



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

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

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Running title: Mechanism for  $\text{ET}_B\text{R}$ -induced  $\text{Ca}^{2+}$  influx.

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## **Abstract**

The mechanism for sustained  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  influx depending on  $\text{G}_{\text{q}/11}$  protein, phospholipase C (PLC),  $\text{Na}^+/\text{H}^+$  exchanger (NHE) and p38 mitogen-activated protein kinase (p38MAPK), whereas P2Y-R-induced sustained  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  influx.

## **Keywords:**

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

G protein-coupled receptors (GPCRs) including endothelin type A receptor (ET<sub>A</sub>R) and ET<sub>B</sub>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<sub>q</sub> protein-coupled receptors induces formation of the second messengers such as inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) via phospholipase C (PLC). Binding of IP<sub>3</sub> to its receptor on endoplasmic reticulum (ER) triggers Ca<sup>2+</sup> release from ER, resulting in a transient increase in intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) (1). According to the classical theory, this is followed by a sustained increase in [Ca<sup>2+</sup>]<sub>i</sub>, resulting from Ca<sup>2+</sup> influx through several types of voltage-independent Ca<sup>2+</sup>-permeable cation channels such as store-operated Ca<sup>2+</sup> channels (SOCCs) (2). On the other hand, DAG activates directly receptor-operated Ca<sup>2+</sup> channels (ROCCs), leading to sustained Ca<sup>2+</sup> influx. Thus, the sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> via SOCCs and ROCCs results from activation of G<sub>q/11</sub>/PLC pathway.

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

To generate CHO cells stably expressing ET<sub>B</sub>R (ET<sub>B</sub>R-CHO), the gene of human ET<sub>B</sub>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/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 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  $\pm$  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 ( $304.9 \pm 31.6$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  with a  $pEC_{50}$  value of  $5.86 \pm 0.08$  and the maximum increase in  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $\mu$ M ATP in ET<sub>B</sub>R-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<sub>B</sub>R-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  $\mu$ M YM-254890), a PLC inhibitor (3  $\mu$ M U-73122), an NHE inhibitor (10  $\mu$ M 5-(N-ethyl-N-isopropyl)amiloride; EIPA), and a p38MAPK inhibitor (50  $\mu$ 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<sub>A</sub>R (6), NHE/p38MAPK-dependent mechanism is involved in the ET<sub>B</sub>R-induced sustained  $Ca^{2+}$  influx.

Recently, we have shown that  $G_{q/11}$ /PLC-independent, p38MAPK-dependent pathway regulates NHE activity after ET<sub>A</sub>R stimulation (6). To clarify intracellular mechanisms responsible for NHE activation by ET<sub>B</sub>R, we used the Cytosensor<sup>TM</sup> 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  $\mu$ 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  $\mu$ M EIPA, indicating the involvement of NHE. To identify upstream signaling molecules in NHE activation by ET<sub>B</sub>R 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<sub>B</sub>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  $\text{Ca}^{2+}$  response induced by  $\text{ET}_A\text{R}$  (5, 6). In addition, activation of p38MAPK by  $\text{ET}_A\text{R}$  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  $\text{ET}_B\text{R}$  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  $\mu\text{M}$  ATP was very weak and transient (Fig. 3A). At 15 min following stimulation, ET-1 induced a concentration-dependent phosphorylation of p38MAPK with a  $\text{pEC}_{50}$  value of  $10.28 \pm 0.12$  and the maximum level of  $879.9 \pm 52.2\%$  ( $n = 6$ , Fig. 3B), but ATP (1  $\mu\text{M}$  - 30  $\mu\text{M}$ ) 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  $\text{ET}_A\text{R}$  (6), YM-254890 (1  $\mu\text{M}$ ) and U-73122 (10  $\mu\text{M}$ ) had little effect on p38MAPK phosphorylation by 1 nM ET-1 (data not shown). Taken together with the above-mentioned Cytosensor<sup>TM</sup> microphysiometer study, these data provide further evidence for the involvement of  $\text{G}_{q/11}/\text{PLC}$ -independent pathway in  $\text{ET}_B\text{R}$ -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  $\text{ET}_A\text{R}$ , both  $\text{ET}_B\text{R}$  and P2Y-R can couple with a member of  $\text{G}_{12}$  family ( $\text{G}_{12}$  and  $\text{G}_{13}$  proteins) in addition to  $\text{G}_{q/11}$  protein (11-13), and activation of  $\text{G}_{12}$  protein is involved in  $\text{ET}_A\text{R}$ -induced NHE activation via p38MAPK, causing a sustained increase in  $[\text{Ca}^{2+}]_i$  (6). Interestingly,  $\text{G}_{13}$  protein is a potential candidate responsible for NHE activation mediated via  $\text{ET}_B\text{R}$  (13), despite stimulation of GPCRs coupled to  $\text{G}_q$  (10) and  $\text{G}_{12/13}$  (14) can activate NHE. In analogy with the case of  $\text{ET}_A\text{R}$  (6), EIPA-sensitive part of the  $\text{ET}_B\text{R}$ -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.



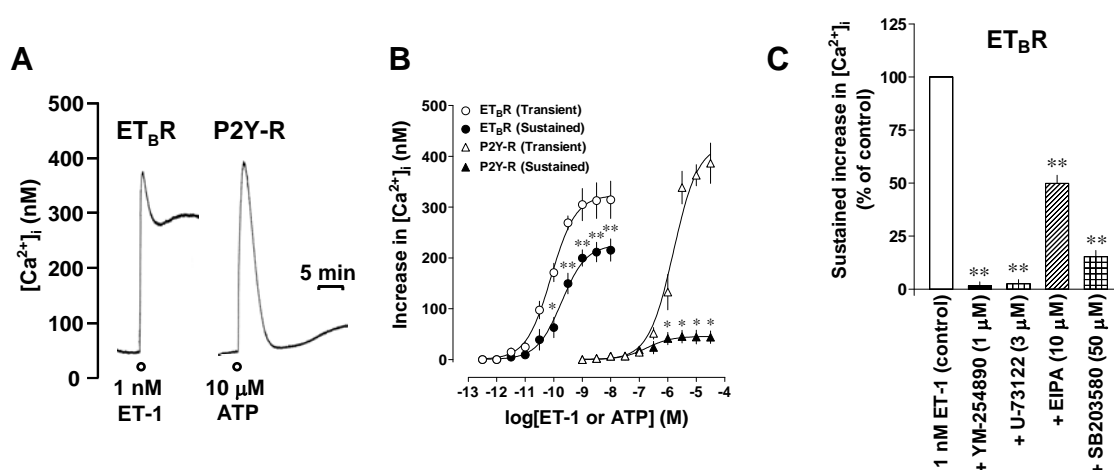
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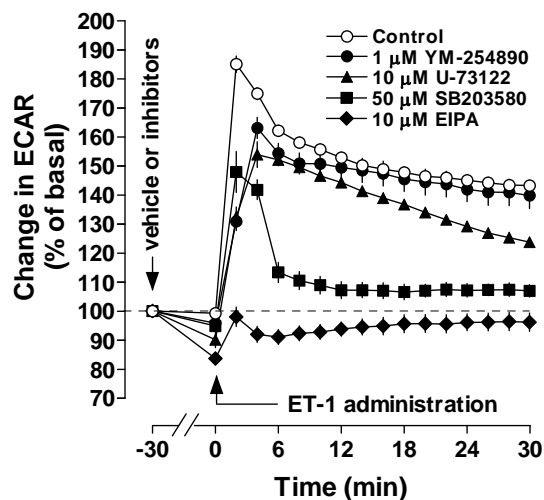
- signaling at the  $\beta_3$ -adrenoceptor produced by 3-(2-Ethylphenoxy)-1-[(1,S)-1,2,3,4-tetrahydronaph-1-ylamino]-2S-2-propanol oxalate (SR59230A) relative to receptor agonists. *Mol Pharmacol.* 2007;72:1359-1368.
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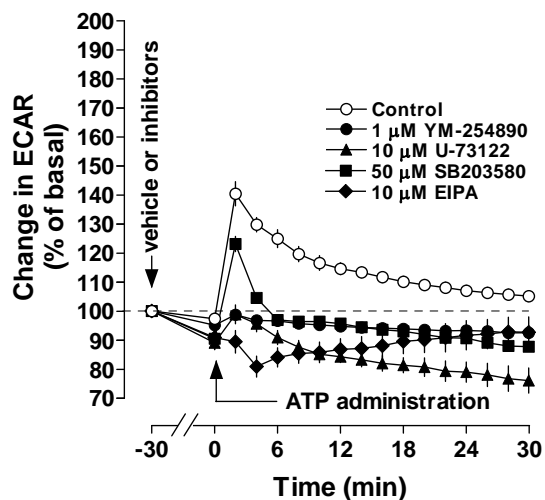
**Fig. 1.**

Characterization of the increases in  $[Ca^{2+}]_i$  induced by ET-1 and ATP in ET<sub>B</sub>R-CHO. (A) representative traces showing the  $[Ca^{2+}]_i$  increases induced by stimulation of ET<sub>B</sub>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  $\pm$  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<sub>B</sub>R



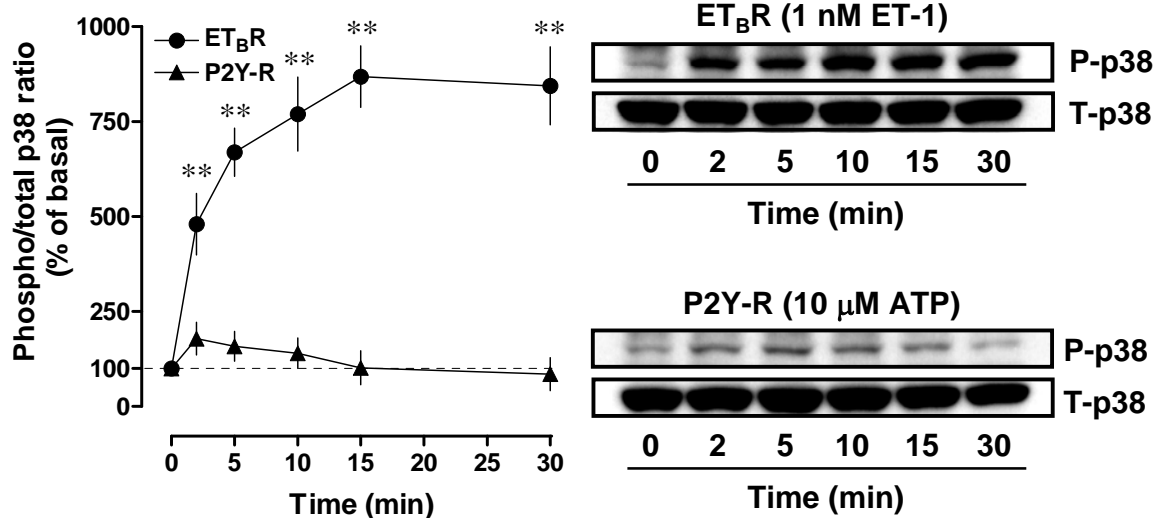
### B. P2Y-R



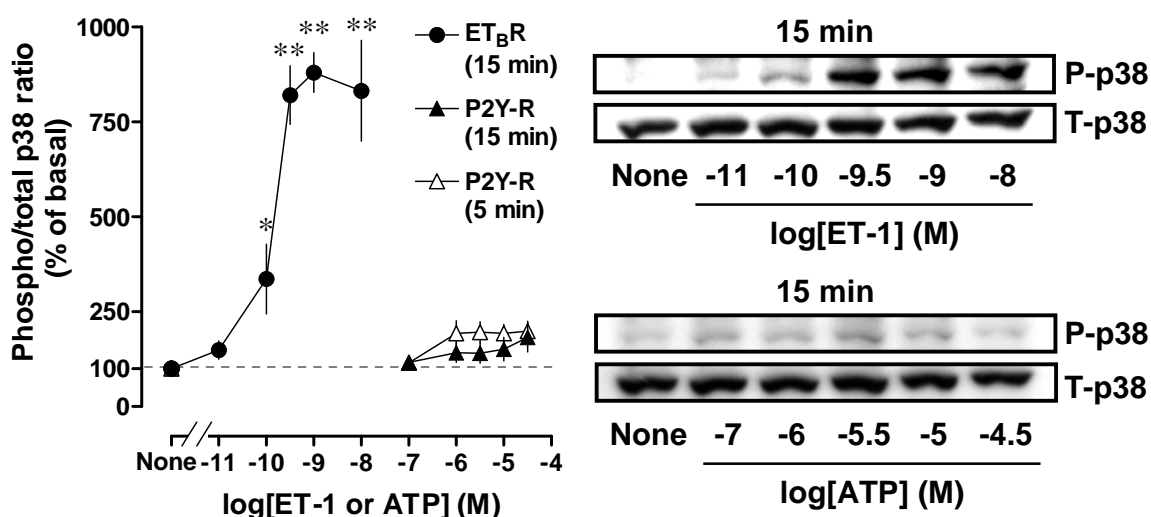
**Fig. 2.**

Characterization of changes in ECAR induced by 1 nM ET-1 (A) and 10  $\mu$ M ATP (B) in ET<sub>B</sub>R-CHO. The change in ECAR was measured by the Cytosensor<sup>TM</sup> 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<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.