Title

Endothelin Type B Receptor–Induced Sustained Ca\textsuperscript{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 (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). [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™ 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™ (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 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 \(\mu\)M YM-254890, a G\(_{q/11}\) inhibitor (data not shown). Notably, the
sustained increase in $[\text{Ca}^{2+}]$ were very small throughout the tested concentrations of ATP (Fig. 1B). Interestingly, although the transient $[\text{Ca}^{2+}]$ increases induced by 1 nM ET-1 and by 10 μM 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 $[\text{Ca}^{2+}]$ increase. Maximally effective concentrations of a Gq/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 Ca$^{2+}$ responses to ET-1 are mediated via Gq/11, PLC, NHE, and p38MAPK. This result in turn implies that like ETAR (6), NHE/p38MAPK-dependent mechanism is involved in the ETBR-induced sustained Ca$^{2+}$ influx.

Recently, we have shown that Gq/11/PLC-independent, p38MAPK-dependent pathway regulates NHE activity after ETAR stimulation (6). To clarify intracellular mechanisms responsible for NHE activation by ETBR, we used the Cytosensor™ 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 ETBR 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 Gq/11/PLC-independent, p38MAPK-dependent pathway for ETBR and via Gq/11/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™ 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\(^{2+}\)], (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\(^{2+}\)], is mediated by NHE, that is in turn activated via p38MAPK: the remaining part of the [Ca\(^{2+}\)], 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 G12/13 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 G12/13 protein. Further studies will be required to confirm the possible involvement of G12 and/or G13 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\(_{q/11}\)(G12)/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 G12/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\(_B\)R-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\(^{2+}\)]\(_i\). 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<sub>B</sub>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_{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.