M) induced no significant increase. To determine upstream regulatory molecules for p38MAPK, the effects of inhibitors for G\(_{q/11}\) and PLC on the ET-1-induced p38MAPK phosphorylation were examined. Like p38MAPK activation via ET\(_A\)R (6), YM-254890 (1 \(\mu\)M) and U-73122 (10 \(\mu\)M) had little effect on p38MAPK phosphorylation by 1 nM ET-1 (data not shown). Taken together with the above-mentioned Cytosensor\textsuperscript{TM} microphysiometer study, these data provide further evidence for the involvement of G\(_{q/11}\)/PLC-independent pathway in ET\(_B\)R-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\(_A\)R, both ET\(_B\)R and P2Y-R can couple with a member of G\(_{12}\) family (G\(_{12}\) and G\(_{13}\) proteins) in addition to G\(_{q/11}\) protein (11-13), and activation of G\(_{12}\) protein is involved in ET\(_A\)R-induced NHE activation via p38MAPK, causing a sustained increase in [Ca\(^{2+}\)]\(_i\) (6). Interestingly, G\(_{13}\) protein is a potential candidate responsible for NHE activation mediated via ET\(_B\)R (13), despite stimulation of GPCRs coupled to G\(_q\) (10) and G\(_{12/13}\) (14) can activate NHE. In analogy with the case of ET\(_A\)R (6), EIPA-sensitive part of the ET\(_B\)R-induced sustained increase in [Ca\(^{2+}\)]\(_i\) is mediated by NHE, that is in turn activated via p38MAPK: the remaining part of the [Ca\(^{2+}\)]\(_i\) increase which is resistant to EIPA is considered to be mediated through ROCCs and/or SOCCs, whose activation requires both G\(_{q/11}\)/PLC and p38MAPK. An
The activator of p38MAPK may be G_{12/13} protein. In contrast, the minimum level of the sustained increase in \([\text{Ca}^{2+}]\), 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 \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\) increase (6), these results imply that G_{q/11}/PLC-independent, p38MAPK-dependent activation of NHE plays an important role in the sustained \(\text{Ca}^{2+}\) response to stimulation of ET_{B}R in addition to ET_{A}R.
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signaling at the $\beta_3$-adrenoceptor produced by 3-(2-Ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-2S-2-propanol oxalate (SR59230A) relative to receptor agonists. Mol Pharmacol. 2007;72:1359-1368.


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

Characterization of changes in ECAR induced by 1 nM ET-1 (A) and 10 μM ATP (B) in ET_B-R-CHO. The change in ECAR was measured by the Cytosensor™ 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 ± S.E.M of the results obtained from 5 experiments.
Characterization of p38MAPK phosphorylation in response to ET-1 and ATP in ETB-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.