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_BR-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_BR) 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_BR 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_BR-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 (IP_3) and diacylglycerol (DAG) via phospholipase C (PLC). Binding of IP_3 to its receptor on endoplasmic reticulum (ER) triggers Ca^{2+} release from ER, resulting in a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (1). According to the classical theory, this is followed by a sustained increase in $[Ca^{2+}]_i$, resulting from Ca^{2+} influx through several types of voltage-independent Ca^{2+}-permeable cation channels such as store-operated Ca^{2+} channels (SOCCs) (2). On the other hand, DAG activates directly receptor-operated Ca^{2+} channels (ROCCs), leading to sustained Ca^{2+} influx. Thus, the sustained increase in $[Ca^{2+}]_i$ via SOCCs and ROCCs results from activation of $G_{q/11}$/PLC pathway.

Recently, we have reported that in addition to Ca^{2+} influx through SOCC and ROCC activated via $G_{q/11}$/PLC (3, 4), ET_AR induced $G_{q/11}$/PLC-independent activation of sustained Ca^{2+} influx which is not mediated through either SOCC or ROCC: the Ca^{2+} influx is mediated by the reverse mode of Na^+/Ca^{2+} 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 G_{12} protein (6). Because $G_{q/11}$-coupled receptors can couple to other G proteins, these findings imply that mechanisms for sustained Ca^{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 $G_{q/11}$/PLC-independent, p38MAPK-dependent NHE activation in sustained Ca^{2+} influx to other GPCRs coupled with $G_{q/11}$/PLC and $G_{12/13}$, we examined whether this mechanism is involved in the Ca^{2+} 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_BR 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_BR 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_BR-CHO, stimulation of ET_BR 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 μM 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 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 $[Ca^{2+}]_i$ 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 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 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 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 $G_{q/11}$/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 ± 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_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 Cytosensor™ microphysiometer study, these data provide further evidence for the involvement of $G_{q/11}$/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 $G_{q/11}$ protein (11-13), and activation of G_{12} protein is involved in ET_AR-induced NHE activation via p38MAPK, causing a sustained increase in $[Ca^{2+}]_i$ (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 $G_{12/13}$ (14) can activate NHE. In analogy with the case of ET_AR (6), EIPA-sensitive part of the ET_BR-induced sustained increase in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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_BR-mediated p38MAPK activation. In addition, the reasons for these differences in signaling cascade and sustained Ca^{2+} response between ET_BR 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_AR 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_BR, respectively. Therefore, there is the possibility that the difference in intracellular signaling mechanism between ET_BR 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_BR but not P2Y-R. Taken together with our previous reports indicating the participation of G_{12}/p38MAPK/NHE cascade in the ET_AR-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_BR in addition to ET_AR.

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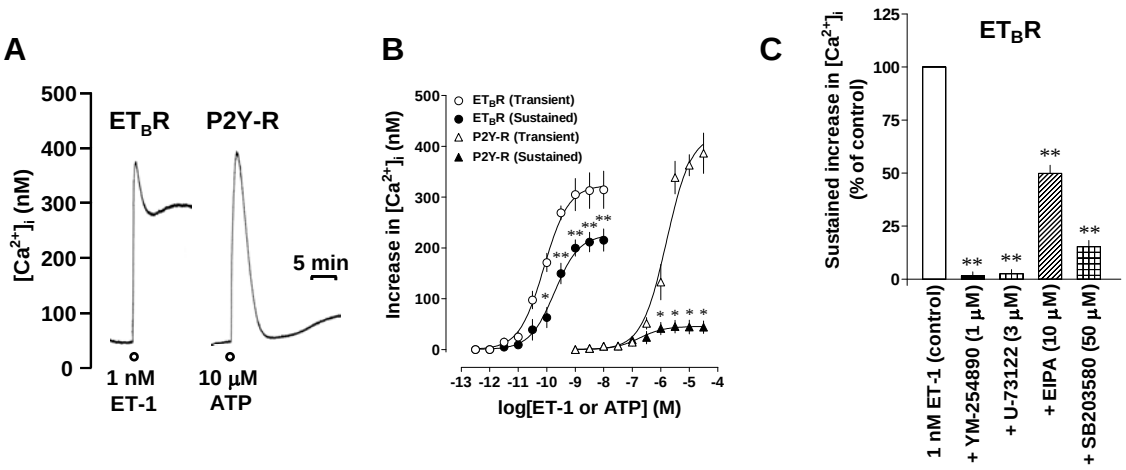

Fig. 1.

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 ± S.E.M of the results obtained from 5 experiments. $^{**}P < 0.01$, versus its control (1 nM ET-1 alone, open column).

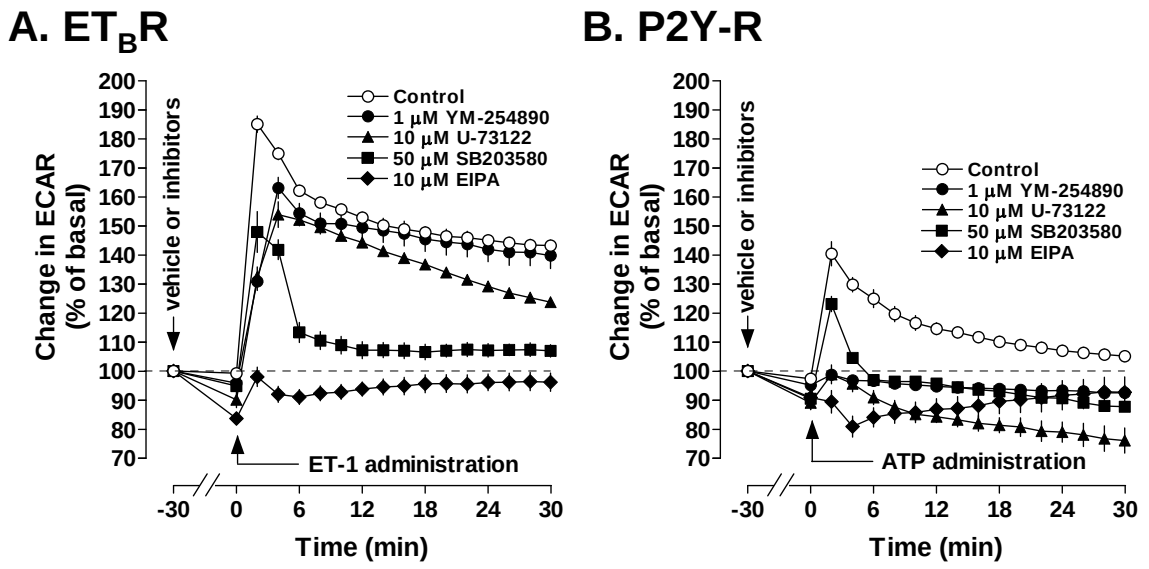

Fig. 2.

Characterization of changes in ECAR induced by 1 nM ET-1 (A) and 10 μM ATP (B) in ET_BR-CHO. The change in ECAR was measured by the Cytosensor^{TM} 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.

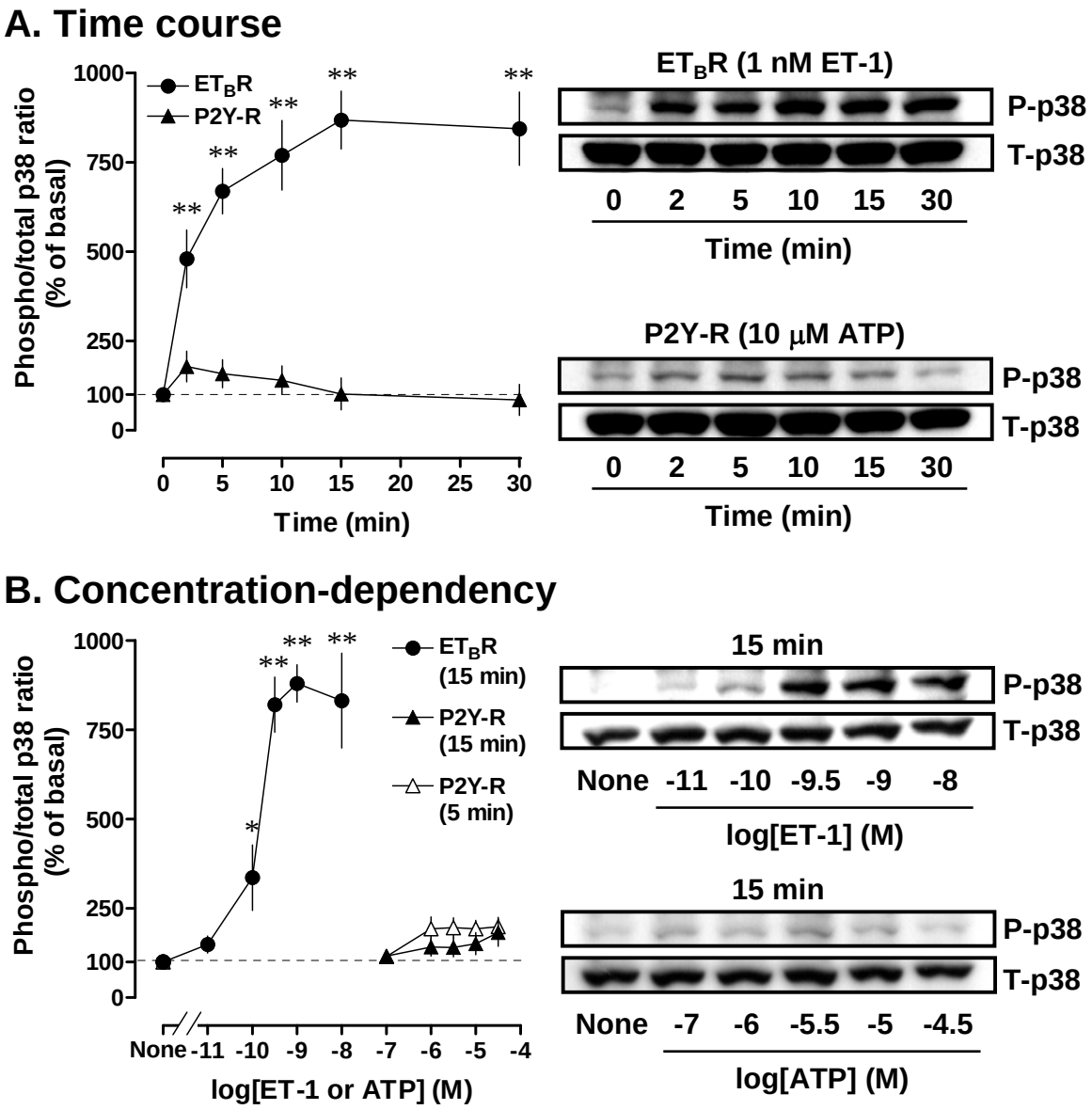

Fig. 3.

Characterization of p38MAPK phosphorylation in response to ET-1 and ATP in ET_BR-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.