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ORIGINAL ARTICLE

Inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity by extracellular Ca^{2+} entry and Ca^{2+} release from the endoplasmic reticulum in renal proximal tubule cells

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The kidney is important in the long-term regulation of blood pressure and sodium homeostasis. Stimulation of ETB receptors in the kidney increases sodium excretion, in part, by decreasing sodium transport in the medullary thick ascending limb of Henle and in collecting duct. However, the role of ETB receptor on Na^+–K^+ ATPase activity in renal proximal tubule (RPT) cells is not well defined. The purpose of this study is to test the hypothesis that ETB receptor inhibits Na^+–K^+ ATPase activity in rat RPT cells, and investigate the mechanism(s) by which such an action is produced. In RPT cells from Wistar–Kyoto rats, stimulation of ETB receptors by the ETB receptor agonist, BQ3020, decreased Na^+–K^+ ATPase activity, determined by ATP hydrolysis (control=0.38 ± 0.02, BQ3020=0.26 ± 0.03, BQ788=0.40 ± 0.06, BQ3020+BQ788=0.37 ± 0.04, $n=5$, $P<0.01$). The ETB receptor-mediated inhibition of Na^+–K^+ ATPase activity was dependent on an increase in intracellular calcium, because this effect was abrogated by a chelator of intracellular-free calcium (BAPTA-AM; 5×10^{-3} M 15 min^{-1}), Ca^{2+} channel blocker (10^{-6} M 15 min^{-1} nicardipine) and PI3 kinase inhibitor (10^{-7} M per wortmannin). An inositol 1,4,5-trisphosphate (IP3) receptor blocker (2-aminoethyl diphenyl borate; 10^{-4} M 15 min^{-1}) also blocked the inhibitory effect of the ETB receptor on Na^+–K^+ATPase activity (control=0.39 ± 0.06, BQ3020=0.25 ± 0.01, 2-APB=0.35 ± 0.05, BQ3020+ 2-APB=0.35 ± 0.06, $n=4$, $P<0.01$). The calcium channel agonist (BAY-K8644; 10^{-6} M 15 min^{-1}) inhibited Na^+–K^+ ATPase activity, an effect that was blocked by a phosphatidylinositol-3 kinase inhibitor (10^{-7} M 15 min^{-1} wortmannin). In rat RPT cells, activation of the ETB receptor inhibits Na^+–K^+ ATPase activity by facilitating extracellular Ca^{2+} entry and Ca^{2+} release from endoplasmic reticulum.
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Keywords: ETB receptor; kidney; Na^+–K^+ ATPase activity; renal proximal tubule

INTRODUCTION

The kidney is important in the long-term regulation of blood pressure and is the major organ involved in the regulation of body sodium homeostasis.[1–3] The proximal tubule and medullary thick ascending limb of Henle are preeminent in the overall regulation of sodium balance in essential hypertension.[4,5] Indeed, several studies have shown that human essential hypertension and rodent genetic hypertension are associated with increased sodium transport in the renal proximal tubule (RPT) and medullary thick ascending limb of Henle.[3–5]

Endothelins are a family of isopeptides (ET1, ET2 and ET3) transduced by at least two receptor subtypes (ETA and ETB).[6,7] Renal tissue expresses both endothelin receptors, and endothelin is synthesized by renal tubules, wherein it regulates sodium transport.[8] Emerging evidence suggests that ETB receptor has an important role in the regulation of sodium balance and blood pressure.[4,5,9–11]

The effects of ETB on sodium transport in RPT cells seem to be complex. Both inhibitory and stimulatory effects of endothelin on sodium hydrogen exchanger 3 (NHE3) activity have been reported in the RPT.[12–14] Short-term stimulation of ETB receptors in opossum kidney cells, an RPT cell line, activates NHE3.[12] In contrast, chronic treatment of the same opossum kidney cells by endothelin has an opposite effect on NHE3 activity.[13] Decreasing intracellular sodium by the inhibition of NHE3 can result in a secondary inhibition of Na^+–K^+ ATPase. We hypothesize that activation of ETB receptor, independent of NHE3, has an inhibitory effect

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on Na^+–K^+ ATPase activity in RPT cells. The purpose of this study is to determine the effect of ETB receptor on Na^+–K^+ ATPase activity, and the mechanism(s) by which such an action is produced. The results reported here suggest that in rat RPT cells, the ETB receptor inhibits Na^+–K^+ ATPase activity, which involves Ca^{2+} entry, activation of phosphatidylinositol-3 (PI3) kinase and an increase in inositol 1,4,5-trisphosphate (IP3), which triggers Ca^{2+} release from the endoplasmic reticulum (ER) to further increase intracellular Ca^{2+} concentration.

METHODS

Cell culture

Immortalized RPT cells from 4- to 8-week-old Wistar–Kyoto rats were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C,[15–17] cultured at 37 °C in a 95% air/5% CO_2 atmosphere in DMEM/F-12 with transferrin (5 μg ml^{-1}), insulin (5 μg ml^{-1}), epidermal growth factor (10 ng ml^{-1}), dexamethasone (4 μg ml^{-1}) and 5% fetal bovine serum (Sigma, St Louis, MO, USA). For subculturing, cells were dissociated with 0.1% trypsin–EDTA, split 1:4 and subcultured in Costar plates with 21 cm^2 growth areas (Costar, Badhoevedorp, the Netherlands). The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. In all the experiments, cells were maintained in fetal bovine serum-free medium for 3 h.

Human renal proximal tubule cells: Histologically, normal sections of fresh human kidneys from normotensive patients (n=6; mean age, 65 years; 3 men, 3 women) who had unilateral nephrectomy because of renal carcinoma or trauma were grown in culture. All patients signed a consent form agreeing that the tissues taken from them are the property of the Department of Pathology and that such tissues can be used for study. All studies were approved by the Institutional Review Board of the University of Virginia Center for the Health Sciences.

Human RPT cells,[18] passages 6 and 7, were incubated at 37°C in 95% O_2/5% CO_2 under polarized conditions on Transwells inserted on 12-well plates in medium with 5% fetal bovine serum consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with selenium (5 ng ml^{-1}), insulin (5 μg ml^{-1}), transferrin (5 μg ml^{-1}), hydrocortisone (36 ng ml^{-1}), triiodothyronine (4 pg ml^{-1}) and epidermal growth factor (10 ng ml^{-1}).[18]

Na^+–K^+ ATPase activity assay

ATP hydrolysis. Rat RPT cells were treated with vehicle (dH_2O), an ETB receptor agonist (BQ3020, Sigma) or an ETB receptor antagonist (BQ788, Sigma)[19,20] at indicated concentrations and durations of incubation. Na^+–K^+ ATPase activity was determined as the rate of inorganic phosphate released in the presence or absence of ouabain.[21] To prepare membranes for Na^+–K^+ ATPase activity assay, RPT cells cultured in 21 cm^2 plastic culture dishes were washed twice with 5 ml chilled phosphate-free buffer (3.36 mM NaCl, 0.54 mM $NaHCO_3$, 0.4 mM KCl and 0.12 mM $MgCl_2$ scraped in phosphate-free buffer) and were centrifuged at 3000 *g* for 10 min. The cells were then placed on ice and lysed in 2 ml of lysis buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$ and 5 mM $MgCl_2$). Cellular lysates were centrifuged at 3000 *g* for 2 min to remove intact cells, debris and nuclei. The resulting supernatant was suspended in an equal volume of 1 M sodium iodide, and the mixture was centrifuged at 48 000 *g* for 25 min. The pellet (membrane fraction) was washed twice and suspended in 10 mM Tris and 1 mM EDTA (pH 7.4). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 1 mg ml^{-1}. The membranes were stored at −70 °C until further use. To measure Na^+–K^+ ATPase activity, 100 μl aliquots of membrane fraction were added to an 800 μl reaction mixture (75 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 6 mM sodium azide, 1 mM Na_4EGTA, 37.5 mM imidazole, 75 mM Tris HCl and 30 mM histidine; pH 7.4) with or without 1 mM ouabain (final volume=1 ml) and preincubated for 5 min in a water bath at 37 °C. Reactions were initiated by adding Tris-ATP (4 mM) and terminated after 15 min of incubation at 37 °C by adding 50 μl of 50% trichloracetate. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures containing ouabain. To quantify the amount of phosphate produced, 1 ml of coloring reagent (10% ammonium molybdate in 10 N sulfuric acid + ferrous sulfate) was added to the reaction mixture. The mixture was then combined thoroughly and centrifuged at 3000 *g* for 10 min. Formation of phosphomolybdate was determined spectrophotometrically at 740 nm against a standard curve prepared from K_2HPO_4. Na^+–K^+ ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity and expressed as nmol phosphate released per mg protein per min.

To eliminate the effect of proteases and phosphatases, we added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} each of leupeptin and aprotinin) and phosphatase inhibitor (50 μM sodium orthovanadate) to all solutions used after drug/vehicle incubations.[22]

Sodium green tetraacetate uptake. To determine the effect of the ETB receptor on Na^+–K^+ ATPase activity, we measured the uptake of sodium green as reported by Sasaki *et al.*[23] in the absence (total transport) and presence (ouabain-sensitive transport) of ouabain. We also used another proximal tubule cell line to determine whether ETB receptor inhibits Na^+–K^+ ATPase activity in cells other than those from rats. Human renal proximal tubular cells[18] were cultured to confluence under polarized conditions on Transwells inserted on 12-well plates. The cells were incubated in culture medium with or without 50 μM ouabain for 1 hour at 37 °C, washed gently with PBS three times and treated for 15 min at 37 °C with vehicle (PBS, control), 10 nM BQ3020, or 10 nM BQ3020 and 10 nM BQ788, as indicated. After washing, cells were loaded for 30 min at room temperature with the cell permeant sodium indicator, sodium green tetraacetate (5 μM, Molecular Probes, Eugene, OR, USA), in DMEM/F12 medium without phenol red. The cells were washed gently with PBS three times, and the fluorescence emission (excitation 485 nm, emission 535 nm) of each Transwell was read in a Victor 3 V plate reader (Perkin Elmer, Vienna, VA, USA). Ouabain-sensitive transport was expressed as percentage of total sodium transport.

Determination of the second messenger(s) involved in the ETB receptor-mediated inhibition of Na^+–K^+ ATPase activity

To determine the second messenger(s) involved in the ETB receptor-mediated inhibition of Na^+–K^+ ATPase activity, several agonists or antagonists were used, including cell permeable, myristoylated peptide inhibitor of PKC (peptide 19–31),[24,25] PKA 14–22 amide[26] (Calbiochem Company, Darmstadt, Germany), calcium channel blocker, nicardipine (Sigma),[27,28] the PI3 kinase inhibitor, wortmannin (Tocris, Ellisville, MO, USA)[29,30] and the IP3 receptor blocker, 2-aminoethyl diphenyl borate (2-APB) (10^{-4} M 15 min) (Sigma).[31]

Measurement of intracellular calcium ($[Ca^{2+}]_i$) concentration

Twenty-four hours before the experiments, Wistar–Kyoto cells were harvested and seeded into 7.5 cm^2 petri dishes (Falcon, Franklin Lakes, NJ, USA). Wistar–Kyoto cells were loaded with the calcium indicator Fura-2AM (5 μM) in Hepes-buffered saline. Changes in $[Ca^{2+}]$i in individual cells were measured using an Aquacosmos system with band-pass filters for 340 and 380 nm. $[Ca^{2+}]$i was calculated from the Fura-2 fluorescence ratio (F340/F380) using linear regression between adjacent points on a calibration curve generated by measuring F340/F380 in at least seven calibration solutions containing $[Ca^{2+}]$ between 0 and 854 nM. The ETB receptor-mediated changes in $[Ca^{2+}]$i after stimulation with BQ3020 or with individual reagents in Ca^{2+}-free and Ca^{2+} concentration were measured as previously described.[32]

Statistical analysis

Data are expressed as mean ± s.e.m. Comparison within groups was carried out by repeated measures analysis of variance (ANOVA) and comparison among groups was carried out by factorial ANOVA and Duncan's test. A value of $P<0.05$ was considered significant.

RESULTS

Activation of ETB receptor inhibits Na^+–K^+ ATPase activity in RPT cells

An ETB receptor agonist, BQ3020, inhibited Na^+–K^+ ATPase activity in a concentration- and time-dependent manner. The inhibitory effect

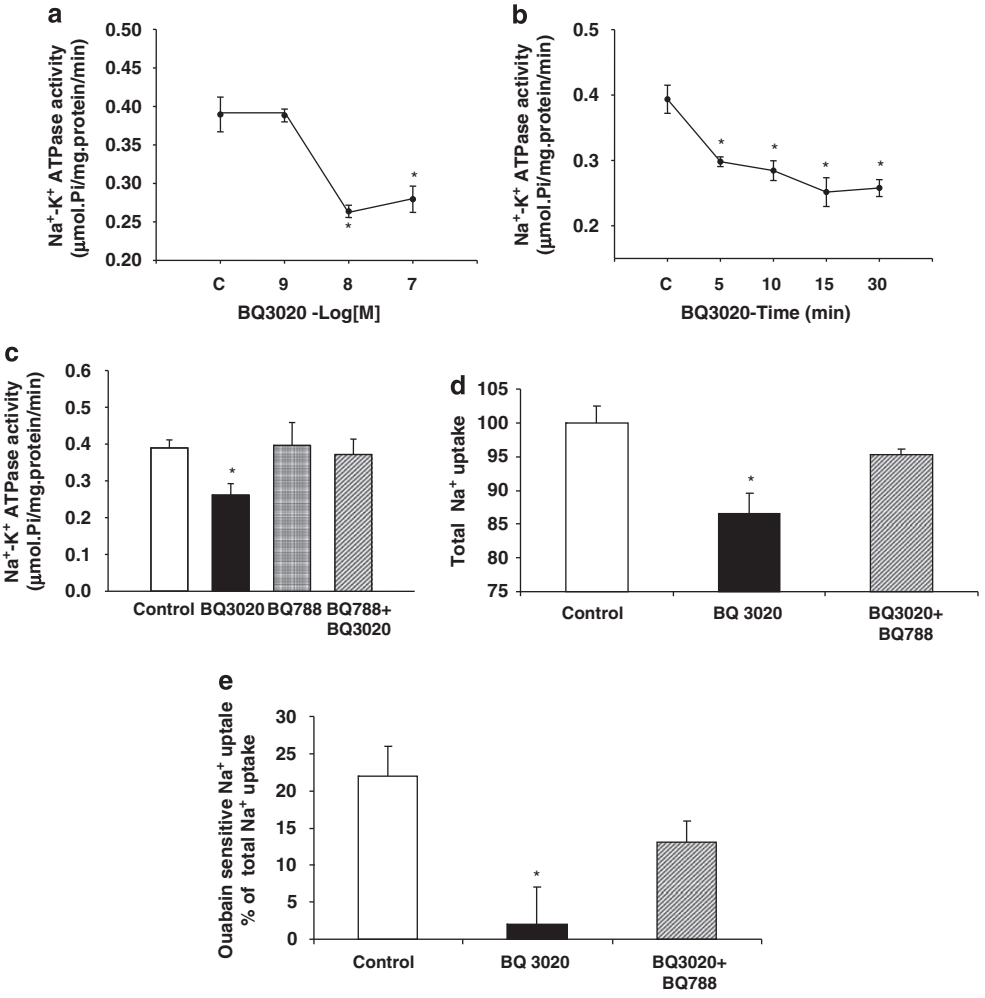

Figure 1 Effect of ETB receptor stimulation on Na^+–K^+ ATPase activity in RPT cells from Wistar–Kyoto rats. (**a**) Concentration–response of Na^+–K^+ ATPase activity in RPT cells incubated with the ETB receptor agonist, BQ3020, for 15 min. Results are expressed as micromol phosphate released per mg protein per min (n=8, $^*P<0.01$ *vs.* control (C), ANOVA, Duncan's test). (**b**) Time course of Na^+–K^+ ATPase activity in RPT cells incubated with the ETB receptor agonist, BQ3020 (10^{-8} M), at varying durations of incubation. Results are expressed as micromol phosphate released per mg protein per min (n=5, $^*P<0.01$ *vs.* control (C), ANOVA, Duncan's test). (**c**) Effects of an ETB receptor agonist (BQ3020, 10^{-8} M 15 min^{-1}) and an ETB receptor antagonist (BQ788, 10^{-8} M 15 min^{-1}) on Na^+–K^+ ATPase activity in RPT cells. Results are expressed as micromol phosphate released per mg protein per min (n=5, $^*P<0.01$ *vs.* others, ANOVA, Duncan's test). (**d** and **e**) Effects of an ETB receptor agonist (BQ3020, 10^{-8} M 15 min^{-1}) and an ETB receptor antagonist (BQ788, 10^{-8} M 15 min^{-1}) on Na^+–K^+ ATPase activity in human RPT cells. The Na^+–K^+ ATPase activity was determined by the uptake of sodium green. Na^+–K^+ ATPase activity was determined as the difference between sodium green uptake in the absence (total activity, (**d**)) and presence (ouabain insensitive, (**e**)) of ouabain (50 μM) (n=4 control/vehicle, n=5, BQ3020, n=3 BQ3020 and BQ788), $^*P<0.01$ *vs.* that of others, ANOVA, Duncan's test.

was evident at 10^{-8} M, noted as early as 5 min, and maintained for at least 30 min (Figures 1a and b).

Specificity of BQ3020 as an ETB receptor agonist was determined using the ETB receptor antagonist, BQ788. Consistent with the results shown in Figures 1a and b, BQ3020 (10^{-8} M 15 min^{-1}) inhibited Na^+–K^+ ATPase activity. BQ788 (10^{-8} M 15 min^{-1}), by itself, had no effect on Na^+–K^+ ATPase activity, but reversed the inhibitory effect of BQ3020 on Na^+–K^+ ATPase activity (control=0.38 ± 0.02, BQ3020=0.26 ± 0.03, BQ788=0.40 ± 0.06, BQ3020+BQ788=0.37 ± 0.04, n=5, $P<0.01$) (Figure 1c).

To further confirm the inhibitory effect of BQ3020 on Na^+–K^+ TPase activity in RPT cells, we determined Na^+–K^+ ATPase activity by the intracellular uptake of sodium green. Similar to the results in Figures 2b and c, BQ3020 (10^{-8} M 15 min^{-1}) inhibited Na^+–K^+ ATPase activity, which was partially blocked by the ETB receptor antagonist, BQ788 (10^{-8} M 15 min^{-1}) (Figures 1d and e). These effects were observed in human RPT cells,[33] indicating that the inhibitory effect of ETB on Na^+–K^+ ATPase activity is observed in RPT cells, other than those from rats.

Intracellular Ca^{2+} is involved in the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity

Endothelin, in part through ETB receptors, has been shown to activate Ca^{2+} channels, leading to an increase in $[Ca^{2+}]i$.[34,35] We therefore determined whether intracellular Ca^{2+} is involved in the ETB receptor-mediated inhibition of Na^+–K^+ ATPase activity. Rat RPT cells were first treated with BAPTA-AM (5×10^{-3} M)[34,36] (Biomol Research Labs Plymouth Meeting, PA, USA), a chelator of intracellular-free calcium. In a Ca^{2+}-free solution, in the presence of BAPTA-AM, the inhibitory effector of ETB receptor on Na^+–K^+ ATPase activity was no longer evident (control=0.39 ± 0.02, BQ3020=0.27 ± 0.02, BAPTA-AM= 0.40 ± 0.03, BQ3020+ BAPTA-AM=0.39 ± 0.04, n=5) (Figure 2).

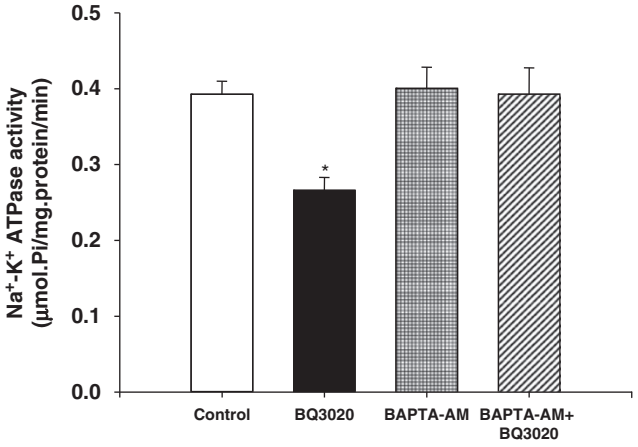

Figure 2 Effect of ETB receptor agonist, BQ3020 (10^{-8} M 15 min^{-1}), on Na^+–K^+ ATPase activity in the presence of BAPTA-AM (5×10^{-3} M) (a chelator of intracellular-free calcium, n=5) in RPT cells from Wistar–Kyoto rats. Results are expressed as micromol phosphate released per mg protein per min (*$P<0.01$ *vs.* others, ANOVA, Duncan's test).

We also tested the effects of a PKA inhibitor (14–22 amide), and of a PKC inhibitor, PKC peptide 19–31. However, neither 14–22 amide nor peptide 19–31 could block the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity (data not shown).

To prove further the role of intracellular calcium in the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity, we studied the effect of stimulation of the ETB receptor on intracellular calcium concentration. We found that BQ3020 increased intracellular calcium, an effect that was blocked by nicardipine (10^{-6} M 15 min^{-1}), 2-APB (10^{-4} M 15 min^{-1}), wortmannin (10^{-7} M 15 min^{-1}) or BAPTA-AM (5×10^{-3} M 15 min^{-1}) (Figures 3a and b).

Both extracellular Ca^{2+} entry and ER Ca^{2+} release take part in the signaling of ETB receptor-inhibited Na^+–K^+ ATPase activity

Intracellular Ca^{2+} concentration depends on extracellular Ca^{2+} entry and ER Ca^{2+} release. To determine whether the Ca^{2+} channel at the plasma membrane was involved in the ETB-mediated inhibition of Na^+–K^+ ATPase activity, a Ca^{2+} channel blocker, nicardipine (10^{-6} M 15 min^{-1}) (Sigma),[27] was added to the incubation medium and the effect of the ETB receptor agonist, BQ3020 (10^{-8} M 15 min^{-1}),

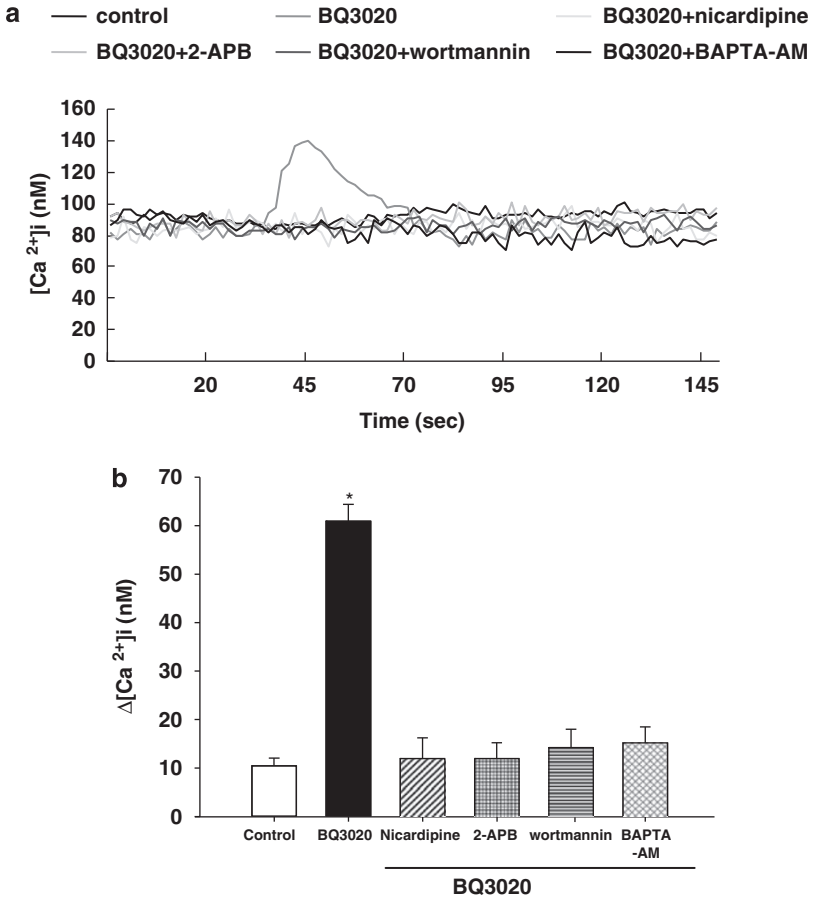

Figure 3 Effect of the ETB receptor agonist, BQ3020, on intracellular calcium concentration in the presence or absence of pharmacological agents in Wistar–Kyoto cells. Wistar–Kyoto cells were treated with BQ3020 (10^{-8} M 15 min^{-1}) and intracellular calcium concentration was determined by laser confocal microscopy. The stimulatory effect of BQ3020 on intracellular calcium concentration was also tested in the presence of nicardipine (10^{-6} M 15 min^{-1}), wortmannin (10^{-7} M 15 min^{-1}), BAPTA-AM (5×10^{-3} M 15 min^{-1}) and 2-APB (10^{-4} M 15 min^{-1}) (n=5). Representative tracings are show in (**a**) and graphs of the data are show in (**b**) ($^{\Delta}[Ca^{2+}]i$ in Figure 4b shows the difference in calcium concentration between 0 and 45 s). *$P<0.05$ *vs.* that of others, ANOVA, Duncan's test. A full color version of this figure can be found at the *Hypertension Research* journal online.

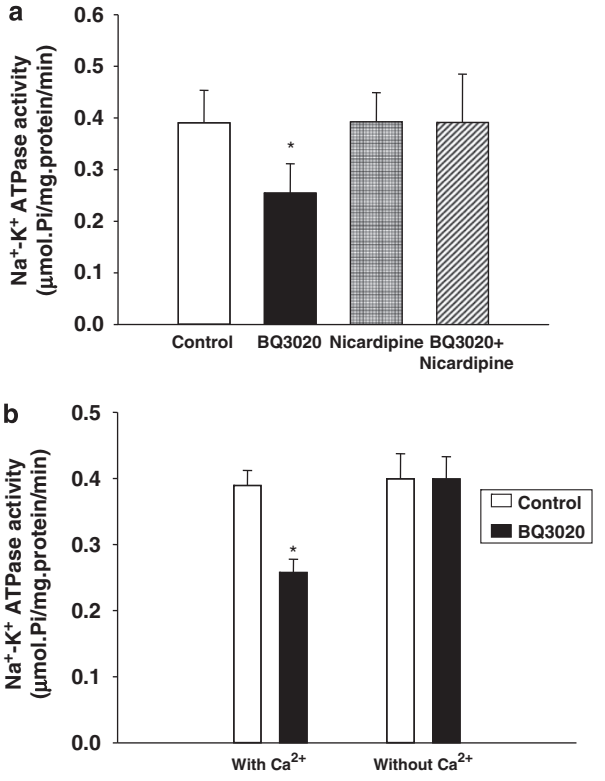

Figure 4 Effect of ETB receptor BQ020 (10^{-8} M 15 min^{-1}) on Na^+–K^+ ATPase activity in the presence of nicardipine (10^{-6} M; n=6) (**a**) or in the medium with or without calcium (**b**) (n=4) in RPT cells from Wistar–Kyoto rats. Results are expressed as micromol phosphate released per mg protein per min (*P<0.01 *vs.* that of others, ANOVA, Duncan's test).

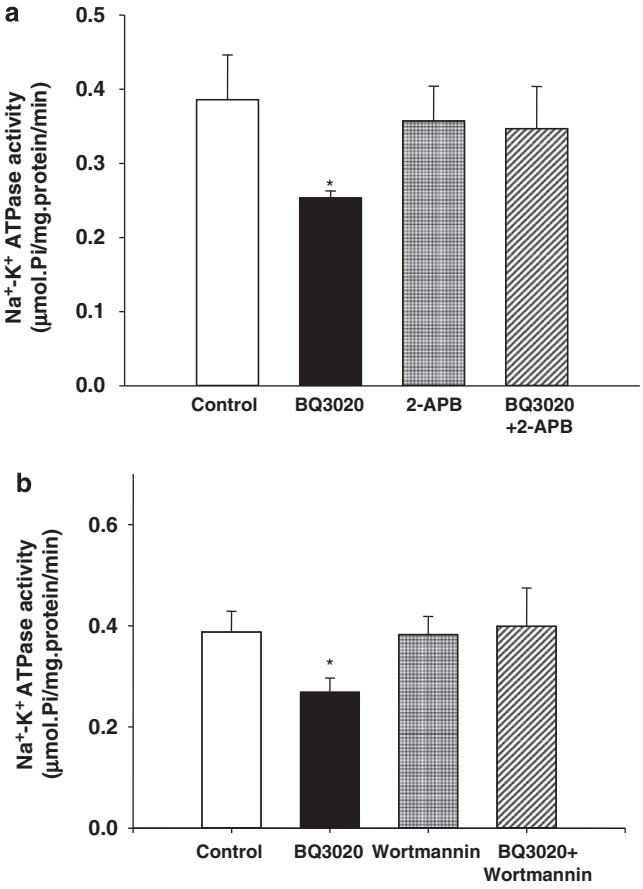

Figure 5 Effect of ETB receptor BQ3020 (10^{-8} M 15 min^{-1}) on Na^+–K^+ ATPase activity in the presence of an IP3 receptor blocker, 2-aminoethyl diphenyl borate (2-APB; 10^{-4} M; n=4) (**a**), or the PI3 kinase inhibitor wortmannin (10^{-7} M; n=5) (**b**) in RPT cells from Wistar–Kyoto rats. Results are expressed as micromol phosphate released per mg protein per min (n=4, *P<0.01 *vs.* that of others, ANOVA, Duncan's test).

was retested. Nicardipine, by itself, had no effect on Na^+–K^+ ATPase activity, but blocked the inhibitory effect of BQ3020 on Na^+–K^+ ATPase activity (Figure 4a), which suggests that the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity requires extracellular Ca^{2+} entry (control=0.39 ± 0.06, BQ3020=0.25 ± 0.05, nicardipine=0.39 ± 0.06, BQ3020+ nicardipine=0.39 ± 0.09, n=6, P<0.01). To confirm the relative contribution of extracellular Ca^{2+} on the inhibitory effect of BQ3020, studies were performed in a Ca^{2+}-free medium. Similar to the abrogation of the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity in the presence of calcium channel blockers, the Ca^{2+}-free medium also prevented the inhibitory effect of BQ3020 on Na^+–K^+ ATPase activity (culture medium with calcium: control=0.39 ± 0.02, BQ3020=0.26 ± 0.02; culture medium without calcium: control=0.40 ± 0.04, BQ3020=0.40 ± 0.03, n=4) (Figure 4b).

Entry of extracellular Ca^{2+} into the cell leads to Ca^{2+} release from the ER through the IP3 receptor, resulting in a further increase in $[Ca^{2+}]i$.[37,38] To determine the effect of ER Ca^{2+} release on the ETB receptor-mediated inhibition of Na^+–K^+ ATPase activity, we used an IP3 receptor blocker, 2-APB (10^{-4} M 15 min^{-1}) (Sigma),[31,39] to treat RPT cells in the presence of the ETB receptor agonist, BQ3020 (10^{-8} M 15 min^{-1}). 2-APB, by itself, had no effect on Na^+–K^+ ATPase activity, but blocked the inhibitory effect of BQ3020 on Na^+–K^+ ATPase activity (control=0.39 ± 0.06, BQ3020=0.25 ± 0.01, 2-APB=0.35 ± 0.05, BQ3020+2-APB=0.35 ± 0.06, n=4, P<0.01) (Figure 5a).

Intracellular signaling by many cell surface receptors requires the generation of IP3. PI3 kinase is an important enzyme in the production of IP3.[40] To determine whether PI3 kinase is involved in ETB action, the PI3 kinase inhibitor, wortmannin,[41] was used. In the presence of wortmannin (10^{-7} M 15 min^{-1}), the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity was blocked (control=0.39 ± 0.04, BQ3020=0.27 ± 0.03, wortmannin=0.38 ± 0.04, BQ3020+wortmannin=0.40 ± 0.08, n=5, P<0.01)(Figure 5b).

The studies, so far, have shown that, in RPT cells, the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity involved both extracellular Ca^{2+} entry and Ca^{2+} release from ER. However, the upstream signal in this effect is not clear. Activation of calcium channels by BAY-K8644 (10^{-6} M 15 min^{-1}) (Sigma) inhibited Na^+–K^+ ATPase activity (control=0.39 ± 0.04, BAY-K8644=0.19 ± 0.04, wortmannin=0.38 ± 0.04, BAY-K8644+wortmannin=0.38 ± 0.03, n=5, P<0.01) (Figure 6),[42], which was blocked by the PI3 kinase inhibitor, wortmannin. These results indicate that extracellular Ca^{2+} entry was needed to trigger ER Ca^{2+} release, which subsequently inhibited Na^+–K^+ ATPase activity in RPT cells.

DISCUSSION

ETB receptor has an important role in the regulation of blood pressure.[10,11,43] At the whole-animal level, a naturally occurring or induced deletion of the ETB receptor gene in rats results in

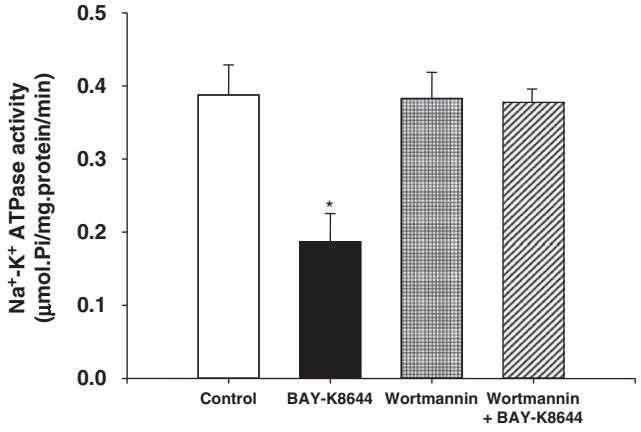

Figure 6 Effect of BAY-K8644 (10^{-6} M 15 min^{-1}), a calcium channel agonist, on Na^+–K^+ ATPase activity in the presence of wortmannin (10^{-7} M), a PI3 kinase inhibitor, in RPT cells from Wistar–Kyoto rats. Results are expressed as micromol phosphate released per mg protein per min (n=5, $^*P<0.01$ *vs.* that of others, ANOVA, Duncan's test).

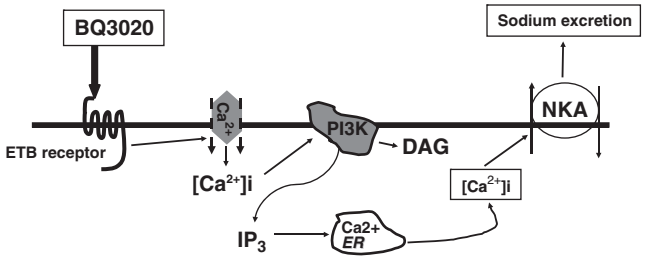

Figure 7 Schematic representation of the signaling pathways linked to ETB receptor-mediated inhibition of Na^+–K^+ ATPase activity (NKA) in RPT cells. IP3, inositol 1,4,5-trisphosphate; PI3K, phosphatidylinositol-3 kinase; DAG, diacylglycerol; ER, endoplasmic reticulum.

salt-sensitive hypertension.[10,44] ETB blockade produces hypertension that is exaggerated by salt intake.[11] ETB receptors are also involved in the hypertension that occurs in spontaneously hypertensive rats and after the administration of deoxycorticosterone acetate and NaCl,[45] but not in angiotensin II-induced hypertension.[9] Systemic ETB blockade produces hypertension in mice, which is maintained by ETA receptors.[46] These findings strongly suggest that the ETB receptor, by itself, or in conjunction with ETA receptors, can regulate blood pressure as a consequence of its vasodilator and natriuretic effects. However, under certain circumstances, ETB receptors, acting on vascular smooth muscle cells, can also increase blood pressure.[47–49] Therefore, the eventual blood pressure resulting from ETB receptor activation depends upon which action of ETB predominates.

ETB receptors, expressed in RPT cells, in the medullary thick ascending limb of Henle and collecting duct, can decrease the reabsorption of sodium and water.[13,14] Previous studies have shown that diuretic and natriuretic responses to endothelin-1 precursor big ET-1 can be inhibited by ETB blockade;[43] activation of the ETB receptor decreases sodium transport in the medullary thick ascending limb of Henle and collecting duct.[9,10,44] However, the ETB receptor has also been reported to stimulate NHE3 in RPTs/cells.[12–14]

The major regulation of sodium transport across RPT is provided by two key proteins: NHE3, located at the brush border membrane, and Na^+–K^+ ATPase, located at the basolateral membrane. Although endothelin has been shown to inhibit fluid and bicarbonate transport by reducing Na^+–K^+ ATPase activity in the rat proximal straight tubule,[33] the role of ETB receptor on Na^+–K^+ ATPase activity in RPT cells is not well defined. We now report that activation of the ETB receptor decreases Na^+–K^+ ATPase activity in RPT cells.

Na^+–K^+ ATPase activity is regulated by intracellular calcium.[50] In agreement with previous reports, $[Ca^{2+}]$i mediates the inhibitory effect of ETB on Na^+–K^+ ATPase activity.[50,51] RPT cells treated with an intracellular calcium chelator, BAPTA-AM, in a Ca^{2+}-free solution prevents the inhibitory effect of ETB receptor on Na^+–K^+ ATPase activity. In many cell types, Ca^{2+} signaling induced by neurotransmitters or hormones is a biphasic phenomenon.[52,53] In the early phase, neurotransmitter or hormone binding to a specific receptor at the cell surface activates G proteins and IP3 kinase, resulting in the generation of IP3. IP3 then binds to IP3 receptors on the ER-triggering release of Ca^{2+} from intracellular stores.[38] In a later phase, the increase in $[Ca^{2+}]$i stimulates the intracellular movement of extracellular Ca^{2+}. Our results show that both mechanisms are involved in the signaling pathway by which the ETB receptor inhibits Na^+–K^+ ATPase activity, because this effect is prevented by an L-type Ca^{2+} channel blocker (nicardipine) and a Ca^{2+}-free medium. However, these experiments cannot determine whether Ca^{2+} entry from the extracellular space, or Ca^{2+} released from the ER, initiates the ETB effect. Previous studies have shown that the sequence of events may vary depending on cell type. For example, in cardiac endothelial cells, the Ca^{2+} cascade is initiated by Ca^{2+} released from ER, followed by a sustained Ca^{2+} entry across the plasma membrane.[53] In contrast, in cardiomyocytes, extracellular Ca^{2+} entry across the plasma membrane is the initiating event.[49,54] In thyroid carcinoma cells, PI3 kinase is involved as calcium activation increases PI3 kinase activity.[55] Our results show that the L-type Ca^{2+} channel agonist, BAY-K8644, inhibits Na^+–K^+ ATPase activity, which is blocked by an inhibitor of PI3 kinase. These results suggest that Ca^{2+} entry through the plasma membrane is the triggering event after ETB receptor occupation, which is subsequently followed by Ca^{2+} release from the ER (Figure 7).

In summary, we have shown that activation of the ETB receptor inhibits Na^+–K^+ ATPase activity in RPT cells; the inhibitory effect is mediated by an increase in intracellular calcium that is initially due to extracellular Ca^{2+} entry and is followed by Ca^{2+} release from ER.

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