Voltage Dependence of Facilitated Arginine Flux Mediated by the System y^+ Basic Amino Acid Transporter†

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Received September 18, 1992; Revised Manuscript Received March 12, 1993

ABSTRACT: Two-microelectrode voltage clamp was used to measure membrane currents resulting from flux of cationic amino acids in *Xenopus* oocytes expressing the cloned dual-function ecotropic murine leukemia virus receptor/system y^+ transporter. At membrane potentials ranging from +20 mV to −120 mV, arginine influx obeyed Michaelis–Menten kinetics. At concentrations from 0.01 to 1 mM, influx increased exponentially with membrane hyperpolarization (*e*-fold increase/−59 mV). Efflux from oocytes preloaded with arginine increased exponentially with depolarization (*e*-fold increase/+52 mV). Kinetic analysis based on an *iso uni uni* facilitated transport model suggests that the effect of voltage on steady-state flux arises largely from charge movement across the membrane field during the conformational transition of the unliganded transporter which switches the substrate binding site from one membrane face to the other. This charge movement would facilitate rapid increases in intracellular arginine concentrations in response to hyperpolarization, a property which could play a role in modulating nitric oxide synthesis in some types of cells.

The mouse cell-surface receptor for ecotropic leukemia retroviruses (ecoR) is encoded by a cDNA whose predicted amino acid sequence contains 12–14 potential membrane-spanning domains (Albritton et al., 1989). Following injection of RNA transcribed from the receptor cDNA into *Xenopus* oocytes, a basic amino acid uptake activity is expressed (Kim et al., 1991; Wang et al., 1991). The uptake induced in oocytes exhibits many of the characteristics of the cellular transport activity known as system y^+, which was first identified in cultured Ehrlich cells (Christensen & Antonioli, 1969). This activity is found in a wide range of cell types and serves as the predominant route of entry for arginine, lysine, and ornithine [for a review, see White (1985)]. Although system y^+ is sodium-independent, basic amino acid uptake can be inhibited by certain neutral amino acids such as homoserine and cysteine in a sodium-dependent fashion (Christensen et al., 1969; White, 1985). Application of basic amino acids to voltage-clamped oocytes expressing the system y^+ transporter results in a dose-dependent, saturable inward current; the magnitude of the current correlates with radiolabeled amino acid uptake in a manner suggesting that one positive charge is carried into the cell per molecule of substrate (Wang et al., 1991). Characteristic of the cellular transport system y^+, inward currents carried by arginine, lysine, and ornithine are sodium-independent; inward currents are also observed following superfusion with the neutral amino acids homoserine and cysteine, but not in the absence of sodium (Wang et al., 1991). Studies of transport of radiolabeled basic amino acids mediated by system y^+ suggest a facilitated diffusion mechanism which involves binding and release of substrate at sites exposed alternately to the extracellular and intracellular membrane faces (White, 1985). Because operation of the y^+ transporter can be measured in real time by recording membrane currents under voltage clamp conditions, it offers some advantages over radiotracer methods for kinetic studies. The present study involves the use of this technique to determine the voltage and concentration dependence of arginine flux mediated by the cloned y^+ transporter expressed in *Xenopus* oocytes, and the results are analyzed within the framework of an *iso uni uni* kinetic model of facilitated transport. The voltage-sensitive behavior of the transporter is discussed in the context of the role it plays as the major route of entry for the precursor of the messenger molecule nitric oxide.

EXPERIMENTAL PROCEDURES

Oocyte Expression and Voltage Clamp Recording. The ecoR cDNA was subcloned in the pGEM3Z plasmid and transcribed *in vitro* with SP6 polymerase (Wang et al., 1991); approximately 50 ng of capped RNA was injected into defolliculated stage V–VI oocytes. Two-microelectrode voltage clamp recordings were made 3–12 days later at 20–22 °C with a Dagan TEV-200 clamp amplifier filtered at 20 Hz. Data were acquired by a TL-1 interface controlled by computer using the pCLAMP Clampex program (Axon Instruments). The electrode solutions contained 3 M KCl; the bath solution (ND96) contained (in mM) NaCl (96), HEPES–NaOH, pH 7.5 (5), KCl (2), $CaCl_2$ (1.8), and $MgCl_2$ (1). Voltage-clamped oocytes were continuously superfused with this solution; for uptake experiments, the bath solution was changed to one containing the indicated concentration of substrate. Oocytes were superfused with substrate until peak currents were obtained (typically 10–20 s). Successive applications were made in order of increasing concentration at 5–10-min intervals. Current–voltage curves were obtained by applying voltage ramps of 4-s duration before addition of substrate and again during the peak response; substrate-specific currents were then determined by computer subtraction (Clampfit, Axon Instruments). For efflux studies, a current–voltage curve was obtained immediately before a 15–20-min application of 1 mM arginine, and this was subtracted from the current–voltage curve obtained during the peak outward current following washout of arginine. Kinetic parameters were obtained by least-squares fitting the substrate specific currents (I) to the expression $I = I_{max}[S]/(K_M + [S])$ where [S] is the substrate concentration and I_{max} and K_M are constants.

Computer Simulation of Transport. A simulation to model voltage-dependent steady-state flux was developed from the

† Supported by NIH Grants DA03160 to R. A. North and CA25810 to D. Kabat.

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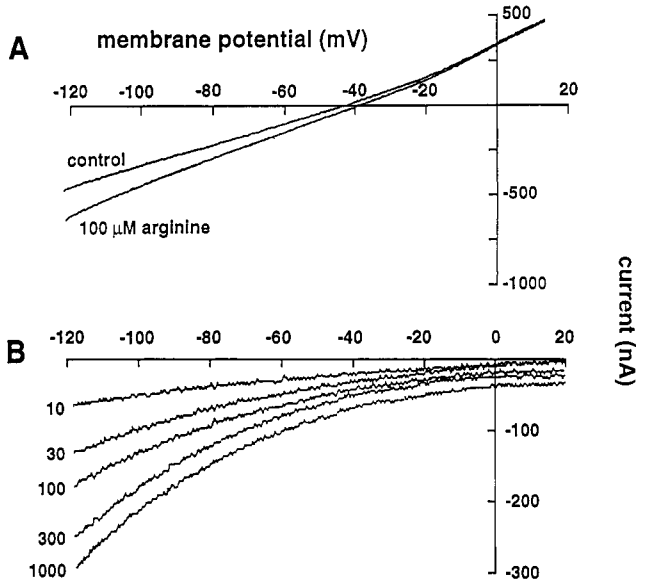

FIGURE 1: Voltage dependence of transporter inward currents. (A) Current measured during a voltage ramp (−120 mV to +20 mV) applied to a representative oocyte expressing ecoR before (control) and during peak response to application of 100 μM arginine. (B) Digitally subtracted arginine-dependent current–voltage curves for the concentrations of arginine indicated (μM).

iso uni uni kinetic model (Lieb & Stein, 1974) using SCoP software (Simulation Resources, Inc., Berrien Springs, MI). The forward and backward rate constants were constrained by the principle of detailed balance. The rate constants were assumed to be symmetric exponential functions of voltage of the form $f_i = f_i^0 \exp(\delta_i Z_i eV/2kT)$ and $b_i = b_i^0 \exp(-\delta_i Z_i eV/2kT)$ where f_i and b_i are the forward and backward rate constants for step i, V is the membrane potential, e is the fundamental charge, k is the Boltzmann constant, T is the absolute temperature, f_i^0 and b_i^0 are the rate constants with zero transmembrane potential, and Z_i is the number of elementary charges transferred in step i across the fraction δ_i of the electric field (Lauger & Stark, 1970). Movement of one positive charge across the membrane electric field was assumed to occur during a symmetric translocation of charged substrate in steps 1 and 2 (extracellular binding and intracellular unbinding). A variable charge movement intrinsic to the conformational changes of the polypeptide during these steps is added; this "transition charge movement" is returned during step 3 (the conformational transition of the empty transporter), resulting in net translocation of one positive charge during a cycle.

RESULTS AND DISCUSSION

In voltage-clamped oocytes expressing the ecotropic retrovirus receptor, addition of arginine caused inward currents which were both concentration- and voltage-dependent (Figure 1). At a given concentration, the inward arginine current increased in an exponential fashion with membrane hyperpolarization. The voltage dependence of the current did not vary significantly with concentration in the range from 0.01 to 1 mM arginine: the current induced by 1 mM arginine increased e-fold per 59.4 ± 2.1 mV while that induced by 10 μM arginine increased e-fold per 58.1 ± 3.3 mV (n = 15). At depolarized potentials, the currents appeared to approach a limiting value but did not reverse. Similar results were obtained with currents induced by 1 mM lysine (e-fold increase per 60.9 ± 1.5 mV, n = 3) and ornithine (e-fold increase per 60.2 ± 1.5 mV, n = 3). The voltage dependence of the kinetic

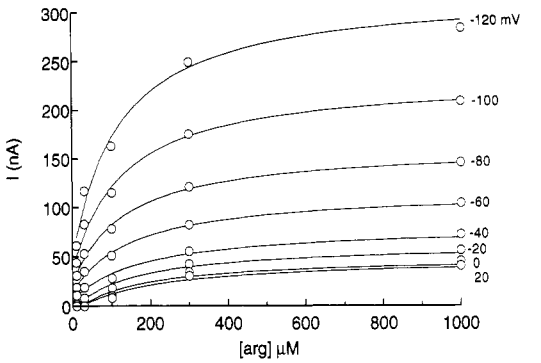

FIGURE 2: Concentration dependence of the arginine influx current at various membrane potentials. Data from a representative oocyte voltage-clamped at the indicated potentials are fit by least-squares to the Michaelis–Menten equation.

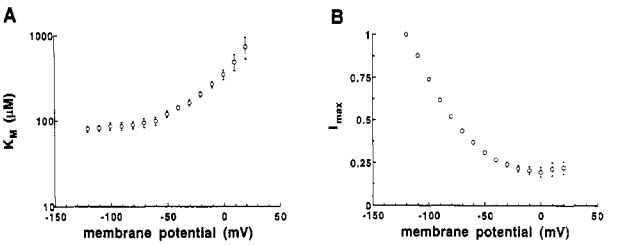

FIGURE 3: Voltage dependence of arginine transport kinetic parameters. (A) Effect of membrane potential on the K_M of the inward current induced by arginine. (B) Effect of membrane potential on I_{max}, normalized to the value at −120 mV. Data shown are mean ± SEM individually fit to the Michaelis–Menten equation (n = 6).

parameters K_M and I_{max} was determined by fitting the concentration-response data at different membrane potentials to the Michaelis–Menten equation (Figure 2). The I_{max} for arginine influx increased with hyperpolarization whereas the K_M value increased with depolarization (Figure 3).

Cellular transport of basic amino acids is bidirectional. In order to investigate efflux, oocytes expressing y^+ were allowed to accumulate arginine. As shown in Figure 4A, during extended superfusion the inward current declined and an outward current relative to the original holding current developed after termination of superfusion. The voltage dependence of the efflux current in oocytes preloaded with arginine was investigated by application of voltage ramps (Figure 4B). The steady-state arginine efflux current increased exponentially with membrane depolarization e-fold per 52 ± 6.2 mV (n = 5).

An analysis of the effects of membrane potential on cationic amino acid flux mediated by the system y^+ transporter can proceed from the assumption that transport occurs by facilitated diffusion with no direct link to cellular energy sources or ion cotransport (White, 1985; Kim et al., 1991; Wang et al., 1991). [^{3}H]Arginine flux mediated by system y^+ in fibroblasts and hepatoma cells is well described by a simple *iso uni uni* kinetic scheme (Lieb & Stein, 1974; White & Christensen, 1982). The key feature of this kinetic model is the existence of two conformational states of the carrier which allow binding and release of substrate at sites exposed alternately to the extracellular or intracellular membrane faces (Figure 5). A complete transport cycle for zero-trans influx involves substrate binding to state T_o at the external membrane face, unbinding at the internal face from T_i, and a conformational transition of the unliganded transporter returning to state T_o. In general, membrane potential will influence the rate constants for any step i in the transport cycle of Figure 5 which involves movement of a quantity of charge Z across a fraction δ of the membrane field. If the activation energy

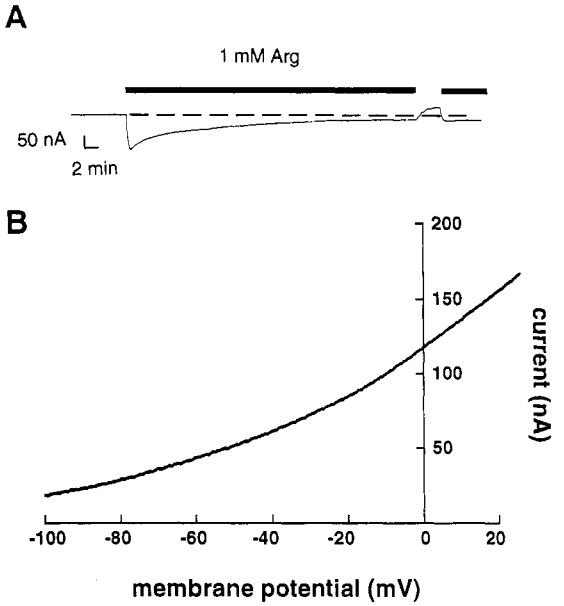

FIGURE 4: Outward currents in cells preloaded with arginine. (A) An oocyte expressing y^+ was voltage-clamped at −30 mV and superfused for the duration indicated by the bar with 1 mM arginine. Following termination of arginine superfusion, an outward current results. (B) Arginine efflux current–voltage relation in another oocyte preloaded by a 20-min incubation in 1 mM arginine. Difference current obtained by subtraction of current record before and after arginine washout (see Experimental Procedures).

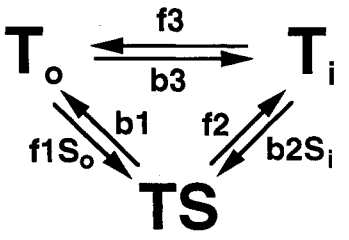

FIGURE 5: Two-state *iso uni uni* model of facilitated transport. Subscripts i and o refer to inside and outside the cell; f_i and b_i are the forward and backward rate rate constants for the corresponding step i in the transport cycle.

barrier between the states is assumed to be symmetric, this voltage dependence is given by

$$f_i = f_i^0 \exp(-\delta_i Z_i eV/2kT) \quad (1)$$

$$b_i = b_i^0 \exp(\delta_i Z_i eV/2kT) \quad (2)$$

where f_i^0 and b_i^0 are the rate constants with zero transmembrane potential, e is the fundamental charge, V is the membrane potential, k is the Boltzmann constant, and T is the absolute temperature (Lauger & Stark, 1970). The present results demonstrate that operation of the cloned y^+ transporter is intrinsically electrogenic as a consequence of the uptake (Figure 1) or outflow (Figure 4) of its cationic substrate arginine. Assuming that one net charge is translocated during a transport cycle, the effect of membrane potential on the ratios of the kinetic parameters K_M and I_{max} for influx and efflux can be calculated from the Nernst and *iso uni uni* velocity equations:

$$K_{eq} = \frac{[arg]^{in}}{[arg]^{out}} = \frac{K_M^{in} I_{max}^{out\rightarrow in}}{K_M^{out} I_{max}^{in\rightarrow out}} = \exp(eV/kT) \quad (3)$$

where K_M^{out} and K_M^{in} are the Michaelis constants at the extracellular and intracellular faces, respectively, and $I_{max}^{out\rightarrow in}$ and $I_{max}^{in>out}$ are the maximum currents for zero-trans influx and efflux, respectively. Although no *a priori* predictions about the voltage dependence of any individual parameter can be made, equilibrium distribution ratios and flux of radiolabeled y^+ substrates in cell lines have been reported which are generally consistent with the predicted effects of membrane potential (White & Christensen, 1982; Bussolati et al., 1989).

The present study has used the voltage clamp technique to directly measure the effect of membrane potential on steady-state flux mediated by the cloned system y^+ transporter. Among the molecular mechanisms which could underlie the observed effects of membrane potential on the kinetic parameters are actions arising from "ion well" effects on binding and unbinding of a charged substrate to a site on the protein which senses a fraction δ of the membrane electric field. As membrane potential is made positive, the K_M^{out} increases (Figure 3), consistent with such an effect for binding the cationic arginine substrate. However, the effects of membrane potential are likely to arise from the voltage dependence of rate constants in addition to those for binding and unbinding external arginine, because the voltage dependence of influx is not significantly affected by varying the concentration of arginine. Further evidence for additional voltage-dependent steps in the transport cycle comes from the lack of saturation of the arginine transport rate at hyperpolarized potentials (Figures 1 and 3). Electrogenic y^+ transport differs in this respect from transport mediated by the voltage-dependent glucose (Umbach et al., 1991) and GABA (Kavanaugh et al., 1992) transporters, both of which approach a maximal rate at hyperpolarized potentials. Saturation occurs at membrane potentials where the rate-limiting step in transport becomes independent of voltage [see Lauger (1991)]. There is no evidence for saturation of arginine flux with voltage; it continues to increase exponentially even at −180 mV, near the experimental limit of the voltage clamp amplifier (data not shown). Nonsaturating voltage dependence would be expected to arise only if each forward rate constant (eq 1) in the cycle had a Boltzmann coefficient >1 at negative membrane potentials. This would require that the coefficients of the corresponding backward rate constants (eq 2) be >1 at positive potentials, leading to nonsaturating efflux at depolarized potentials, as is observed (Figure 4). A simple model consistent with this behavior would involve both the internal and external arginine cation binding sites being within the membrane electric field, resulting in voltage-dependent binding and unbinding (Figure 5, steps 1 and 2). In addition, such a model requires movement of negative charge from inside to outside (or alternatively, positive charge from outside to inside) during the transition of the unliganded transporter for the zero-trans influx cycle (Figure 5, step 3). If the conformational return of the unliganded transporter were not associated with any charge movement, then steady-state flux would approach a maximum value determined by the limiting voltage-independent rate constant for this transition. In order for steady-state flux of a substrate with a valence of +1 to be nonsaturating at all voltages, this transition of the protein would require a movement of charge corresponding to $0 < Z\delta < 1$.

The voltage-dependent behavior of steady-state flux predicted by the *iso uni uni* kinetic model can be examined by solving the state equations with varying exponentially voltage-dependent rate constants (see Experimental Procedures). Figure 6 (top) shows the results of computed simulations to examine the effect of membrane potential on steady-state influx assuming that there is no charge movement ($\delta Z = 0$) during the conformational transition of the empty transporter;

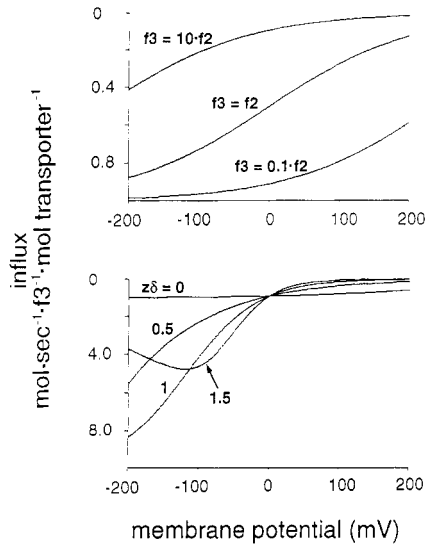

FIGURE 6: Computer simulation of voltage-dependent influx based on the *iso uni uni* kinetic model of Figure 5 (see Experimental Procedures). (Top) Steady-state influx–voltage relation with voltage-independent rate constants $f3$ and $b3$ (i.e., no transition charge movement in step 3). In this case, as the quantity $f3/f2$ (indicated for each curve) is decreased, influx saturates at more depolarized potentials. (Bottom) Effect of varying charge movement during step 3, the return of the empty substrate binding site to the external membrane face for the case when $f3/f2 = 0.1$. A charge movement ($Z\delta$, indicated next to the corresponding influx–voltage curve) during this step strongly affects the voltage dependence of flux. When $Z\delta > 1$, a negative slope factor results.

in this case, the voltage dependence of flux arises only from the translocation of the positively charged arginine molecule across the membrane electric field during steps 1 and 2. In this case, influx increases with hyperpolarization to approach a saturating value reflecting the voltage-independent rate-limiting constant $f3$ (Figure 6, top). The membrane potential range over which saturation occurs depends on the ratios of the rate constants $f2$ (the dissociation rate constant for substrate at the inside face) and $f3$ (the first-order rate constant for the transition of the empty transporter). With no charge movement in step 3, the behavior predicted by the model is similar to the observed behavior only in the case when the dissociation rate constant for substrate at the inside face, $f2$, is less than the first-order rate constant for the transition of the empty transporter, $f3$ (Figure 6, top). However, kinetic analysis of trans effects on radiolabel influx mediated by system y^+ suggests that $f2$ must be greater than $f3$ (White, 1985; and unpublished observations). In the case when $f2 > f3$, only when a charge movement $Z\delta$ occurs during the return of the unloaded transporter does nonsaturating voltage-dependent influx result (Figure 6, bottom). In this case, the steady-state influx becomes directly proportional to $f3$ and exhibits the same voltage dependence. From eq 1 and the measured dependence of influx (e-fold/−59 mV), an apparent charge movement of −0.86 e can be calculated to accompany the return of the binding site to the outside face. The difference in the voltage dependence of efflux (e-fold/52 mV; $\delta Z = 0.97$) suggests the possibility of an asymmetry in the energy barrier for the transition between the unliganded states [see Lauger (1991)].

These results suggest that a charge translocation δZ of approximately 0.9e occurs across the membrane dielectric during the conformational reorientation of the unliganded transporter, and that this charge movement significantly affects the voltage dependence of steady-state cationic amino acid flux mediated by system y^+. As a consequence, alterations of membrane potential in the physiological range not only affect the arginine equilibrium distribution ratio but also affect the rate at which the new equilibrium is reached. The question arises whether this property of the transporter might have any physiological role. One important arginine-dependent signaling pathway is the biosynthesis of nitric oxide (Palmer et al., 1987, 1988; Marletta et al., 1988), a molecule with an increasing number of recognized biological functions in vascular tissue and brain [see Knowles and Moncada (1992) and Bredt and Snyder (1992)]. Probably the best studied role of nitric oxide is in relaxation of vascular smooth muscle following its release from vascular endothelium (Palmer et al., 1987, 1988). The availability of extracellular arginine can limit agonist-induced nitric oxide synthesis in cultured vascular endothelial cells (Palmer et al., 1988) and neuroblastoma cells (Forstermann et al., 1990). Evidence from *in vivo* studies also suggests that influx of arginine may play a modulatory role in the nitric oxide response (Morikawa et al., 1992; Aisaka et al., 1989). Arginine influx into vascular endothelium appears to be mediated by system y^+ (Mann et al., 1989, 1991), and Northern blots show mRNA is expressed in rat capillaries, peripheral nerve, and brain (Stoll et al., 1992). The rapid nitric oxide-mediated dilation response to vasoactive agonists is accompanied by marked (up to 90 mV) hyperpolarization of vascular endothelium (Oleson et al., 1988; Nagao & Vanhoute, 1991; Mehrke et al., 1991). The physiological significance of this hyperpolarization is not clear, but the results of this study show that one consequence would be a rapid increase in arginine influx; indeed, [^{3}H]arginine uptake as well as nitric oxide synthesis has been shown to be increased in vascular endothelial cells by vasoactive agonist application (Bogle et al., 1991). Because increased arginine flux concomitant with hyperpolarization could conceivably play a role in modulating sustained nitric oxide release, further studies to investigate the functional interactions of the y^+ transporter with the nitric oxide system will be of interest.

ACKNOWLEDGMENT

I thank R. Alan North and David Kabat for discussion and critical reading of the manuscript, Hao Wang and Esther Dechant for generously preparing and providing plasmids, and Yan-na Wu for injecting RNA.

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