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Functional characterization of human monocarboxylate transporter 6 (*SLC16A5*) \*

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**Abbreviations:**

ES, estrone-3-sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MCT, monocarboxylate transporter; OATP, organic anion transporting polypeptide; OAT, organic anion transporter; PAH, *p*-aminohippuric acid; PCR, polymerase chain reaction; TAT1, T-type amino acid transporter 1

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## Abstract

Human monocarboxylate transporter 6 (MCT6) has recently been isolated and its tissue distribution has been established at the mRNA level, but its functional properties remain unknown. The aim of this study is to investigate the transport properties of MCT6. When expressed in *Xenopus laevis* oocytes, MCT6 transported [<sup>3</sup>H]bumetanide in a pH- and membrane potential-sensitive, but not proton gradient dependent manner, with the K<sub>t</sub> value of 84 μM. Furthermore, MCT6 transported various drugs such as probenecid and nateglinide. Neither [<sup>14</sup>C]L-lactic acid nor [<sup>3</sup>H]L-tryptophan, typical substrates of other MCT isoforms, was transported by MCT6. Four loop diuretics, *i.e.* furosemide, piretanide, azosemide and torasemide, thiazides, probenecid, glibenclamide and nateglinide inhibited the MCT6-mediated uptake of [<sup>3</sup>H]bumetanide. In contrast, short-chain carboxylic acids, such as L-lactic acid and succinic acid did not inhibit the MCT6 mediated uptake of bumetanide. These results suggest that the substrate specificity of MCT6 is distinct from those of other MCTs. Bumetanide would be a good tool for investigating the functional properties of MCT6. It is probable that MCT6 is involved in the disposition of various drugs, including bumetanide.

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## Introduction

Human monocarboxylate transporter 6 (MCT6; *SLC16A5*) is a member of the MCT family, which comprises 14 members (Halestrap and Meredith, 2004), and was first cloned from a placenta cDNA library (Price et al., 1998). MCT6 shares 38% identity with MCT4 at the amino acid level. Northern blot analysis revealed that MCT6 mRNA is highly expressed in the kidney and placenta (Price et al., 1998), but expression at the protein level and the function of MCT6 are still unknown.

The first member of the MCT family was cloned from a mutated Chinese hamster ovary cell line with enhanced mevalonate uptake (Kim et al., 1992). Subsequently, the wild-type protein was demonstrated to catalyze proton-linked transport of L-lactic acid and pyruvic acid, and was named monocarboxylate transporter 1 (MCT1) (Garcia et al., 1994a). Based on the nucleotide sequence of hamster MCT1, MCT1 orthologs have been cloned from human, rat, and mouse (Garcia et al., 1994b; Takanaga et al., 1995; Jackson et al., 1995; Carpenter et al., 1996). It has been reported that MCT1 co-transporters  $H^+$  with L-lactic acid (Kim et al., 1992; Bröer et al., 1997; Bröer et al., 1998). MCT1 (*SLC16A1*), MCT2 (*SLC16A7*), MCT3 (*SLC16A8*) and MCT4 (*SLC16A3*) all transport both L-lactic acid and pyruvic acid (Lin et al., 1998; Grollman et al., 2000; Manning et al., 2000). T-type amino acid transporter 1 (TAT1, *SLC16A10*),

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another member of the MCT family, transports aromatic amino acids such as L-tryptophan (Kim et al., 2001; Kim et al., 2002). More recently, the rat ortholog of MCT8 (*SLC16A2*) has been shown to transport thyroid hormones (Friesema et al., 2003). The function of the other MCT family members remains unknown.

In the present study, we examined the substrate specificity of MCT6 using a *Xenopus* oocyte expression system. We also investigated the transport properties of MCT6 by using bumetanide, a newly identified substrate of MCT6.

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## Materials and Methods

### Materials

[<sup>3</sup>H]Bumetanide (1.85 GBq/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [<sup>3</sup>H]Probenecid was kindly provided by Fuji Biomedix Co., Ltd. (Saitama, Japan). [<sup>14</sup>C]Nateglinide and nateglinide were kindly supplied by Ajinomoto Co., Inc. (Tokyo, Japan). Nonradioactive bumetanide was purchased from MP Biomedicals (Irvine, CA, USA). Azosemide, torasemide and piretanide were kindly supplied by Sanwa Kagaku Kenkyusho Co. (Nagoya, Japan), Taisho Toyama Pharmaceutical Co. (Tokyo, Japan) and Nippon HEXAL Co. (Tokyo, Japan), respectively. Furosemide was purchased from Wako Pure Chemicals (Osaka, Japan). pSP64T-mKV1.3-EC plasmid was obtained from American Type Culture Collection (Manassas, VA). All other chemicals were of the highest grade commercially available.

### Methods

#### Construction of pSP64T-hMCT6

pSP64T vector containing a *Bgl* II restriction site was constructed from the pSP64T-mKV1.3-EC plasmid by digesting with *Bst*E II, blunting with Klenow fragment, and

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ligating with a *Bgl* II linker. Next, pSP64T was digested with *Bgl* II and ligated with a *Not* I linker.

The plasmid containing *MCT6* cDNA (clone KAT01693, Genbank accession no. **AK000416**) was obtained from New Energy and Industrial Technology Development Organization (NEDO) human cDNA project. *MCT6* cDNA was amplified by polymerase chain reaction (PCR). PCR primers were designed to contain the 5'- and 3'-untranslated region sequences of *MCT6*. The forward primer was 5'-AAG ATT AAG CGG CCG CAA GAA AAG AGC ACT TCC CTG C-3' and the reverse primer was 5'-TTA GTA TGC GGC CGC GGT CAA CAA TTT GCC CAA CTC-3'. Each primer contains a *Not* I restriction site (underlined). The PCR products were digested with *Not* I and ligated into the pSP64T-*Not* I vector. Finally, we obtained pSP64T-hMCT6. The identity of products was confirmed by means of sequencing.

### **cRNA synthesis and preparation of *Xenopus* oocytes**

The capped cRNA of MCT6 was transcribed *in vitro* from *Bam* HI-linearized pSP64T-hMCT6 with SP6 RNA polymerase. Oocytes were prepared from ovaries removed from female *Xenopus laevis* as previously described (Kobayashi et al., 2004). Oocytes (stage

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V-VI) were injected with cRNA and incubated in Barth's buffer (88 mM NaCl, 1 mM KCl, 0.33 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.41 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.82 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.4 mM  $\text{NaHCO}_3$ , 10 mM HEPES, 50 ng/mL gentamicin sulfate, pH 7.6) at 18°C for 2 to 3 days.

### **Uptake study**

For uptake experiments, groups of 8-20 oocytes were transferred into 24-well multi-dishes and preincubated in uptake buffer (15 mM HEPES, 82.5 mM NaCl, 2.5 mM KCl, 1 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM  $\text{MgCl}_2$ , pH 7.4 adjusted with Tris). The uptake buffer containing radiolabeled drugs (400  $\mu\text{L}$ ) was added to initiate the uptake after removal of preincubation buffer, and the mixture was incubated at room temperature (20-23°C) at pH 7.4 or pH 6.0, except in the pH dependency experiment. pH levels of uptake buffer were adjusted with MES/Tris (pH 5.5, 6.0 and 6.5) or HEPES/Tris (pH 7.4 and 8.0). To examine the effects of  $\text{Na}^+$  and  $\text{Cl}^-$ , NaCl in the buffer was replaced with N-methyl-D-glucamine, mannitol, or sodium gluconate. The high potassium uptake buffer contained 15 mM NaCl, 120 mM KCl, 1 mM  $\text{CaCl}_2$ , and 10 mM HEPES or MES. The bicarbonate-containing uptake buffer was prepared by replacing 15 mM NaCl with 15 mM  $\text{NaHCO}_3$  and was gassed with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ . To equilibrate intracellular pH (pHi) to external pH (pHo), we used the high potassium uptake



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buffer containing 20  $\mu\text{M}$  nigericin and 10  $\mu\text{M}$  valinomycin (Emoto et al., 2002; Sasaki et al., 1992). The uptake was stopped by the addition of ice-cold uptake buffer, and oocytes were washed three times. The oocytes were divided into 4-8 scintillation vials and dissolved in 250  $\mu\text{L}$  of 10% sodium lauryl sulfate. The solution was slowly shaken for 1 hour. A scintillation cocktail was added and the radioactivity was counted using a liquid scintillation counter. Since uptake of all compounds in Table 1 by uninjected oocytes was essentially the same as that of oocytes injected with water both at pH 7.4 and pH 6.0, uninjected oocytes were used as the control instead of water-injected oocytes throughout this study. Values of uptake of [ $^3\text{H}$ ]bumetanide, [ $^3\text{H}$ ]probenecid, [ $^3\text{H}$ ]prostaglandin  $\text{F}_{2\alpha}$ , and [ $^{14}\text{C}$ ]nateglinide by uninjected oocytes at pH 7.4 were  $0.06 \pm 0.01$ ,  $0.51 \pm 0.03$ ,  $0.19 \pm 0.02$ , and  $0.25 \pm 0.02$  (mean  $\pm$  SE, three separate experiments), respectively, and were not significantly different from those by water-injected oocytes:  $0.10 \pm 0.01$ ,  $0.58 \pm 0.01$ ,  $0.26 \pm 0.02$ , and  $0.26 \pm 0.01$ , respectively. The uptake by oocytes (uptake;  $\mu\text{L}/\text{oocyte}$ ) is presented as the ratio of radioactivity in the sample (dpm/oocyte) to the initial concentration in the uptake buffer (dpm/ $\mu\text{L}$ ).

MCT6-specific uptake was calculated by subtraction of the uptake by uninjected oocytes from that by MCT6 cRNA-injected oocytes. To estimate the kinetic parameters of the

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saturable uptake via MCT6, the Michaelis-Menten equation (1) was fitted to the experimental data by means of nonlinear least-squares regression analysis (Yamaoka et al., 1981).

$$J/C = J_{\max}/(Kt + C) \quad \text{----equation (1)}$$

where J and C represent the uptake rate (pmol/oocyte/30 min) and the concentration of substrate ( $\mu\text{M}$ ), respectively.  $J_{\max}$  and Kt represent the maximum uptake rate for the saturable component and the half-maximal concentration.

For inhibition curves, the following sigmoidal inhibition equation (2) was fitted to the data by means of nonlinear least-squares regression analysis.

$$E = 100 - 100 \cdot C / (IC_{50} + C) \quad \text{---- equation (2)}$$

where E,  $IC_{50}$  and C represent the relative uptake rate of substrate (% of control), the concentration of inhibitor which gives 50% inhibition and the concentration of inhibitor, respectively.

### **Statistical analysis**

Statistical analysis was performed using the unpaired Student's *t*-test or analysis of

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variance (ANOVA) followed by Dunnett's test. A difference between means was considered statistically significant when the *P* value was less than 0.05

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## Results

### Transport of organic compounds via MCT6

[<sup>14</sup>C]Bumetanide was found to be taken up specifically by oocytes injected with MCT6 cRNA (MCT6-expressing oocytes) in comparison with uninjected control oocytes, showing that bumetanide is a substrate of MCT6. We therefore examined the uptake of various compounds via MCT6 using bumetanide as positive control. Uptake of [<sup>3</sup>H]estrone-3-sulfate (ES; a substrate of organic anion transporter (OAT) 3, OAT4 and organic anion transporting polypeptide (OATP) 1A2, OATP1B1, OATP1B3, OATP2B1, OATP3A1) (Kullak-Ublick et al., 2001; Hagenbuch and Meier, 2004), [<sup>14</sup>C]*p*-aminohippuric acid (PAH; a typical substrate of OAT1, OAT3 and OAT4) (Koepsell and Endou, 2004), [<sup>3</sup>H]valproic acid, [<sup>14</sup>C]salicylic acid, [<sup>3</sup>H]hexanoic acid and [<sup>14</sup>C]L-leucine by MCT6-expressing oocytes was similar to that by control oocytes at both pH 7.4 and pH 6.0 (Table 1). The uptake of [<sup>3</sup>H]valproic acid was lower than that by control oocytes. However, uptake of [<sup>14</sup>C]nateglinide or [<sup>3</sup>H]prostaglandin F<sub>2α</sub> by MCT6-expressing oocytes was significantly higher than that by control oocytes (Table 1). The uptake rate of [<sup>14</sup>C]nateglinide at 8 μM was higher than that at 57 μM, but still lower than that of [<sup>3</sup>H]bumetanide (Table 1). The values of uptake of [<sup>3</sup>H]probenecid by MCT6-expressing oocytes at pH 7.4 and pH 6.0 were significantly higher and lower,

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respectively, than those by control oocytes (Table 1). MCT6 did not mediate the uptake of [<sup>14</sup>C]L-lactic acid, a typical substrate of MCT1-MCT4, or [<sup>3</sup>H]tryptophan, a typical substrate of TAT1 (data not shown). Uptake of [<sup>3</sup>H]bumetanide by MCT1- and MCT4-expressing oocytes were similar to that by control oocytes (data not shown).

### **Functional characteristics of MCT6**

The uptake of [<sup>3</sup>H]bumetanide via MCT6 linearly increased up to 60 minutes and reached a steady state at 90-120 min (Figure 1A). In the following experiments, the uptake was examined at 30 min. When the concentration dependence of [<sup>3</sup>H]bumetanide uptake by MCT6-expressing oocytes was examined, the uptake was saturable with regard to substrate concentration (Figure 1B). Kinetic analysis with the Michaelis-Menten equation (1) yielded a K<sub>t</sub> value of 84.2 (56.3, 125.9; mean - SD, mean + SD) μM and a J<sub>max</sub> value of 45.2 ± 21.88 pmol/oocyte/30 min (mean ± SD of ten separate experiments). Figure 1C shows the effect of extracellular pH on the uptake of [<sup>3</sup>H]bumetanide via MCT6. At each pH between 8.0 to 5.5, the uptake by the MCT6-expressing oocytes was higher than that by control oocytes. The uptake rate of [<sup>3</sup>H]bumetanide via MCT6 increased with decreasing extracellular pH.

The K<sub>t</sub> and J<sub>max</sub> values at pH 6.0 were 83.4 (64.9, 107.2; mean - SD, mean + SD) μM

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and  $83.1 \pm 27.0$  pmol/oocyte/30 min (mean  $\pm$  SD of four separate experiments), respectively.

Although the  $K_t$  values were comparable at pH 7.4 and pH 6.0, the  $J_{\max}$  value at pH 6.0 was about twice that at pH 7.4. Therefore, the increase of uptake activity of MCT6 for [ $^3\text{H}$ ]bumetanide at acidic extracellular pH can be ascribed to an increased maximum transport rate, rather than to increased affinity for MCT6.

When NaCl in uptake buffer was replaced with N-methyl-D-glucamine, mannitol, or sodium gluconate in order to investigate the involvement of sodium ion and chloride ion, there was no significant change of uptake of [ $^3\text{H}$ ]bumetanide by MCT6 at pH 7.4 (Table 2). We also examined the effect of membrane potential on the uptake of [ $^3\text{H}$ ]bumetanide by MCT6. Under the condition of intracellular  $\text{K}^+$  concentration ( $[\text{K}^+]_{\text{in}}$ ) = extracellular  $\text{K}^+$  concentration ( $[\text{K}^+]_{\text{out}}$ ), attained by preincubation with the high potassium buffer containing 10  $\mu\text{M}$  valinomycin, the uptake of [ $^3\text{H}$ ]bumetanide by MCT6 was significantly higher than that in standard uptake buffer (Figure 2A), suggesting that MCT6-mediated transport of bumetanide is dependent on membrane voltage. As shown in Figure 2B, extracellular acidic pH also increased the uptake of [ $^3\text{H}$ ]bumetanide by MCT6 under the condition of  $[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$ . These results indicates that the uptake of [ $^3\text{H}$ ]bumetanide by MCT6 is pH- and membrane potential-sensitive.

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Furthermore we investigated the mechanism of the pH effect on MCT6 activity. Under the condition of  $[K^+]_{in} = [K^+]_{out}$ , the uptake of [ $^{14}C$ ]L-lactic acid by MCT1, which is proton-coupled, was significantly reduced by removing the proton gradient with nigericin (Figure 2C), but the uptake of [ $^3H$ ]bumetanide by MCT6 was not (Figure 2D). These results imply that a proton gradient is not the driving force for MCT6.

We also examined the effect of  $HCO_3^-$  on the MCT6-mediated [ $^3H$ ]bumetanide uptake under the condition of high extracellular  $K^+$  concentration. The uptake of [ $^3H$ ]bumetanide via MCT6 for 30 min after preincubation in high  $K^+$  and  $HCO_3^-$  containing buffer for 30 min was  $0.91 \pm 0.11 \mu L/oocyte$  (mean  $\pm$  SE, n=5) and was not significantly different from that after preincubation in high  $K^+$  but not  $HCO_3^-$  containing buffer,  $0.95 \pm 0.06 \mu L/oocyte$ .

### **Inhibitory effect of various compounds on the uptake of bumetanide mediated by MCT6**

Figure 3 shows the inhibitory effects of several loop diuretics (furosemide, piretanide, azosemide and torasemide) on the uptake of [ $^3H$ ]bumetanide. All of these drugs concentration-dependently inhibited the MCT6-mediated uptake of [ $^3H$ ]bumetanide. The  $IC_{50}$  values of furosemide, piretanide, azosemide and torasemide were  $46.1 \pm 16.4 \mu M$ ,  $163.2 \pm 74.7$

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$\mu\text{M}$ ,  $20.8 \pm 5.7 \mu\text{M}$  and  $13.0 \pm 9.4 \mu\text{M}$ , respectively (estimate  $\pm$  SE). Table 3 shows the inhibitory effects of various compounds other than loop diuretics on the MCT6-mediated uptake of [ $^3\text{H}$ ]bumetanide. L-Lactic acid (a typical substrate of MCT1-MCT4), pyruvic acid (a substrate of MCT1, MCT2 and MCT4), succinic acid and citric acid did not affect the MCT6-mediated uptake of [ $^3\text{H}$ ]bumetanide. The effects of cationic compounds were subsequently examined. The MCT6-mediated uptake of [ $^3\text{H}$ ]bumetanide was inhibited by cimetidine but not by choline. In addition, PAH and ES inhibited the MCT6-mediated uptake of [ $^3\text{H}$ ]bumetanide. Thiazides, probenecid, glibenclamide and nateglinide also inhibited the uptake of [ $^3\text{H}$ ]bumetanide.



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## Discussion

Price *et al.* identified human MCT6 cDNA and examined the tissue distribution of MCT6 mRNA by Northern blot analysis (Price et al., 1998), but the present paper is the first to describe the functional characteristics of MCT6.

We found that MCT6 transports bumetanide, but not L-lactic acid, a typical substrate of MCT1-MCT4, or L-tryptophan, a typical substrate of TAT1. In contrast, MCT1 and MCT4 transported L-lactic acid but not bumetanide. These results suggest that the substrate specificity of MCT6 is different from those of other MCTs, such as MCT1-MCT4 and TAT1. The substrates of other MCTs are endogenous compounds such as L-lactic acid, thyroid hormone and aromatic amino acids (Halestrap and Meredith, 2004), and there is no evidence that the other MCTs mediate the transport of therapeutic drugs. In contrast, MCT6 mediated the transport of bumetanide, probenecid and nateglinide, and was inhibited by various drugs (Tables 1, 3). Therefore, it seems likely that MCT6 participates in the membrane transport of various drugs. There are similar peptide sequences of rat (ex. XM\_346905) and mouse (ex. XM\_126601) in the GenBank database (<http://www.ncbi.nlm.nih.gov>). Further studies will be required to elucidate the difference in MCT6-mediated drug transport between humans and rodents. In addition, the physiological and pharmacokinetic roles of human MCT6 remain to

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be elucidated.

The uptake of [ $^3\text{H}$ ]probenecid by MCT6-expressing oocytes at pH 6.0 was lower than that by control oocytes. Further investigation is necessary to compare the properties of probenecid uptake by MCT6 with those of bumetanide uptake, and to understand the action of pH on the transport activity of MCT6.

MCT6 did not transport PAH and ES, typical substrates of organic anion transporters, such as OATs and OATPs, which are involved in the transport of various anionic drugs (Table 1). These results suggest that the substrate specificity of MCT6 is different from those of OATs and OATPs. The MCT6-mediated uptake of [ $^3\text{H}$ ]bumetanide was inhibited by PAH and ES (Table 3). Kinetic analysis of the inhibitory effects of PAH and ES remains to be performed.

Four loop diuretics structurally related to bumetanide, *i.e.* furosemide, piretanide, azosemide and torasemide, inhibited the MCT6-mediated uptake of [ $^3\text{H}$ ]bumetanide (Figure 3), as did several thiazides, probenecid, glibenclamide and nateglinide. In contrast, short-chain carboxylic acids, such as L-lactic acid and succinic acid did not inhibit the MCT6-mediated uptake of bumetanide (Table 3). All known substrates of other MCT isoforms have a carboxylate moiety. However, MCT6 was inhibited by azosemide and torasemide, which have an anionic sulfonamide moiety but not a carboxylate moiety, and cimetidine, an organic cation,

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but not by short-chain carboxylic acids, suggesting that a carboxylate group may not be essential for affinity to MCT6.

It has been demonstrated that the transport of L-lactic acid by MCT1 is proton-coupled (Poole and Halestrap, 1993). MCT2, MCT3 and MCT4 transport monocarboxylates in a pH-dependent manner. On the other hand, transport mediated by MCT8 and TAT1 has been shown to be pH-insensitive (Halestrap and Meredith, 2004; Kim et al., 2002). The uptake of bumetanide via MCT6 was shown to be sodium- and chloride- independent, and pH- and membrane potential-sensitive, but not proton gradient-dependent. Under the condition of membrane depolarization, the presence of bicarbonate or diminution of the inward proton gradient did not affect the uptake of bumetanide via MCT6, suggesting that MCT6 is neither a proton-coupled transporter nor an  $\text{OH}^-$  or  $\text{HCO}_3^-$  exchanger. When the membrane potential was depolarized, the uptake of bumetanide via MCT6 was increased. Our results suggest that MCT6-mediated transport of bumetanide will be the movement with net charge(s). Therefore, under physiological conditions, MCT6 may be involved in the facilitated diffusion of organic anions along the electrochemical potential gradient. To test this hypothesis, further electrophysiological studies are needed.

The transepithelial transport of bumetanide in the kidney has been suggested to

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involve hOAT1 - hOAT3 localized on the basolateral side and hOAT4 localized on the apical side of the proximal tubules (Hasannejad et al., 2004; Kobayashi et al., 2005). Since *MCT6* mRNA is expressed predominantly in the kidney (Price et al., 1998), *MCT6* may be involved in the renal secretion or reabsorption of bumetanide. The  $K_t$  values of bumetanide for hOAT2, hOAT3 and hOAT4 were 7.52, 1.59 and 0.31  $\mu\text{M}$ , respectively (Hasannejad et al., 2004; Kobayashi et al., 2005), but the affinity of bumetanide for *MCT6* ( $K_t$  value at pH 7.4; 84  $\mu\text{M}$ ) was lower. The clinical plasma concentration and plasma protein binding of bumetanide were reported to be about 0.27  $\mu\text{M}$  and 95%, respectively (Davies et al., 1974; Pentikainen et al., 1977). Consequently, the estimated plasma unbound concentration of bumetanide is 0.014  $\mu\text{M}$ , which is lower than  $K_t$  values of *MCT6*, hOAT2, hOAT3 and hOAT4. Therefore, none of these transporters should be saturated, and they are all possibly involved in the renal secretion and/or reabsorption of bumetanide. The renal localization of *MCT6* and its quantitative contribution to the renal secretion and/or reabsorption of bumetanide needs further study.

Even under the condition of membrane depolarization, the uptake of bumetanide by *MCT6* was elevated by extracellular acidic pH. However, our results indicate that the pH gradient across the cell membrane is not the driving force of *MCT6*. The mRNA of *MCT6* is highly expressed in placenta and kidney. In kidney, the intracellular pH of tubular

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epithelial cells is lower than the pH of the extracellular fluid, and urine is acidic under normal conditions. In placenta, fetal blood is slightly more acidic (pH 7.3) than maternal blood (pH 7.4). In addition,  $\text{Na}^+/\text{H}^+$  exchangers on the luminal and basolateral membrane of tubular epithelium and brush-border membrane of trophoblast cells produce a microacidic environment. Although the physiological meaning of the pH-sensitivity of MCT6 activity is still unknown, MCT6 could be regulated by the acidic environment in these tissues.

In conclusion, we identified bumetanide for the first time as an MCT6 ligand. MCT6 transports bumetanide in a pH- and membrane potential-sensitive, but not proton gradient-dependent manner, and the substrate specificity of MCT6 is different from those of the other MCTs. It is probable that MCT6 is involved in the disposition of various drugs, including bumetanide. Bumetanide should be a good tool for investigating the functional properties of MCT6 and the differences of substrate recognition sites among MCT isoforms.

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### **Footnotes**

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## Figure legends

Figure 1. Time course (A), concentration dependency (B) and pH dependency (C) of the uptake of [<sup>3</sup>H]bumetanide by MCT6.

Uptake of 0.1 μM [<sup>3</sup>H]bumetanide into MCT6-expressing (closed circles; A and C) or uninjected control oocytes (open circles) was measured at room temperature and pH 7.4 (A,B) for 30 min (C). MCT6-mediated uptake (open squares) was calculated by subtracting the uptake amount of control oocytes from that of MCT6-expressing oocytes. Each point represents the mean ± SEM (n = 3-5, \*; p < 0.05 vs. uninjected). The solid line in (B) represented the fitting line obtained from the data fitted with equation 1 using nonlinear least-squares regression analysis.

Figure 2. Membrane potential-sensitive and pH-sensitive [<sup>3</sup>H]bumetanide uptake via MCT6.

(A) After preincubation in the standard uptake buffer (2.5 mM K<sup>+</sup>) or the high potassium uptake buffer (120 mM K<sup>+</sup>) containing 10 μM valinomycin at pH 7.4 for 30 min, uptake of [<sup>3</sup>H]bumetanide into MCT6-expressing or uninjected control oocytes for 30 min was measured in the same buffer. MCT6-mediated uptake is shown (mean ± SEM, three separate experiments, \*; p < 0.05 vs. control). (B) After preincubation in the high potassium uptake buffer (120 mM K<sup>+</sup>) containing 10 μM valinomycin at pH 7.4 for 30 min, uptake of [<sup>3</sup>H]bumetanide into MCT6-expressing or uninjected control oocytes for 10 min was measured at pH 7.4, 6.0 or 5.5. MCT6-mediated uptake is shown (mean ± SEM, n=5). (C), (D) After preincubation, uptake of [<sup>14</sup>C]L-lactic acid by MCT1-expressing oocytes (C) and uptake of [<sup>3</sup>H]bumetanide by MCT6-expressing oocytes was measured under each condition as follows; open triangles, preincubation and uptake buffers were the high potassium buffer containing 10 μM valinomycin at pH 7.4 ([K<sup>+</sup>]<sub>in</sub> = [K<sup>+</sup>]<sub>out</sub>, pHi ≈ pHo); closed triangles, preincubation and

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uptake buffers were the high potassium buffer containing 10  $\mu\text{M}$  valinomycin and 20  $\mu\text{M}$  nigericin at pH 7.4 ( $[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$ ,  $\text{pHi} = \text{pHo}$ ); open circles, After preincubation in the high potassium buffer containing 10  $\mu\text{M}$  valinomycin at pH 7.4, uptake was measured in the high potassium buffer containing 10  $\mu\text{M}$  valinomycin at pH 6.0 ( $[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$ ,  $\text{pHi} > \text{pHo}$ ); closed circles, preincubation and uptake buffers were the high potassium buffer containing 10  $\mu\text{M}$  valinomycin and 20  $\mu\text{M}$  nigericin at pH 6.0 ( $[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$ ,  $\text{pHi} = \text{pHo}$ ). MCT1- or MCT6-mediated uptake is shown (mean  $\pm$  SEM,  $n=5$ , \*;  $p < 0.05$  vs. the value in the absence of nigericin).

Figure 3. Inhibitory effects of loop diuretics on the uptake of [ $^3\text{H}$ ]bumetanide by MCT6.

MCT6-expressing and control oocytes were incubated with uptake buffer containing 0.1-0.2  $\mu\text{M}$  [ $^3\text{H}$ ]bumetanide and the indicated concentrations of inhibitors at room temperature, pH 7.4, for 30 min. MCT6-mediated uptake was calculated by subtracting the uptake amount of uninjected control oocytes from that of MCT6-expressing oocytes (mean  $\pm$  SEM,  $n = 3-5$ ). Equation 2 was fitted to the data using nonlinear least-squares regression analysis (estimate  $\pm$  SE), and the result is represented by the solid line.

**Table 1. Uptake of various compounds by MCT6**

	Concentration ( $\mu\text{M}$ )	Uptake ( $\mu\text{L}/\text{oocyte}$ ) at pH7.4		Uptake ( $\mu\text{L}/\text{oocyte}$ ) at pH6.0	
		Uninjected	MCT6-expressing	Uninjected	MCT6-expressing
[ $^3\text{H}$ ]Bumetanide	0.1	$0.04 \pm 0.02$	$0.77 \pm 0.02$ *	$0.11 \pm 0.01$	$0.83 \pm 0.09$ *
[ $^3\text{H}$ ]Estrone-3-sulfate	0.01	$0.07 \pm 0.03$	$0.08 \pm 0.001$	$0.13 \pm 0.01$	$0.17 \pm 0.02$
[ $^{14}\text{C}$ ] <i>p</i> -Aminohippuric acid	5	$0.10 \pm 0.03$	$0.10 \pm 0.006$	$0.09 \pm 0.01$	$0.12 \pm 0.01$
[ $^3\text{H}$ ]Probenecid	0.5	$0.53 \pm 0.01$	$0.76 \pm 0.06$ *	$2.44 \pm 0.22$	$1.88 \pm 0.12$ *
[ $^3\text{H}$ ]Prostaglandin $\text{F}_{2\alpha}$	0.001	$0.26 \pm 0.04$	$0.39 \pm 0.02$ *	$0.69 \pm 0.05$	$1.21 \pm 0.07$ *
[ $^3\text{H}$ ]Valproic acid	0.01	$0.62 \pm 0.10$	$0.58 \pm 0.93$	$6.53 \pm 0.17$	$6.43 \pm 0.18$
[ $^{14}\text{C}$ ]Salicylic acid	5	$0.34 \pm 0.03$	$0.32 \pm 0.01$	$2.34 \pm 0.25$	$2.47 \pm 0.19$
[ $^3\text{H}$ ]Hexanoic acid	0.03	$2.20 \pm 0.17$	$2.25 \pm 0.11$	$5.77 \pm 0.69$	$4.97 \pm 0.27$
[ $^{14}\text{C}$ ]L-Leucine	4	$1.67 \pm 0.27$	$2.08 \pm 0.10$	$3.60 \pm 0.55$	$3.67 \pm 0.16$



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[ <sup>14</sup> C]Nateglinide	57	0.20 ± 0.01	0.41 ± 0.05 *	2.12 ± 0.08	2.54 ± 0.05 *
[ <sup>14</sup> C]Nateglinide	8	0.20 ± 0.04	0.90 ± 0.07 *,a	ND	ND

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Uptake of each compound in MCT6-expressing oocytes or uninjected control oocytes was measured at room temperature and pH 7.4 or pH 6.0, for 30 min. Each value represents the mean ± SEM of 3-5 separate experiments. Significance of differences between the uptake in uninjected oocytes and MCT6-expressing oocytes was determined by the unpaired Student's *t*-test (\*; *p* < 0.05).

<sup>a</sup> Uptake was measured for 60 min. ND: not determined.

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Table 2. Effects of extracellular Na<sup>+</sup>, and K<sup>+</sup>, and Cl<sup>-</sup> on the uptake of [<sup>3</sup>H]bumetanide by MCT6

	Uptake		
	(% of control)		
NaCl (control)	100		
<b>Na<sup>+</sup> free</b>			
N-Methyl-D-glucamine	97.6	±	7.5
<b>Na<sup>+</sup> and Cl<sup>-</sup> free</b>			
Mannitol	128.9	±	19.2
<b>Cl<sup>-</sup> free</b>			
Gluconate	130.2	±	16.7

The uptake of [<sup>3</sup>H]bumetanide into MCT6-expressing oocytes or uninjected control oocytes was performed at room temperature and pH 7.4 for 30 min in the standard uptake buffer (NaCl) or Na<sup>+</sup> and/or Cl<sup>-</sup> free buffer. Each value represents the mean ± SEM of three separate experiments.

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**Table 3. Inhibitory effects of anions and cations on the uptake of [<sup>3</sup>H]bumetanide by**

**MCT6**

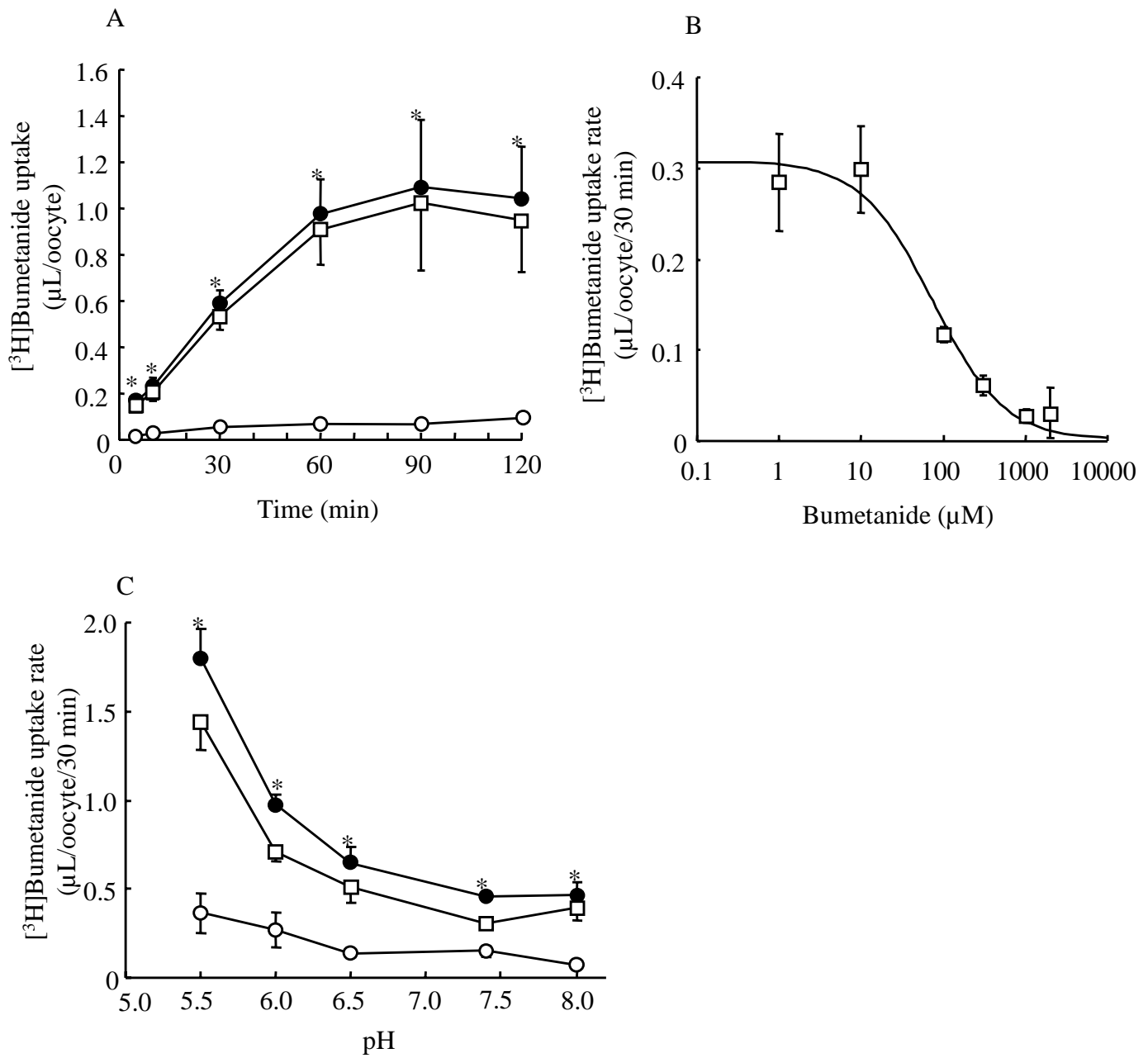
inhibitor	Concentration (mM)	MCT6-specific uptake (% of control )	
		pH 7.4	pH 6.0
L-Lactic acid	5	123.8 ± 11.4	76.8 ± 11.7
Pyruvic acid	5	95.1 ± 12.4	75.7 ± 5.6
Succinic acid	5	101.8 ± 14.2	73.0 ± 7.3
Citric acid	5	128.2 ± 8.9	99.2 ± 0.5
Choline	5	126.4 ± 14.1	76.3 ± 6.2
Cimetidine	5	40.4 ± 2.6 *	58.1 ± 7.9 *
<i>p</i> -Aminohippuric acid	5	30.9 ± 7.7 *	83.9 ± 1.9
Estrone-3-sulfate	5	11.6 ± 4.0 *	21.3 ± 8.1 *
Chlorothiazide	0.5	52.0 ± 6.7 *	77.5 ± 9.7
Hydrochlorothiazide	0.5	71.0 ± 5.1 *	68.6 ± 5.1 *
Trichlormethiazide	0.5	20.7 ± 2.6 *	64.2 ± 5.5 *
Probenecid	0.5	46.3 ± 2.4 *	82.6 ± 14.2
Glibenclamide	0.1	31.3 ± 4.3 *	56.3 ± 9.4 *
Nateglinide	0.001	40.8 ± 2.5 *	47.1 ± 10.3 *

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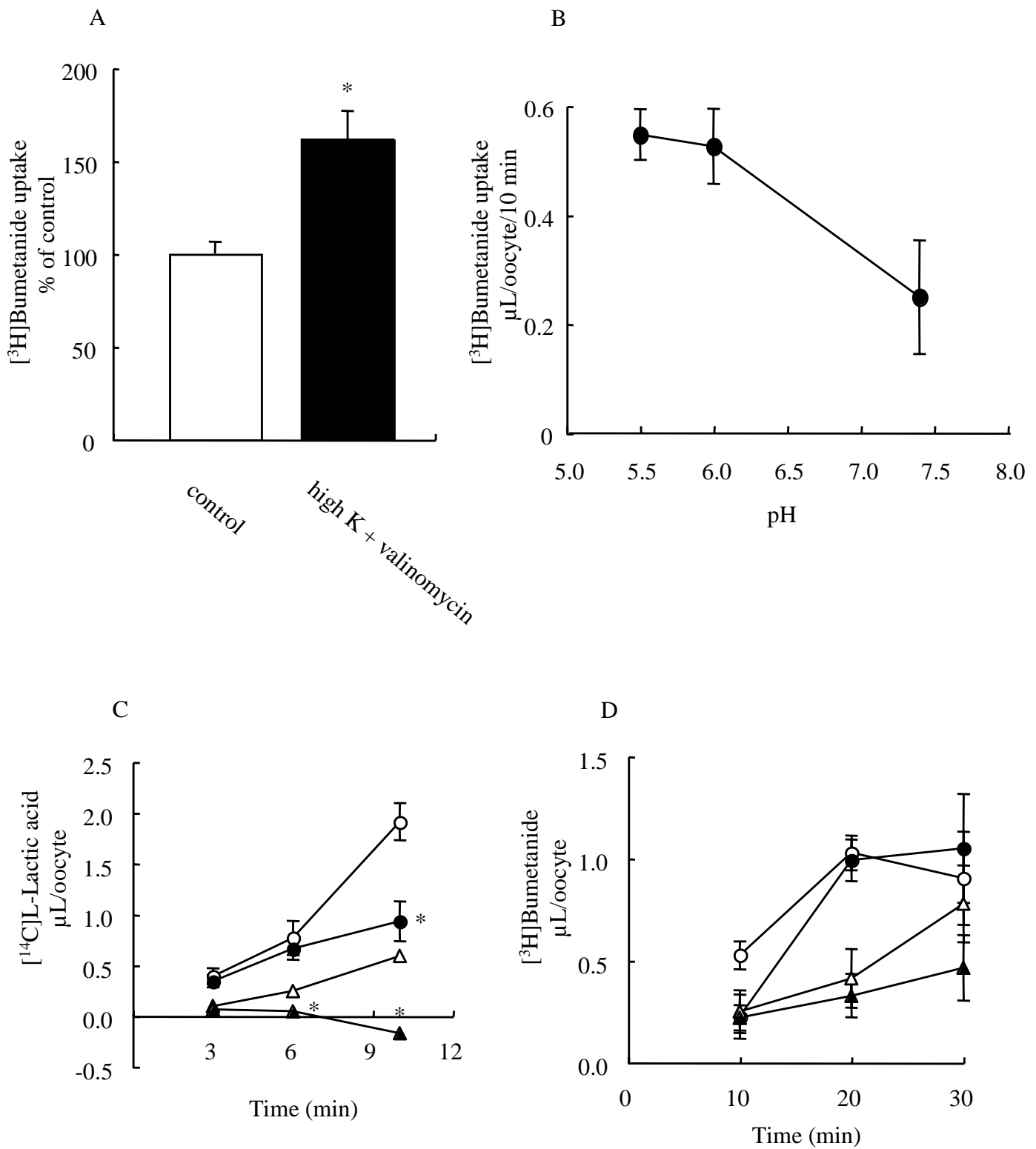
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Uptake rate of 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]bumetanide in MCT6-expressing oocytes or uninjected oocytes was measured at room temperature and pH 7.4 or pH 6.0 for 30 min in the presence or absence of inhibitor. MCT6-specific uptake was obtained by subtracting the uptake amount of uninjected oocytes from that of MCT6-expressing oocytes. The results are shown as percentages of control uptake measured in the absence of inhibitor. Each value represents the mean  $\pm$  SEM (n = 4-10, \* $p$  < 0.05 vs control).

**Figure 1**



**Figure 2**



**Figure 3**

