Modulation of Monocarboxylic Acid Transporter-1 Kinetic Function by the cAMP Signaling Pathway in Rat Brain Endothelial Cells*

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MCT1 (monocarboxylic acid transporter 1) facilitates bidirectional monocarboxylic acid transport across membranes. MCT1 function and regulation have not been characterized previously in cerebral endothelial cells but may be important during normal cerebral energy metabolism and during brain diseases such as stroke. Here, by using the cytoplasmic pH indicator 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxyethyl ester, the initial rates of monocarboxylate-dependent cytoplasmic acidification were measured as an indication of MCT1 kinetic function in vitro using the rat brain endothelial cell (RBE4) model of blood-brain transport. The initial rate of L-lactate-dependent acidification was significantly inhibited by 5–10-min incubations with agonists of intracellular cAMP-dependent cell signaling pathways as follows: dibutyryl cAMP, forskolin, and isoproterenol. Isoproterenol reduced \( V_{\text{max}} \) but did not affect \( K_m \) values. The effects of forskolin were completely reversed by the protein kinase A inhibitor H89, whereas H89 alone increased transport rates. Cytoplasmic cAMP levels, measured by radioimmunoassay, were increased by forskolin or isoproterenol, and the effect of isoproterenol was inhibited by propranolol. MCT1-independent intracellular pH control mechanisms did not contribute to the forskolin or H89 effects on MCT1 kinetic function as determined with amiloride, monocarboxylate-independent acid loading, or the transport inhibitor \( \alpha \)-cyano-4-hydroxycinnamate. The data demonstrate the direct modulation of MCT1 kinetic function in cerebral endothelial cells by agents known to affect the \( \beta \)-adrenergic receptor/adenylyl cyclase/cAMP/protein kinase A intracellular signaling pathway.

MCT1 (monocarboxylic acid transporter 1) facilitates the diffusion of monocarboxylic acids such as L-lactic acid, pyruvic acid, and keto acids across biological membranes. It is an obligatory symporter that carries a dissociated proton monocarboxylate pair with each transport cycle (for review see Refs. 1–3). Based on sequence homology, MCT1 has been characterized as a member of the monocarboxylic acid transporter family of solute carriers (SLC16 family (4)) that includes up to 14 subtypes (MCT1–14), although the monocarboxylate substrate specificity has been demonstrated for only 4 (5). MCT1 is the prominent monocarboxylic acid transporter in the cerebral microvascular endothelium, where it is present on the luminal and abluminal membranes of vascular endothelial cells (6, 7). Here MCT1 is proposed to be the major regulator of bidirectional monocarboxylic acid transport between the brain and the blood, and may have pharmacologic importance for treating brain diseases such as stroke (2, 8).

The vast majority of strokes are associated with severe cerebral lactic acidosis, which is a key factor leading to permanent brain cell damage (for review see Ref. 9). Decreased activity of MCT1 in the cerebral microvasculature may be a critical determinant of acid-related cell damage during stroke, and this is evident because lactic acid is not cleared from brain during cerebral ischemia (10). Similarly, overexpression of MCT1 mRNA in brain is correlated with a reduction in cell damage following experimental transient focal cerebral ischemia in rodents. Therefore, factors that regulate the kinetic function of MCT1 in the cerebral microvascular endothelium may be important in mediating the extent of lactic acidosis and therefore brain damage during stroke.

The regulation of MCT1 function is suggested by its shared structural similarity with other transporters that are kinetically regulated by specific intracellular signaling mechanisms. These include sodium/hydrogen exchangers (11, 12), sodium/calcium exchangers (13), and glucose transporters (14, 15). Regulation of MCT1 by phosphorylation is suggested by the presence of sequence motifs that have a high phosphorylation potential in its intracellular protein domain. Recently, hormone-induced translocation of MCT1 from cytoplasm to plasma membrane has been shown to regulate MCT1-dependent butyrate uptake in a cell line from human intestine, suggesting the possibility of such a regulatory mechanism for cerebral vascular MCT1 (16). Although the Michaelis-Menten kinetics and inhibition of MCT1 have been clearly defined by a number of studies in other cells (17–19), neither its basic kinetics nor its functional regulation in cerebral microvascular cells has been investigated.

Here we report the modulation of MCT1 kinetic function in the RBE4 cell model of rat cerebral endothelial cells by agents known to affect the \( \beta \)-adrenergic/adenylyl cyclase/protein kinase A-intracellular signal transduction pathway. The elucidation of this regulation improves our understanding of the roles that monocarboxylate and MCT1 have in normal brain energy metabolism. It also suggests the pharmacological modulation of MCT1 kinetic function as a novel target for drug-based therapies to reduce the damaging effects of lactic acidosis during cerebral ischemia and stroke.

EXPERIMENTAL PROCEDURES

RBE4 Cell Culture—Immortalized rat brain endothelial cells (RBE4), a gift from F. Roux (20), were cultured in equal parts minimum essential medium \( \alpha \) and F-10 Nutrient (Ham’s), supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic (Invitrogen), 0.3 mg/ml genetocic (Sigma), and 1.0 ng/ml basic fibroblast growth factor (Amersham

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**Modulation of MCT1 Function**

**TABLE 1**

| Transporter | Primer 1 | Primer 2 |
|-------------|----------|----------|
| MCT1        | Forward, 5'-ATG TAT GCC GGA GGT CCT ACT-3' | Reverse, 5'-CCA ATG GTC GCT TCT TGT AGA-3' |
| MCT2        | Forward, 5'-CTG GTC ATG TAC CGA GGA-3' | Reverse, 5'-AAG CCG AGG GTG AGG TAA AGT-3' |
| MCT4        | Forward, 5'-GCA TTC TCT TTG GTA AGA CGT-3' | Reverse, 5'-GTG AAT TTT TTG CTC GTG CAG-3' |
| MCT7        | Forward, 5'-AAG GCC CAG CAG CGT GTA ACA-3' | Reverse, 5'-GAG GCC CAG CGT GTA ACA-3' |
| MCT8        | Forward, 5'-ATC TGG CCA TTC AGG TGA CAC-3' | Reverse, 5'-CTT AGG CAG CTG CCT ACT-3' |

Biosciences). Cells were cultured directly on 75-mm² polystyrene tissue culture flasks (Falcon) or 25-mm round coverglass and passaged less than 12 times before use. All imaging experiments were conducted before cells reached confluence.

**Gene Expression by Real Time PCR**— Primer pairs for real time PCR were designed using Oligo 6.4 software (Molecular Biology Insights, Cascade, CO) (Table 1). Total RNA was extracted from RBE4 cells using an RNeasy mini kit (Qiagen). CDNA was reverse-transcribed from total RNA using an Omniscript™ kit (Qiagen) with oligo(dT) and random hexamer primers. Reactions for quantitative reverse transcriptase PCR were performed on a LightCycler™ instrument using the LightCycler™ DNA Master SYBR® Green I kit (Roche Applied Science). Before quantitative measurements of cDNA were performed, PCR conditions for each primer set were optimized, and a melting curve analysis was used to confirm that each primer pair produced a single product. Standard curves for candidate cDNAs were prepared from a series of five 10-fold serial dilutions of target cDNA. The quantity of DNA in each sample was normalized to 18 S rRNA or glyceraldehyde phosphate dehydrogenase.

**Immunocytochemical Analysis of MCT1 Protein Expression**—A polyclonal antibody was raised against a peptide coding the 15 carboxyl-terminal amino acids of rat monocarboxylic acid transporter 1 (MCT1, LQNSGPDPEEESV), isolated from chicken egg yolks, affinity-purified, and used in immunocytochemical studies (6). The antibody recognizes a protein migrating with an apparent molecular mass of 46–50 kDa on immunoblots of brain membrane proteins from adult and suckling rats (data not shown) (6). The avidin-biotin-peroxidase complex immunocytochemistry elite kit (Vector Laboratories, Burlingame, CA) was employed with color development for 1–4 min in 3,3′-diaminobenzidine (Dako, Carpinteria, CA). To provide controls for nonspecific staining, the antigenic peptide was added to the primary antibody at 20 μg/ml before use.

**Cell Loading with pH Indicator Dye**—RBE4 cells, growing on glass, were incubated for 10–15 min at room temperature with 3.5 μM 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM) (Molecular Probes, Inc., Eugene, OR) in HEPES-buffered saline (HBS). HBS consisted of the following (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 20 HEPES, 5 sucrose, pH 7.35 ± 0.05 (KOH). Coverslips were transferred to fresh HBS and incubated for 30 min at room temperature in order to allow maximal de-esterification of the indicator and stabilization of the cytoplasmic H⁺ concentration ([H⁺]). Incubation was continued on ice for a minimum of 15 min prior to imaging.

**Determination of pH-dependent Fluorescence**—BCECF fluorescence was determined using an inverted Nikon Eclipse TE300 fluorescence microscope with a 10× objective, a 490 ± 20-nm excitation filter combined with a 1.0 neutral density filter, a 440 ± 20-nm excitation filter, and a 535 ± 25-nm emission filter cube (Omega Optical, Inc., Brattleboro, VT). A Sutter 10-position filter wheel with a LUDL controller was used to control the excitation filtering. Fluorescence images were obtained using a SIT68 video camera (Dage-MTI, Inc.) and computerized using a MuTech MV1000 frame grabber and MetaFluor Imaging System software, version 4.6 (Universal Imaging Corp., West Chester, PA). Cells were excited for 100 ms at each wavelength, with a sample rate of ~1 image pair per 1.8 s. Background fluorescence was subtracted from a cell-free region of each image during acquisition. The image pairs were stored digitally for off-line analysis using MetaFluor Imaging System software (above) and Microlab™ Origin™ graphical analysis software (Microlab Software, Inc., Northampton, MA).

**Calibration of BCECF Fluorescence to pH**—Ratiometric images were calibrated to indicate the approximate pH, from image regions corresponding to individual cells by using the method of Thomas et al. (21). Briefly, cells were perfused with HBS substituted with 135 mM KCl and 5 mM NaCl, containing the K⁺/H⁺-exchange ionophore nigericin (10–20 μM), in solutions of different pH values (adjusted with NaOH). Nigericin equilibrates the intracellular and extracellular pH in the presence of high K⁺ (21). At the end of each experiment, the fluorescence ratio from nigericin-treated cells was determined at eight pH values ranging from pH 6.2 to 8.2. The calibration data were fitted to a sigmoidal dose-response curve using Microcal Origin™ software to determine the maximum (Rₘₐₓ) and minimum (Rₘᵦₙ) fluorescence ratios and the pKₐ of the dye in each cell. The 495/440 excitation ratio data (R) was transformed to determine the approximate pHᵢ, at each time point and for each individual cell in all experiments, using the following equation: 

\[ \text{pHᵢ} = \text{pK} + \log((R-Rₘᵦₙ)/(Rₘₐₓ-R)) \]

As an example, the sigmoidal fit for the average R versus extracellular pH in nigericin from 20 cells (±S.E.) is illustrated; however, in practice these data were determined for each individual cell (Fig. 3A).

**Initial Rates of Acidification**—All experiments were conducted at room temperature. Media were exchanged within 2 s by aspirating the imaging chamber (Warner Instruments RC-218BRW) and flushing it with 10–20 chamber volumes (5 ml) of fresh medium. The initial rates of cytoplasmic acidification (vᵢ) were determined using Microcal Origin™ software by line fitting the first four or more points of pHᵢ time data occurring in the earliest, most linear region of a response to bath-applied substrate and were expressed as pH units/s (Fig. 3B). Michaelis-Menten kinetic parameters (Vₘₐₓ and Kₘ) were determined by plotting the extracellular substrate concentration ([S]) versus vᵢ, and fitting the data to the following Michaelis-Menten equation: 

\[ vᵢ = (Vₘₐₓ[S])/(Kₘ + [S]) \]

using Microcal Origin™ software.

**Cytoplasmic cAMP Concentrations**— Cytoplasmic cAMP levels were measured by radioimmunoassay. Briefly, subconfluent RBE4 cells growing in 35-mm culture dishes (Corning) were treated at room temperature with drugs in HBS for 5–10 min in the presence or absence of the phosphodiesterase inhibitor, 3-isobutyl 1-methylxanthine (25 μM). The media were aspirated and replaced with 1 ml of absolute ethanol, and the cells were scraped, sonicated, and pelleted, and the level of cAMP in the supernatant was measured using a TRK-432 kit (Amersham Biosciences) following the manufacturers’ recommended protocol. cAMP levels were expressed as picomoles of cAMP per 35-mm culture dish.
Modulation of MCT1 Function

To measure the function of MCT1, we used ratiometric fluorescence video microscopy of cells loaded with the pH indicator BCECF-AM, and we measured the responses to extracellularly applied monocarboxylate substrates of MCT1. All experimentation was conducted in bicarbonate-free buffer to minimize the activity of bicarbonate-dependent pH regulatory mechanisms. This method has been shown previously to give very reliable estimates of MCT1 kinetic parameters and is comparable in efficacy to methods using intracellular pH-sensitive electrodes and uptake of L-[14C]lactate (17–19, 24).

BCECF-AM-loaded RBE4 cells showed cytoplasmic fluorescence that was even and bright. Fluorescence ratios of BCECF-loaded cells, excited at 495/440 nm, were calibrated to indicate the approximate pH, using the method of Thomas et al. (21). In these experiments, the data exhibited minimal variation and were tightly fit by the theoretical parameters used in the calibration, further indicating the high efficacy of this method (Fig. 3) (21). Resting pH was stable for periods longer than ½ h, whereas experiments were typically completed within 10 min. pH,

Drugs and Solutions—All drugs, unless otherwise noted, were purchased from Sigma. Drugs were dissolved in ethanol, dimethyl sulfoxide, HBS, or water as recommended by the manufacturer and diluted to their final concentrations in HBS, and the pH was adjusted if necessary (KOH). In no case did any vehicle at its final concentration cause measurable effects on RBE4 cell pH (data not shown). The [Cl] and osmolarity of media containing transporter substrates were kept constant by substituting the substrates for an equivalent portion of NaCl.

Statistics—All statistics and curve fitting were performed using Microcal Origin software and applied as indicated in the figure legends or text.

RESULTS

Functional MCT1 Is Expressed in RBE4 Cells—Strong immunoreactivity against MCT1 protein was present in RBE4 cell cultures using a polyclonal antibody that is specific for MCT1 (6). Staining was evident throughout the plasma membrane of all cells and was completely inhibited by the antigenic peptide, confirming that the RBE4 cells expressed robust levels of MCT1 protein (Fig. 1). mRNA for MCT1 was the predominant monocarboxylate transporter transcript expressed in these cells, as shown by quantitative RT-PCR analysis (Fig. 2). The MCT2 RT-PCR product was minimally detected at less than 15% of the MCT1 product. It was not surprising that MCT2 mRNA would be low in these cells because MCT2 protein is not detectable in the rat cerebral endothelium from which RBE4 cells are derived (22, 23). MCT1 is, however, the only detectable monocarboxylate transporter in rat brain endothelium in situ (6). These data suggested that MCT2 is relatively insignificant in RBE4 cells and was further supported by our kinetic analysis (see below). MCT4, -7, and -8 PCR product levels were very low in RBE4 cells, between 0.16 and 4% of the MCT1 product (Fig. 2). The combined data suggested that RBE4 cells would be an excellent model for the study of MCT1 protein function.

To determine the function of MCT1, we used ratiometric fluorescence video microscopy of cells loaded with the pH indicator BCECF-AM, and we measured the responses to extracellularly applied monocarboxylate substrates of MCT1. All experimentation was conducted in bicarbonate-free buffer to minimize the activity of bicarbonate-dependent pH regulatory mechanisms. This method has been shown previously to give very reliable estimates of MCT1 kinetic parameters and is comparable in efficacy to methods using intracellular pH-sensitive electrodes and uptake of L-[14C]lactate (17–19, 24).

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was unaffected by repetitive exchanges of the bath with fresh HBS. Rapid exchange of the bathing medium with HBS containing 20 mM L-lactate induced an immediate acidification of the cytoplasm of all cells that was completely reversed upon reperfusion with lactate-free HBS (Fig. 3B). As a measure of MCT1 kinetic function, the initial rate of \( v_i \) was determined by linear regression of the earliest, most linear portion of this response in each cell and was expressed in pH units/s (Fig. 3B).

Plots of the average \( v_i \) measured in groups of 19–40 cells, ± S.E., were determined at different concentrations of MCT1 substrates and fit to the Michaelis-Menten equation (Fig. 4). A summary of kinetic data (Table 2) indicates that RBE4 cells responded to the MCT1 substrates, L-lactate, D-3-hydroxybutyrate, and pyruvate very consistently with previously published kinetic studies that characterized the Michaelis-Menton parameters for MCT1 function using both BCECF and pH-sensitive intracellular electrodes (17–19). These data supported our RT-PCR data suggesting that MCT2 would not be significant in RBE4 cells, because \( K_m \) values for MCT2, as determined previously with these substrates, are only between 8 and 21% of \( K_m \) values for MCT1 (25).

FIGURE 4. Michaelis-Menten plots from RBE4 cell responses to L-lactate (squares), pyruvate (open circles), and D-3-hydroxybutyrate (triangles). Each data point shows the average \( v_i/(pH/h) \) ± S.E. (n = 19–40 cells per data point). Each data set was fit to the Michaelis-Menten equation (curved lines) to estimate \( K_m \) and \( V_{\text{max}} \). For L-lactate \( K_m = 3.99 \) mM and \( V_{\text{max}} = 0.0226 \) pH/s. For pyruvate \( K_m = 2.02 \) mM and \( V_{\text{max}} = 0.0143 \) pH/s. For D-3-hydroxybutyrate, \( K_m = 11.98 \) mM and \( V_{\text{max}} = 0.0218 \) pH/s. In the presence of 5 mM CHC, the maximum rate of acidification was 0.00173 pH/s (polygon) and had a linear \( v_i \) dependence when tested at multiple concentrations of L-lactate (not shown).

### TABLE 2

| MCT1 Kinetic Parameters for MCT1 in RBE4 and Other Cell Types |
|-------------------------------|-------------------|-------------------|-------------------|
| Cell type                     | \( K_m (\text{mM}) \) | \( V_{\text{max}} \) % L-lactate | \( K_m (\text{mM}) \) | \( V_{\text{max}} \) % L-lactate | \( K_m (\text{mM}) \) | \( V_{\text{max}} \) % L-lactate |
|--------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| RBE4 cell                      | 4.0               | 100               | 2.0               | 63               | 12.0              | 96               |
| Ehrlich-Lettre tumor cell (17) | 4.5               | 100               | 0.7               | 64               | 10.1              | 105              |
| MCT1/oocyte (18)               | 3.5               | 100               | 1.0               | 45               | 12.5              | 109              |
| Primary rat hepatocytes (19)   | 4.7               | 100               | 1.3               | 63               | 24.7              | 94               |

Because PKA activation is downstream of cell surface receptor-mediated mechanisms (26, 27), and because cerebral endothelium is known to express \( \beta \)-adrenergic receptors that activate PKA, we tested the hypothesis that elevated cytoplasmic concentrations of cAMP might lead to a modulation of MCT1 kinetic function, we incubated RBE4 cells in the membrane-permeant cAMP analog Br\(_2\)cAMP or the adenylyl cyclase activator forskolin for 5–10 min prior to measuring L-lactate-dependent \( v_i \) values. Neither drug caused a perceptible change in the resting \( \mathrm{pH} \) during the preincubation (not shown); however, the initial rate of acidification with 25 mM L-lactate was significantly reduced by both drugs (Fig. 5A). This result was repeated on more than four separate occasions.

A role for cAMP-dependent protein kinase A (PKA) in slowing lactate-dependent \( v_i \) was suggested because the PKA activator, Br\(_2\)cAMP, inhibited MCT1 function. To determine whether the forskolin-dependent reduction in lactate-dependent \( v_i \) was mediated by PKA, we pre-treated cells with the specific PKA antagonist 20 \( \mu \)M H89, with and without forskolin, for 5 min before measuring responses to 25 mM L-lactate. H89 did not cause a perceptible change in the basal \( \mathrm{pH} \) during the preincubation (not shown). However, inclusion of H89 in the forskolin preincubation completely reversed the effects of forskolin (Fig. 5B). Interestingly, H89 alone caused a significant increase in lactate-dependent \( v_i \), suggesting that PKA may be exerting a tonic slowing effect on MCT1 function under control conditions (Fig. 5B). The above results were repeated on at least three separate occasions and suggest a role for PKA in modulating MCT1 kinetic function in RBE4 cells.

Because PKA activation is downstream of cell surface receptor-mediated mechanisms (26, 27), and because cerebral endothelium is known to express \( \beta \)-adrenergic receptors that activate PKA, we tested the hypothesis that a \( \beta \)-adrenergic receptor agonist would lead to a reduction in the lactate-dependent \( v_i \). Isoproterenol caused a dose-dependent slowing of \( v_i \) consistent with an \( \beta \)-adrenergic receptor-mediated process having an IC\(_{50}\) near 4 \( \mu \)M when tested with 25 mM L-lactate (Fig. 6A). The transport-slowing effect of 100 \( \mu \)M isoproterenol was similar in magnitude to that observed with Br\(_2\)cAMP and forskolin (Figs. 5 and 6). Inclusion of the \( \beta \)-adrenergic receptor antagonist, propranolol (10 mM), in the preincubation with isoproterenol, led to a statistically significant reversal of the isoproterenol-dependent reduction in \( v_i \).
reversal of inhibition was not complete (data not shown). Isoproterenol or propranolol alone did not cause a perceptible change in the basal pH during the preincubation (not shown). Comparison of Michaelis-Menten plots constructed at varying L-lactate concentrations in the presence and absence of 100 μM isoproterenol showed that drug treatment caused a 14% reduction in $V_{\text{max}}$ but only a 2% change in $K_{\text{m}}$ (Fig. 6B). The combined data demonstrated that MCT1 kinetic function in RBE4 cells is modulated by agents known to affect the β-adrenergic receptor/adenyl cyclase/cAMP/PKA intracellular signal transduction pathway.

Forskolin and Isoproterenol Pretreatment Increased cAMP Levels in RBE4 Cells—Cytoplasmic cAMP levels were measured by radiomimunoassay in identical cultures of RBE4 cells. cAMP was not detectable in control cultures but increased when cells were incubated for 5 min in the phosphodiesterase inhibitor 3-isobutyl 1-methylxanthine (IBMX) (Table 3).

![Figure 5](image)

**FIGURE 5.** Forskolin and Bt2cAMP reduce $v_i$ in RBE4 cells responding to 25 mM L-lactate, and H89 increases $v_i$. A, the mean $v_i$ was significantly reduced by 5–10-min pretreatments with 100 μM forskolin or 500 μM Bt2cAMP (Bt2cAMP). B, in a separate set of experiments, $v_i$ was similarly reduced by 100 μM forskolin; however, this effect was reversed by including 20 μM H89 in the preincubation medium. 20 μM H89 alone caused a significant increase in $v_i$; was normalized to the mean $v_i$ determined with control cells for each set of experiments. $n$ = 20 cells for all data points. *p < 0.05. Statistical significance was determined by comparing control to drug-treated groups with a paired Student’s t test, $p = 0.005$.

![Figure 6](image)

**FIGURE 6.** Isoproterenol reduces $v_i$ and affects $V_{\text{max}}$ but not $K_{\text{m}}$. A, log(isoproterenol) (μM, 10 min pretreatment) versus the 25 mM L-lactate-dependent $v_i$, normalized to the maximal $v_i$ determined in the absence of isoproterenol. The curve shows a sigmoidal dose-response fit of the data giving an IC₅₀ of 4.01 μM isoproterenol and a maximum inhibition of about 52%. B, Michaelis-Menten plots in the absence (squares) and presence (circles) of a 10-min pretreatment with 100 μM isoproterenol. $K_{\text{m}}$ and $V_{\text{max}}$ for controls were 4.4 mM L-lactate and 0.029 pH/s respectively, and 4.32 mM and 0.025 pH/s for isoproterenol-pretreated cells. $n$ = 20–40 cells for all data points. *p < 0.05. Statistical significance was determined by comparing control to drug-treated groups with a paired Student’s t test, $p = 0.005$.

![Figure 7](image)

**FIGURE 7.** 10-min pretreatment with 100 μM amiloride did not affect the Bt2cAMP or forskolin-induced inhibition of the 25 mM L-lactate-dependent $v_i$ in RBE4 cells. Amiloride alone did not affect $v_i$. Ten-minute preincubation with 500 μM Bt2cAMP (Bt2cAMP) or 100 μM forskolin alone caused a significant inhibition of $v_i$ that was not significantly changed when amiloride was included in the preincubation mixture. For each bar, $v_i$ was normalized to the control rate determined in the absence of drugs. $n$ = 20 cells for all data points. *p < 0.05. Statistical significance was determined by comparing control to drug-treated groups with a paired Student’s t test, $p = 0.005$.

The data demonstrated that cAMP was produced in RBE4 cells under resting conditions. Five- to 10-min pretreatments in 100 μM isoproterenol or 100 μM forskolin in 3-isobutyl 1-methylxanthine caused cAMP levels to become elevated. This indicated that both agents, at the concentrations used in the fluorescence imaging studies, led to enhanced production of cAMP in the RBE4 cells. The rise in cAMP induced by isoproterenol was greatly reduced by the addition of 10 nM propranolol in the preincubation (Table 3). The combined data showed that forskolin and isoproterenol-mediated slowing of lactate-dependent $v_i$ were associated with elevated cytoplasmic cAMP in RBE4 cells.

**TABLE 3**

| Treatment | cAMP (pmol/35-mm culture well) |
|-----------|-------------------------------|
| None, 5 min | ND                       |
| 25 mM IBMX, 5 min | 16.4                   |
| 100 μM forskolin, 5 min | 68.4                   |
| 100 μM isoproterenol, 10 min | 128.3                  |
| 100 μM isoproterenol + 10 nM propranolol, 10 min | 40.2                   |

**Modulation of L-Lactate-dependent $v_i$ Not Because of MCT1-independent pH, Regulatory Mechanisms—**The cAMP signaling pathway is well characterized as a modulator of other pH regulatory proteins such as the Na⁺/H⁺ exchanger (11, 12). Therefore, we investigated the possibility that forskolin- and Bt2cAMP-mediated effects on $v_i$ might be due to enhanced proton efflux through Na⁺/H⁺ exchangers, rather than by slowed proton influx through MCT1. When tested with 25 mM L-lactate, neither the control $v_i$ nor the slowing of $v_i$ induced by 5–10-min preincubations in Bt2cAMP or forskolin were changed by 10-min preincubations with the well characterized Na⁺/H⁺ exchanger inhibitor 100 μM amiloride (Fig. 7). These data suggest that amiloride-sensitive proton transporters, such as the Na⁺/H⁺ exchanger, were not mediating the forskolin– or Bt2cAMP-dependent slowing of lactate-dependent $v_i$.  

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To examine further the potential role of MCT1 and lactate-independent pH regulatory processes in modulating lactate-dependent \( v_i \), the rates of intracellular alkalinization were measured in RBE4 cells following acid loading by the NH\(_4\)Cl pre-pulse method (29). NH\(_4\)\(^+\) caused a rapid and sustained elevation of pH, and subsequent perfusion with Na\(^+\)-free HBS led to hyperacidification of the cytoplasm. Following this acid loading, the pH recovered very slowly relative to \( v_i \) in Na\(^+\)-free HBS, indicating the presence of a slow Na\(^+\)-independent pH regulatory mechanism in the cells (Fig. 8A). During Na\(^+\) reperfusion, pH alkalinized slowly compared with \( v_i \), suggesting the presence of functional Na\(^+\)-dependent pH regulatory mechanisms, such as Na\(^+\)/H\(^+\) exchangers. However, because the fastest alkalinization rates were less than 10% of the lactate-dependent \( v_i \), lactate-independent pH regulatory mechanisms measured by this method could not have made a significant contribution to lactate-dependent \( v_i \) (Fig. 8A).

More importantly, we investigated whether the rates of these lactate-independent pH regulatory mechanisms were significantly affected by 10-min preincubations in either H89 or forskolin. Following NH\(_4\)Cl acid loading, the rates of intracellular alkalinization, measured in Na\(^+\)-free and Na\(^+\)-containing HBS, were not significantly affected by H89. Moreover, the rates of alkalinization decreased somewhat after pretreatment in forskolin (Fig. 8B). Because forskolin decreased the rate of proton efflux from acid-loaded cells, the effect of this drug on lactate-independent pH regulatory mechanisms could only have resulted in a small underestimation of \( v_i \) (approximately less than 2%). Thus, the effects of H89 and forskolin on lactate-independent pH regulatory mechanisms such as Na\(^+\)/H\(^+\) exchangers could not have been the underlying reason for the effects of these drugs in modulating MCT1 function.

To examine more directly the specificity of H89- and forskolin-mediated slowing of lactate-dependent \( v_i \), MCT1 function was inhibited by a 2-min pretreatment with 5 mM CHC (see above), and the effects of H89 and forskolin were measured. Lactate-dependent \( v_i \) was not significantly affected by either H89 or forskolin in the presence of CHC (Fig. 9). These data demonstrate that modulation of lactate-dependent \( v_i \) by H89 and forskolin, as shown in Fig. 5B, does not occur when MCT1 is inhibited (Fig. 9). Combined, the data in Figs. 7–9, strongly suggest that MCT1-independent pH regulatory mechanisms were not responsible for the modulation of lactate-dependent \( v_i \) observed in RBE4 cells.

DISCUSSION

The β-Adrenergic Receptor/cAMP/PKA Signaling Pathway-regulated MCT1 Kinetic Function in Rat Brain Endothelial Cells—The data presented here support the hypothesis that stimulation of β-adrenergic receptors causes an adenyl cyclase-mediated increase in intracellular cAMP levels and an associated PKA-dependent regulation of MCT1.
kinetic function in cerebral endothelial cells (Fig. 10). As discussed below, this finding contributes importantly to our understanding of the role that monocarboxylates and their transporters fulfill during brain homeostasis in both health and disease.

The β-adrenergic receptor-dependent inhibition of MCT1 kinetic function in RBE4 cells is consistent with a number of studies showing that isoproterenol (30) and cAMP have the general effect of reducing endothelial cell permeability (30–32). Most interesting is that this effect of cAMP is hypothesized to provide protection for brain cells from various forms of stress or disease, albeit by unknown mechanisms. Thus, the inhibition of MCT1 function in cerebral microvascular cells may be part of such neuroprotective mechanisms mediated at the blood-brain barrier (31).

Based on the evidence presented, the signaling pathway between the β-adrenergic receptor and PKA is relatively defined in the RBE4 model (Fig. 10). However, the pathway downstream of PKA that links protein phosphorylation to altered MCT1 activity remains to be elucidated. A reduction in the number of functional transporters and not a more subtle modification of the basic activity of the transporter is a likely mechanism for the kinetic modulation shown here, because the $V_{\text{max}}$, but not $K_{\text{m}}$, value for l-lactate transport was affected by isoproterenol (Fig. 6B). This finding compliments a recent study in a human intestinal cell line showing a hormone-mediated increase in the MCT1 protein through enhanced recruitment of the MCT1 protein to the plasma membrane (16). Whether this mechanism also applies to MCT1 in RBE4 cells or whether another mechanism such as direct phosphorylation of MCT1, modification of an accessory protein such as CD147, or other protein-protein interaction is involved will require further investigations. It is, however, unlikely that gene expression and de novo protein synthesis were important in these studies, because all drug treatments and pretreatments were limited to less than 10 min. Although a forskolin-dependent up-regulation of MCT1/CD147 mRNA and protein synthesis in thyroid cell lines has been reported (33), this required a 60–72-h time course and more likely reflects divergent effects of cAMP on gene expression as compared with other short term biochemical events (34, 35).

Other pH$_i$-regulatory Mechanisms Did Not Contribute to the Effects of Drugs in Slowing MCT1 Kinetics—If MCT1-mediated cytoplasmic acidification was countered by Na$^+$/H$^+$ exchanger-mediated proton efflux from RBE4 cells, then any drug-induced enhancement of the Na$^+$/H$^+$ exchanger function might have given the false impression of slowed MCT1 functional activity. Indeed, studies of the Na$^+$/H$^+$ exchanger NHE-1 showed that forskolin and cAMP can lead to increased Na$^+$/H$^+$ exchanger activity (12). Conversely, these same agents reportedly inhibit the NHE-3 isoform (11, 36). In the present studies, amiloride, a well characterized inhibitor of Na$^+$/H$^+$ exchangers (28), did not significantly affect the lactate-dependent $v_i$ nor its slowing by forskolin or Bt$_2$cAMP. Therefore, Na$^+$/H$^+$ exchangers are unlikely to have contributed significantly to the observed drug effects (Fig. 7).

The presence of additional non-MCT1-mediated pH$_i$ regulatory mechanisms was also evaluated under lactate-independent conditions using the NH$_4$Cl pre-pulse technique (Fig. 8) (29). These experiments revealed that the PKA inhibitor, H89, had a negligible effect on proton efflux from RBE4 cells following monocarboxylate-independent acid loading, whereas forskolin lowered the rates. Therefore, the effects of H89 and forskolin on lactate-independent pH$_i$ regulatory mechanisms could not have been the underlying reason for the reduced lactate-dependent $v_i$ observed with these agents (Figs. 5 and 8). This was further confirmed by the absence of an effect of forskolin or H89 on rates of lactate-dependent acidification in the presence of the MCT1 inhibitor CHC (Fig. 9). Therefore, the combined data (Figs. 7–9), and our use of bicarbonate-free buffer, point to a direct effect of the agents on MCT1 kinetic function, rather than acting through other secondary pH$_i$ regulatory proteins such as Na$^+$/H$^+$ exchangers.

The Importance of Modulation of Cerebral Endothelial MCT1 Kinetic Function in the Normal and Diseased Brain—Unlike monosaccharides, the monocarboxylates are components of every major metabolic pathway in mammalian cells and serve as either a substrate or product of energy metabolism. Movement of short chain monocarboxylates is greatly restricted unless facilitated by a membrane carrier. Therefore, it is not surprising that nearly every mammalian cell possesses membrane transporters for monocarboxylates (37). Clearly, the basic function of MCT1 in the cerebral microvascular endothelium is to facilitate blood-brain transport of monocarboxylic acids; however, the possible regulation of this transport function has not been examined previously at the molecular level in cells of the central nervous system. The dynamic control of MCT1 function in the cerebral microvascular endothelium would be very important in brain energy metabolism because it would regulate the transport of monocarboxylates into and out of the brain, controlling their availability as brain energy substrates. For example, activation of receptors on the cerebral vascular endothelial cells by elevation of stress-induced hormones may restrict MCT1 function. This would lead to increased brain retention of monocarboxylates that are normally exported and enhance energy production during times of brain stress or high metabolic demand by providing additional substrates for the mitochondrial citric acid cycle and oxidative phosphorylation. Therefore, it is hypothesized that the regulation of MCT1 kinetic function in the cerebral microvasculature is an important part of maintaining energy homeostasis for the healthy brain as it works to meet a dynamic energy demand.

During disease conditions such as stroke, MCT1 function in the cerebral microvascular endothelium may play an important role in determining the severity of tissue damage and extent of cell recovery. During ischemia or anoxia, lactic acid levels rise dramatically in the adult brain. Therefore, it is evident that the normal function of MCT1 to transport lactic acid down its concentration gradient is rate-limiting, and regulation of MCT1 activity has important implications and potential consequences. Pharmacological enhancement of MCT1 transport activity in the cerebral microvascular endothelium may be a therapy to reduce the severity of lactic acidosis, and its downstream and damaging effects in stroke patients. This hypothesis is supported by data showing that overexpression of MCT1 protein is neuroprotective under conditions of focal cerebral ischemia in rats.3

Our understanding of the importance of cerebral microvascular MCT1 is only beginning to emerge but has strong potential to help resolve many important questions, including the role of monocarboxylates, and their transporters, in brain energy metabolism, stroke, and other pathological processes. By demonstrating for the first time the regulation of MCT1 kinetic function in rat brain endothelial cells, we have set a precedent for future development.

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REFERENCES
1. Halestrap, A. P., and Price, N. T. (1999) Biochem. J. 343, 281–299
2. Drewes, L. R. (1999) in Brain Barrier Systems, Alfred Benzon Symposium 45 (Paulson, O., Knudsen, G. M., and Moos, T., eds) pp. 285–295, Munksgaard, Copenhagen
3. Pierre, K., and Pellerin, L. (2005) J. Neurochem. 94, 1–14
4. Wain, H. M., Bruford, E. A., Lovering, R. C., Lush, M. J., Wright, M. W., and Povey, S. (2002) _Genomics_ 79, 464–470
5. Halestrap, A. P., and Meredith, D. (2004) _Pfluegers Arch._ 447, 619–628
6. Gerhart, D. Z., Enerson, B. E., Zhdankina, O. Y., Leino, R. L., and Drewes, L. R. (1997) _Am. J. Physiol._ 273, E207–E213
7. Leino, R. L., Gerhart, D. Z., and Drewes, L. R. (1999) _Brain Res. Dev. Brain Res._ 113, 47–54
8. Enerson, B. E., and Drewes, L. R. (2003) _J. Pharmacol. Sci._ 92, 1531–1544
9. Martin, R. L., Lloyd, H. G., and Cowan, A. I. (1994) _Trends Neurosci._ 17, 251–257
10. Drewes, L. R., and Gilboe, D. D. (1973) _J. Biol. Chem._ 248, 2489–2496
11. Kurashima, K., Yu, F. H., Cabado, A. G., Szabo, E. Z., Grinstein, S., and Orlowski, J. (1997) _J. Biol. Chem._ 272, 28672–28679
12. Azarani, A., Orlowski, J., and Goltzman, D. (1995) _J. Biol. Chem._ 270, 23166–23172
13. Fan, J., Shuba, Y. M., and Morad, M. (1996) _Proc. Natl. Acad. Sci. U. S. A._ 93, 5527–5532
14. Cloherty, E. K., Diamond, D. L., Heard, K. S., and Carruthers, A. (1996) _Biochemistry_ 35, 13231–13239
15. Heard, K. S., Fidyk, N., and Carruthers, A. (2000) _Biochemistry_ 39, 3005–3014
16. Boyse, M., Sitaraman, S. V., Liu, X., Bado, A., and Merlin, D. (2002) _J. Biol. Chem._ 277, 28182–28190
17. Carpenter, L., and Halestrap, A. P. (1994) _Biochem. J._ 304, 751–760
18. Broer, S., Schneider, H. P., Broer, A., Rahman, B., Hamprecht, B., and Deitmer, J. W. (1998) _Biochem. J._ 333, 167–174
19. Jackson, V. N., and Halestrap, A. P. (1996) _J. Biol. Chem._ 271, 861–868
20. Roux, F., Durieu-Trautmann, O., Chavert, N., Claire, M., Maillly, P., Bourre, J. M., Strossberg, A. D., and Couraud, P. O. (1994) _J. Cell Physiol._ 159, 101–113
21. Thomas, J. A., Buchbaum, R. N., Zimmiai, A., and Racker, E. (1979) _Biochemistry_ 18, 2210–2218
22. Gerhart, D. Z., Enerson, B. E., Zhdankina, O. Y., Leino, R. L., and Drewes, L. R. (1998) _Glia_ 22, 273–281
23. Pierre, K., Magistretti, P. J., and Pellerin, L. (2002) _J. Cereb. Blood Flow Metab._ 22, 586–595
24. Wang, X., Levi, A. J., and Halestrap, A. P. (1994) _Am. J. Physiol._ 267, H1759–H1769
25. Broer, S., Broer, A., Schneider, H. P., Stegen, C., Halestrap, A. P., and Deitmer, J. W. (1999) _Biochem. J._ 341, 529–535
26. Taylor, S. S., Zheng, J., Radzio-Andzelm, E., Knighton, D. R., Ten Eyck, L. F., Sowadski, J. M., Herberg, F. W., and Yonemoto, W. M. (1993) _Philos. Trans. R. Soc. Lond. B Biol. Sci._ 340, 315–324
27. Tasken, K., Skalhegg, B. S., Tasken, K. A., Solberg, R., Knutsen, H. K., Levy, F. O., Sandberg, M., Orstavik, S., Larsen, T., Johansen, A. K., Vang, T., Schrader, H. P., Reintong, N. T., Torgeresen, K. M., Hansson, V., and Jahnsten, T. (1997) _Adv. Second Messenger Phosphoprotein Res._ 31, 191–204
28. Orlowski, J. (1993) _J. Biol. Chem._ 268, 16369–16377
29. Boron, W. F. (1992) in _The Kidney: Physiology and Pathophysiology_ (Seldin, D. W., and Giebisch, G., eds) 2nd Ed., pp. 219–263, Raven Press, Ltd., New York
30. Zink, S., Rosen, P., and Lemoine, H. (1995) _Am. J. Physiol._ 269, C580–C588
31. Casnocha, S. A., Eskin, S. G., Hall, E. R., and McIntire, L. V. (1989) _J. Appl. Physiol._ 67, 1997–2005
32. Farrell, A., Grollman, E. F., Wang, D., and Philp, N. J. (2003) _Am. J. Physiol._ 285, E1223–E1229
33. Tasken, K., and Aandahl, E. M. (2004) _Physiol. Rev._ 84, 137–167
34. Montminy, M. (1997) _Annu. Rev. Biochem._ 66, 807–822
35. Zhao, H., Wiederkehr, M. R., Fan, L., Collazo, R. L., Crowder, L. A., and Moe, O. W. (1999) _J. Biol. Chem._ 274, 3978–3987
36. Poole, R. C., and Halestrap, A. P. (1993) _Am. J. Physiol._ 264, C761–C782