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<th>Endothelin Type B Receptor–Induced Sustained Ca(^{2+}) Influx Involves G(_q/11)/Phospholipase C–Independent, p38 Mitogen-Activated Protein Kinase–Dependent Activation of Na(^+)/H(^+) Exchanger</th>
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<td>Author(s)</td>
<td>Higa, Tsunaki; Horinouchi, Takahiro; Aoyagi, Hiroyuki; Asano, Hiroshi; Nishiya, Tadashi; Nishimoto, Arata; Muramatsu, Ikunobu; Miwa, Soichi</td>
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<tr>
<td>Citation</td>
<td>Journal of Pharmacological Sciences, 113(3): 276-280</td>
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<tr>
<td>Issue Date</td>
<td>2010-07</td>
</tr>
<tr>
<td>Doc URL</td>
<td><a href="http://hdl.handle.net/2115/47620">http://hdl.handle.net/2115/47620</a></td>
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<td>Type</td>
<td>article (author version)</td>
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<td>File Information</td>
<td>JPS113-3_276-280.pdf</td>
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Short communication

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

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Running title: Mechanism for ET\textsubscript{B}R-induced Ca\textsuperscript{2+} influx.

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Number of words used in Abstract: 100
Number of words used in the main body of the text: 1,716
Number of tables: 0
Number of figures: 3
Number of references: 15
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 (ET\(_{B}\)R) and endogenous P2Y receptor (P2Y-R), endothelin-1 elicited a sustained Ca\(^{2+}\) influx depending on G\(_{q/11}\) 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 ET\(_{B}\)R was mediated via p38MAPK but not G\(_{q/11}\)/PLC, while that by P2Y-R involves only G\(_{q/11}\)/PLC/p38MAPK. These results suggest that G\(_{q/11}\)/PLC-independent NHE activation via p38MAPK plays an important role in ET\(_{B}\)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\textsubscript{A}R) and ET\textsubscript{B}R 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\textsubscript{q} protein-coupled receptors induces formation of the second messengers such as inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG) via phospholipase C (PLC). Binding of IP\textsubscript{3} to its receptor on endoplasmic reticulum (ER) triggers Ca\textsuperscript{2+} release from ER, resulting in a transient increase in intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) (1). According to the classical theory, this is followed by a sustained increase in [Ca\textsuperscript{2+}]\textsubscript{i}, resulting from Ca\textsuperscript{2+} influx through several types of voltage-independent Ca\textsuperscript{2+}-permeable cation channels such as store-operated Ca\textsuperscript{2+} channels (SOCCs) (2). On the other hand, DAG activates directly receptor-operated Ca\textsuperscript{2+} channels (ROCCs), leading to sustained Ca\textsuperscript{2+} influx. Thus, the sustained increase in [Ca\textsuperscript{2+}]\textsubscript{i} via SOCCs and ROCCs results from activation of G\textsubscript{q/11}/PLC pathway.

Recently, we have reported that in addition to Ca\textsuperscript{2+} influx through SOCC and ROCC activated via G\textsubscript{q/11}/PLC (3, 4), ET\textsubscript{A}R induced G\textsubscript{q/11}/PLC-independent activation of sustained Ca\textsuperscript{2+} influx which is not mediated through either SOCC or ROCC: the Ca\textsuperscript{2+} influx is mediated by the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger functionally coupled with Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) via Na\textsuperscript{+} transport (5). Furthermore, NHE activation in response to ET\textsubscript{A}R stimulation is mediated via p38 mitogen-activated protein kinase (p38MAPK) which is activated through G\textsubscript{12} protein (6). Because G\textsubscript{q/11}-coupled receptors can couple to other G proteins, these findings imply that mechanisms for sustained Ca\textsuperscript{2+} 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\textsubscript{q/11}/PLC-independent, p38MAPK-dependent NHE activation in sustained Ca\textsuperscript{2+} influx to other GPCRs coupled with G\textsubscript{q/11}/PLC and G\textsubscript{12/13}, we examined whether this mechanism is involved in the Ca\textsuperscript{2+} influx induced by recombinant ET\textsubscript{B}R and endogenous P2Y receptor (P2Y-R) expressed in CHO cells (7).

To generate CHO cells stably expressing ET\textsubscript{B}R (ET\textsubscript{B}R-CHO), the gene of human ET\textsubscript{B}R
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 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 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 Cytosensor\(^{TM}\) 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 PRISM\(^{TM}\) (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\(^{TM}\) 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\(^{TM}\) 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 \[ \text{[Ca}^{2+}]_i \] 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 \[ \text{[Ca}^{2+}]_i \], 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 \[ \text{[Ca}^{2+}]_i \] with a pEC\(_{50}\) value of 5.86 ± 0.08 and the maximum increase in \[ \text{[Ca}^{2+}]_i \] 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 \[ \text{[Ca}^{2+}]_i \] seems to be mediated via P2Y-R but not P2X-R, since the Ca\(^{2+}\) response was completely inhibited by 1 \(\mu\)M YM-254890, a 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 μM ATP in ET\textsubscript{B-R}-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\textsubscript{B-R}-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 μM YM-254890), a PLC inhibitor (3 μM U-73122), an NHE inhibitor (10 μM 5-(N-ethyl-N-isopropyl)amiloride; EIPA), and a p38MAPK inhibitor (50 μM 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\textsubscript{A-R} (6), NHE/p38MAPK-dependent mechanism is involved in the ET\textsubscript{B-R}-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\textsubscript{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 μM 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 μM EIPA, indicating the involvement of NHE. To identify upstream signaling molecules in NHE activation by ET\textsubscript{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 \(G_{q/11}/\text{PLC-independent, p38MAPK-dependent pathway for ET_B-R and via G_q/11/PLC/p38MAPK-dependent pathway}\).
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\(_{A}\)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 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 ± 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 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 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 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
activator of p38MAPK may be G_{12/13} protein. In contrast, the minimum level of the sustained increase in $[\text{Ca}^{2+}]_{i}$ 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 $[\text{Ca}^{2+}]_{i}$, 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 $[\text{Ca}^{2+}]_{i}$ 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.
Acknowledgements

We thank Astellas Pharma Inc. (Tokyo, Japan) for the generously providing YM-254890. This study was supported in part by Grant-in-Aids for Young Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (T.H.), and for Scientific Research (B) from Japan Society for the Promotion of Science (JSPS) (S.M.), and by grants from Smoking Research Foundation of Japan (S.M.), The Shimabara Science Promotion Foundation (T.H.) and Actelion Pharmaceuticals Japan Ltd. (T.H.).
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Fig. 1.

Characterization of the increases in [Ca\(^{2+}\)]\(_i\) induced by ET-1 and ATP in ET\(_{B}\)-CHO. (A) representative traces showing the [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 [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\(^{2+}\)]. 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_{β}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.
Fig. 3.

Characterization of p38MAPK phosphorylation in response to ET-1 and ATP in ET<sub>B</sub>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.