Endothelins activate Ca\(^{2+}\)-gated K\(^{+}\) channels via endothelin B receptors in CD-1 mouse erythrocytes

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Rivera, Alicia, Michelle A. Rotter, and Carlo Brugnara. Endothelins activate Ca\(^{2+}\)-gated K\(^{+}\) channels via endothelin B receptors in CD-1 mouse erythrocytes. Am. J. Physiol. 277 (Cell Physiol. 46): C746–C754, 1999.—Cell dehydration mediated by Ca\(^{2+}\)-activated K\(^{+}\) channels plays an important role in the pathogenesis of sickle cell disease. CD-1 mouse erythrocytes possess a Ca\(^{2+}\)-activated K\(^{+}\) channel (Gardos channel) with maximal velocity (V\(_{\text{max}}\)) of 0.154 ± 0.02 mmol·l cells\(^{-1}\)·min\(^{-1}\) and an affinity constant (K\(_{\text{0.5}}\)) for Ca\(^{2+}\) of 286 ± 83 nM in the presence of A-23187. Cells pretreated with 500 nM endothelin-1 (ET-1) increased their V\(_{\text{max}}\) by 88 ± 9% (n = 8) and decreased their K\(_{\text{0.5}}\) for Ca\(^{2+}\) to 139 ± 63 nM (P < 0.05; n = 4). Activation of the Gardos channel resulted in an EC\(_{50}\) of 75 ± 20 nM for ET-1 and 374 ± 97 nM for ET-3. Analysis of the affinity of unlabeled ET-1 for its receptor showed two classes of binding sites with apparent dissociation constants of 167 ± 51 and 785 ± 143 nM and with capacity of binding sites of 298 ± 38 and 1,568 ± 211 sites/cell, respectively. The Gardos channel was activated by the endothelin B (ET\(_{B}\)) receptor agonist IRL 1620 and inhibited by BQ-788, demonstrating the involvement of ET\(_{B}\) receptors. Calphostin C inhibited 73% of ET-1-induced Gardos activation and 84% of the ET-1-induced membrane protein kinase C activity. Thus endothelins regulate erythrocyte Gardos channels via ET\(_{B}\) receptors and a calphostin-sensitive mechanism.

Gardos channel; endothelin-1; sickle cell anemia; volume regulation

CELL DEHYDRATION IS AN IMPORTANT STEP IN THE FORMATION OF SICKLE CELLS BECAUSE Hb S POLYMERIZATION MARKEDLY INCREASES WITH SMALL INCREASES IN Hb CONCENTRATION. The Ca\(^{2+}\)-gated K\(^{+}\) channel (Gardos channel) can be activated in vitro by oxygenation-deoxygenation cycles with resulting dehydration of sickle erythrocytes. The imidazole antifungal clotrimazole (CLT) specifically inhibits the Gardos channel and reduces cell dehydration in vivo in a transgenic mouse model of sickle cell disease (12) and in patients (8). It is not known whether other modalities of cell dehydration via activation of the Gardos channel could be relevant for the pathogenesis of sickle cell disease. It has been demonstrated that prostaglandin E\(_{2}\) (PGE\(_{2}\)) can activate the Gardos channels of normal human erythrocytes, but it is not known whether this effect is mediated by Ca\(^{2+}\) entry or direct activation of the channel (29). Prostaglandins have been previously shown to alter size, deformability, and membrane structure by a Ca\(^{2+}\)-dependent mechanism (35). In Hb S-containing cells, these effects would tend to increase cell sickling and favor erythrocyte entrapment and ultimate blockage of the microcirculation.

Recently, it has been found that levels of endothelin-1 (ET-1) in plasma are significantly elevated in sickle cell patients during painful crisis, suggesting a possible role of this endothelial cell product in the pathogenesis of the painful crisis (18, 36). Prostaglandins and endothelins have been shown to alter K\(^{+}\) transport in various cell types (35, 39). ET-1 was shown to activate Ca\(^{2+}\)-gated K\(^{+}\) channels in vascular smooth muscle cells (40).

It has been found that the interaction of ET-1 with its receptor mediates an increase in intracellular Ca\(^{2+}\) that not only leads to a contractile response in myocytes but may also activate Ca\(^{2+}\)-dependent K\(^{+}\) channels (38). However, it has also been reported that ET-1 inhibits Ca\(^{2+}\)-gated K\(^{+}\) channels in rat basilar artery myocytes (39).

The effect of endothelins on erythrocyte ion transport has not been investigated. We postulated that endothelins could affect the function of the erythrocyte Gardos channel and could possibly affect control of cell volume in normal and sickle erythrocytes. However, there are no reports on the possible presence of endothelin receptors on mouse or human erythrocytes. Because mouse models of sickle cell disease have played an important role in the understanding of the pathophysiology of the disease and the design of new therapies, we investigated the functional characteristics of the Gardos channel in normal CD-1 mouse erythrocytes. In this report, we describe the presence of the Gardos channel in CD-1 mouse erythrocytes and its regulation by endothelins.

MATERIALS AND METHODS

Drugs and chemicals. Charybdotoxin (ChTX), ET-1, BQ-788, IRL 1620, PGE\(_{2}\), RANTES (regulated on activation, normal T cell expressed, and secreted), interleukin-10 (IL-10), and IL-8 were purchased from RBI Signal Innovation (Natick, MA). Iberiotoxin (IbTX), Stichodactyla toxin (STX), kalitoxin (KTX), and noxistoxin (NXTX) were purchased from Peptide International (Louisville, KY). Dr. Maria L. Garcia (Merck Research Laboratories, Rahway, NJ) kindly provided margatoxin (MgTX). All peptides were prepared as indicated by the manufacturer and stored at −20°C for <3 mo. The A-23187 ionophore was purchased from Calbiochem-Novabiochem (La Jolla, CA). The iodinated ligand ET-1 and \(^{86}\)Rb were purchased from DuPont-New England Nuclear. All other reagents were purchased from Sigma (St. Louis, MO).

Animals and erythrocyte preparation. Male CD-1 mice (Charles River, MA), 5–8 wk old, were used for these studies. Blood was collected in the presence of Na\(^{+}\)-heparin from ether-anesthetized animals. Blood was passed through cotton to decrease the number of leukocytes and then centrifuged in a Sorvall RC (J ouan) centrifuge for 4 min at 4°C and 2,000
Erythrocytes were washed four times with choline washing solution containing (in mM) 165 choline chloride, 1 MgCl₂, and 10 Tris-MOPS (pH 7.4 at 4°C).

Measurement of ⁸⁶Rb influx. Freshly washed erythrocytes were suspended at a hematocrit of 2% in normal influx media containing 165 mM NaCl, 2 mM KCl, 0.15 mM MgCl₂, 1 mM ouabain, 10 mM Tris-MOPS (pH 7.4 at 22°C), 10 µM bumetanide, and 10 µCi/ml ⁸⁶Rb in the presence or absence of an active peptide. Preincubations with endothelins or other substances were carried out for 20 min at 37°C in an isotonic saline. The same concentrations of active peptides or drugs were also added to the influx media. Free Ca²⁺ in the influx media was buffered to between 0 and 3.5 µM with 1 mM EGTA or citrate buffer as described by Wolff et al. (46). The Ca²⁺ concentration was calculated by using the dissociation constants (Kₐ) for ET-1 or citrate and correcting for ionic strength at pH 7.4 and 0.15 mM MgCl₂. The effects of ET-1 in the absence of A-23187 were tested by preincubating fresh washed mouse erythrocytes with or without the active peptide in saline solution [165 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 0.15 mM MgCl₂, 10 mM Tris-MOPS (pH 7.4, 37°C), 1 mM ouabain, and 10 µM bumetanide] for 15 min. The fluxes were measured in the presence or absence of ET-1 at the same preincubation concentration. For experiments with A-23187, a 5 µM concentration of the ionophore was added at time 0 and aliquots at 0.33, 2, and 5 min were removed and immediately spun down through 0.8 ml of cold medium containing 5 mM EGTA buffer and an underlying cushion of n-butyl phthalate. Supernatants were aspirated, and the tube tip containing the cell pellet was cut off. The erythrocyte-associated radioactivity was counted in a gamma counter (model 41600 HE; Isomedic ICN Biomedicals, Costa Mesa, CA). K⁺ uptake was linear up to 5 min, and fluxes were calculated from the slope of the linear regression as described by Brugnara et al. (4).

ET-1 binding assay. Erythrocytes were washed with choline washing solution and suspended at 10% hematocrit for 1 h at 4°C in a binding solution containing 165 mM NaCl, 2 mM KCl, 0.15 mM MgCl₂, 10 mM Tris-MOPS (pH 8.0, 4°C), and 1 mg/ml BSA. Cells were centrifuged and added to a final concentration of 1 × 10⁶ cells/ml into binding media without BSA containing [²²P]labelled ET-1 in the absence or presence of unlabeled ET-1 as described in the figure legends. In experiments using the antagonist BQ-788 or BQ-123, unlabeled ET-1 was preincubated with or without the active peptide in saline solution [165 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 0.15 mM MgCl₂, 10 mM Tris-MOPS (pH 7.4, 37°C), 1 mM ouabain, and 10 µM bumetanide] for 15 min. The filters were presoaked for 1 h at room temperature in BSA-binding solution (0.1%). The cell-containing filters were counted in a gamma counter. All linear or nonlinear curve fittings were performed as described in the figure legends with Enzyme Fitter (version 1.05; Elsevier-Biosoft), unless otherwise stated.

PKC activity measurements. Blood was centrifuged at 1,500 rpm for 10 min at 4°C to remove plasma anduffy coat (white blood cells). Erythrocytes were washed four times at 1,500 rpm for 5 min at 4°C with washing medium A containing (in mM) 145 NaCl, 5 KCl, 10 HEPES-Tris (pH 7.4), and 0.1 sodium phosphate. Membranes were prepared by hemolysis of cells in a 20-fold excess of lysis medium B containing 10 mM Tris·HCl (pH 7.4), 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 0.01 mM phenylmethylsulfonyl fluoride, and 1 mg/ml leupeptin. The membrane pellet was centrifuged at 18,000 rpm (Sorval SS34 rotor) for 20 min, the supernatant (cytosol) was removed and stored on ice, and the ghosts were washed four times in lysis medium B. Membranes were resuspended to a final concentration of ~1 mg protein/ml lysis buffer. Protein determination was performed by the biuretichinic acid method (Pierce). The Bq-free membrane suspensions were stored in Eppendorf tubes at –70°C until ready for assay.

Protein kinase C (PKC) activity was assayed by measuring the rate of ³²P incorporation into a peptide that is specific for PKC (kit RPN77; Amersham, Arlington Heights, IL). The assay mixture contained 12 mM calcium acetate, 30 mM DTT, 50 mM Tris·HCl (pH 7.5), 900 µM peptide, 0.3 mg/ml phosphatidylserine, and 24 µg/ml phosphor 12-merystate 13-acetate. An aliquot (25 µl) of the membrane suspension was added to this mixture (25 µl), and the reaction was initiated by the addition of [³²P]ATP. After 30 min of incubation at 37°C, the reaction was stopped by the addition of orthophosphoric acid. A 40-µl aliquot of the reaction medium was spotted on a 2.5 × 2.5 cm square of phosphocellulose paper. The papers were washed in 75% (vol/vol) phosphoric acid to eliminate any nonspecific binding and dried, and the ³²P level was determined by scintillation counting. PKC activity was expressed as picomoles of ³²P incorporated into the peptide per microgram of protein per minute.

RESULTS

Ionized Ca²⁺ activation curve of K⁺ influx via the Gardos channel. The activation of the K⁺ influx by cellular Ca²⁺ was studied in the presence of A-23187 to clamp intracellular Ca²⁺ at desired values as shown by Escobales and Canessa (14). Figure 1A shows the dependence of K⁺ influx on extracellular free Ca²⁺ in CD-1 mouse erythrocytes, in the presence or absence of 50 nM ChTX. K⁺ influx increased rapidly and saturated at −0.166 mmol·l cells⁻¹·min⁻¹ when Ca²⁺ was increased up to 3.5 µM. The nonlinear fitting of the experimental points for a sigmoidal function gave a maximal velocity (Vₘax) of 0.158 ± 0.01 mmol·l cells⁻¹·min⁻¹ (n = 8). In the presence of ChTX (50 nM), the K⁺ influx was inhibited to 0.02 ± 0.001 mmol·l cells⁻¹·min⁻¹. The difference between the two curves (Fig. 1B; ChTX-sensitive flux) gave a Vₘₐₓ of 0.154 ± 0.02 mmol·l cells⁻¹·min⁻¹ and affinity constant (Kₐ) for Ca²⁺ of 286 ± 83 nm (n = 8). These data demonstrate the presence of Ca²⁺-activated K⁺ channels (Gardos channels) in CD-1 mouse erythrocytes as previously described for human erythrocytes (6). We tested the effects of other venom toxins on Ca²⁺-activated K⁺ influx in CD-1 mouse erythrocytes in a low-ionic-strength medium containing 18 mM NaCl, 2 mM KCl, 230 mM sucrose, 10 mM Tris·HCl (pH 8.0), 0.01 mM bumetanide, 5 µM A-23187, 54 µM CaCl₂, 1 mM ouabain, and 10 µCi/ml ⁸⁶Rb. A significant inhibition of the Ca²⁺-activated K⁺ influx was observed with KTX (95 ± 5%; 50 nM), MgTX (92 ± 10%; 50 nM), and NtxTX (87 ± 1%; 50 nM). IbTX showed partial inhibitory potency (55 ± 10%; 50 nM). We also tested the effect of CLT, a potent and specific inhibitor of the Gardos channel in human erythrocytes (5, 7). The Ca²⁺-activated K⁺ influx in the presence of A-23187 was completely inhibited by 10 µM CLT in a low-ionic-strength medium (97 ± 5%) and was 92 ± 8% inhibited in normal saline (n = 3). In addition, the CLT metabolites 2-chlorophenyl-diphenylmethanol and 2-chlorophenylmethane (10 µM) were also effective as inhibitors of the Gardos channel in mouse erythrocytes.
Although less potent than CLT, 2-chlorophenyl-4-hydrophenyl-phenyl-methane and/or 2-chlorophenyl-4-hydrophenyl-phenyl-methanol at 10 µM produced significant inhibition (59 ± 5 and 42 ± 7%, respectively) of K\(^+\) influx mediated by the mouse erythrocyte Gardos channel (8).

Vasoactive mediators activate the Gardos channels of mouse erythrocytes. Active agents such as RANTES, platelet activator factor, PGE\(_2\), and interleukins have been shown to mobilize Ca\(^{2+}\) causing an increase in cytosolic Ca\(^{2+}\) in white blood cells (2, 3, 27). Therefore, we hypothesized that these peptides could also activate the Gardos channels in erythrocytes, as had been previously shown for eosinophils (13, 37). We tested the effect of these peptides on the ChTX-sensitive K\(^+\) influx in CD-1 mouse erythrocytes as a function of ionized extracellular Ca\(^{2+}\). As shown in Table 1, RANTES and IL-10 significantly increased by 1.5- to 2-fold the V\(_{max}\) of the channel at 10 and 20 ng/ml, respectively. Whereas IL-10 significantly increased the K\(_{0.5}\) for Ca\(^{2+}\) from 286 ± 83 to 703 ± 17 nM (P < 0.05, n = 3), PGE\(_2\) and IL-8 significantly decreased it to 129 ± 51 and 130 ± 32 nM, respectively. A Hill plot analysis indicated that the Hill coefficients for Ca\(^{2+}\)-activated K\(^+\) influx significantly increased (2-to 3-fold) in cells treated with IL-10, IL-8, and RANTES.

Endothelins activate the Gardos channels of CD-1 mouse erythrocytes. We measured the ChTX-sensitive fraction of the K\(^+\) influx in CD-1 mouse erythrocytes after 20-min pretreatments with and without ET-1 (500 nM). The time course of K\(^+\) uptake was measured at 1 µM free extracellular Ca\(^{2+}\) in the presence of A-23187 with or without ChTX (50 nM) as shown in Fig. 2. Under these experimental conditions, intracellular Ca\(^{2+}\) was damped at its electrochemical equilibrium. In control cells, K\(^+\) uptake was linear up to 5 min (r\(^2\) = 0.98) and was significantly inhibited by 50 nM ChTX. In ET-1-pretreated cells, K\(^+\) uptake doubled in 5 min and was completely inhibited by ChTX. The effect of ET-3 was tested under similar conditions. From Fig. 2, the V\(_{max}\) in cells pretreated with ET-1 was 0.32 ± 0.04 mmol·l cells\(^{-1}\)·min\(^{-1}\); it was 0.28 ± 0.02 mmol·l cells\(^{-1}\)·min\(^{-1}\) in cells pretreated with ET-3. Both fluxes...
Mouse erythrocytes were incubated with and without ET-1 (500 nM) for up to 15 min at room temperature in the absence of A-23187. ^86^Rb (K^+^) uptake was measured during that time in a saline solution containing 1 mM CaCl$_2$ in the presence or absence of ChTX (50 nM). As seen in Fig. 3, the basal ChTX-sensitive K$^+$ influx (0.015 ± 0.002 mmol·l$^{-1}$·min$^{-1}$) was enhanced by the presence of ET-1 to a value of 0.055 ± 0.004 mmol·l$^{-1}$·min$^{-1}$ (n = 3). These findings suggest that ET-1 could mediate regulation of the Gardos channel in vivo.

To study the specific effect of ET-1 on the kinetic properties of the Gardos channel, the dependence of the channel activity on free Ca$^{2+}$ was determined in cells pretreated with and without ET-1 (500 nM). As shown in Fig. 4, the $V_{\text{max}}$ of the system after ET-1 treatment increased twofold and the $K_{0.5}$ decreased from 286 ± 83 to 139 ± 63 nM (n = 4; P < 0.05). A dose-response curve for the activation of ChTX-sensitive K$^+$ influx by ET-1 is shown in Fig. 5. The estimated EC$_{50}$ for ET-1 was 75 ± 20 nM (n = 3). Similar experiments with ET-3 yielded an EC$_{50}$ of 374 ± 97 (n = 3).

ET-1 binds specifically to CD-1 mouse erythrocytes. Because ET-1 receptors have not been described in erythrocytes, we tested whether ET-1 specifically binds to an endothelin receptor in intact erythrocytes. As shown in Fig. 6, $^{125}$I-ET-1 binding to intact erythrocytes reached a plateau in 30 min at 4°C. The presence of 1 µM ET-1 significantly decreased (80%) the total binding. The specific binding of $^{125}$I-ET-1 to mouse erythrocytes was a saturable process consistent with a specific receptor interaction as shown in Fig. 7A. A Scatchard plot analysis revealed the presence of a class of high-affinity binding sites with an apparent association constant of 155 ± 23 pM and a maximal binding capacity of 390 ± 35 sites/cell (0.163 ± 0.01 fmol/2.5 × 10$^8$ cells). A competition assay of the radiolabeled ET-1 (100 pM) with ET-1 revealed a maximal inhibition at ~1.5 µM. Analysis of these experiments showed two distinct binding sites with $K_d$ values of 167 ± 51 and 787 ± 143 nM, and maximal binding values of 298 ± 38 and 1,568 ± 211 sites/cell, respectively.

A comparison between levels of $^{125}$I-ET-1 binding in the presence of ET-1, BQ-788 (a selective antagonist of ETA receptors), and BQ-123 (a selective antagonist of ETB receptors) is shown in Fig. 8. ET-1 displaced labeled ET-1 with an IC$_{50}$ of 600 ± 125 nM, which was lower affinity than that for BQ-788 (IC$_{50}$: 200 ± 53 nM) and higher than that for BQ-123 (IC$_{50}$: 750 ± 42 nM). These data suggest that in mouse erythrocytes the
specific binding of ET-1 can be accounted for by the presence of both ETA and ETB receptor subtypes. ETB receptors mediate endothelin effect on Gardos channels in mouse erythrocytes. To test whether endothelin’s action on the Gardos channel was mediated by an ETB receptor, we measured the effect of the ET B receptor agonist IRL 1620 on the Gardos channel activity in CD-1 erythrocytes (Fig. 9). IRL 1620 (500 nM) elicited a significant increase in the Gardos-mediated $86Rb$ influx from 0.156 $\pm$ 0.01 to 0.223 $\pm$ 0.003 mmol·l$^{-1}$·min$^{-1}$ ($P < 0.05; n = 3$), which was not significantly different from that induced by 500 nM ET-1 (0.220 $\pm$ 0.01 mmol·l$^{-1}$·min$^{-1}$). Thus the effect of ET-1 on Gardos channel activity could be mediated by ETB receptors. The effect of ETB receptor antagonist BQ-788 on the ET-1-induced activation of the Gardos channel was also studied. Preincubation of erythrocytes with both ET-1 (500 nM) and BQ-788 (1 µM) for 20 min at 37°C significantly decreased the ET-1-induced activation of the Gardos channel by 85%. Similarly, Gardos channel activation by the ETB receptor agonist IRL 1620 was significantly suppressed by BQ-788. As shown in Fig. 9, BQ-788 by itself did not induce inhibition of the Gardos channel.

Endothelin-induced activation of the Gardos channel is blocked by the PKC inhibitor calphostin C. Human erythrocytes express only two isoforms of PKC, $\xi$ and $\alpha$. It has been shown that the Gardos channel is modulated by PKC$\alpha$ under low-oxygen conditions in human sickle cells (15). Elevation of intracellular Ca$^{2+}$ by A-23187 has been shown to increase the translocation of PKC$\alpha$ to the cell membrane. Because ET-1 has been demonstrated to activate PKC in vascular smooth muscle (19), we have investigated the effect of a PKC inhibitor, calphostin C, on the ET-1-induced Gardos channel in CD-1 mouse erythrocytes. Calphostin C is an inhibitor of PKC that binds to the phorbol/diacylglycerol site and has little effect on other protein kinase activity (9). Erythrocytes were pretreated with calphostin C (10 µM) and 500 nM ET-1 for 20 min at 37°C. As shown in Fig. 10A, calphostin C significantly decreased (86 $\pm$ 11%; $n = 3$) the ET-1-induced activation of the Gardos channel.

PKC activity is increased in ET-1-treated cells. PKC enzymatic activity in membranes from cells treated...
with 300 nM ET-1 for 15 min at 37°C or not treated was measured. Membranes were prepared as described in MATERIALS AND METHODS. As shown in Fig. 10B, the basal activity of PKC increased by 84 ± 3% (n = 3) in ET-1-treated cells. This increase was significantly inhibited (66 ± 2%; n = 3) by calphostin C, suggesting that ET-1 increased specifically the activity of PKC.

DISCUSSION

We have shown that CD-1 mouse erythrocytes express Ca\(^{2+}\)-activated K\(^+\) influx that is specifically inhibited by ChTX. This toxin has been shown to be a highly specific inhibitor of the Ca\(^{2+}\)-activated K\(^+\) channels (Gardos channels) in human erythrocytes and other mammalian cells (6, 32). In mouse erythrocytes, activation kinetics of the Gardos channel by extracellular Ca\(^{2+}\) in the presence of A-23187 indicated a very high affinity for Ca\(^{2+}\). Kinetic analysis indicated a K\(_{0.5}\) for Ca\(^{2+}\) of <300 nM (286 ± 83 nM; n = 8) and a Hill coefficient of 2.7 ± 0.3. It is possible that in mouse erythrocytes the activation of the Gardos channel by Ca\(^{2+}\) may involve more than one active site as previously described for human erythrocytes (46).

The Gardos channels of mouse erythrocytes display a sensitivity for peptide toxins similar to that displayed by human erythrocytes (6, 17). Venom peptide toxins ChTX, MgTX, and KTX are highly effective in inhibiting the mouse channel in normal saline solution. MgTX is much less potent in human erythrocytes (4). IbTX, a specific inhibitor of the high-conductance Gardos channel in excitable cells (11), shows only partial inhibitory effect at 50 nM on the mouse Gardos channel (55%), as well as in human erythrocytes. STX displayed significant inhibition of the Gardos channel and displacement of bound ChTX in human erythrocytes (6). Likewise, STX toxin can also inhibit the CD-1 Gardos channel under similar conditions. Three different types of the Gardos channel in human erythrocytes have been reported (28), which could account for the different inhibitory potencies of these toxins in mouse and human erythrocytes. We speculate that the lack of inhibition of MgTX in human erythrocytes at physiological conditions may represent the absence of a subtype Gardos channel present in mouse erythrocytes. Vandorp et al. (43) recently cloned the cDNA encoding the Gardos channel from a murine erythroleukemia cell line and showed that the amino acid sequence is 88%
endothelins activate Ca\textsuperscript{2+}-gated K\textsuperscript{+} channel

Previous studies have indicated that activation of ET-1 receptors mobilizes intracellular Ca\textsuperscript{2+} stores in nonerythroid cells (1, 30, 42). The ET-1- and ET-3-induced relaxation of trachea smooth muscle cells is mediated by ChTX-sensitive K\textsuperscript{+} channels (20). Also, patch-clamp techniques have been used to describe the activation of the Ca\textsuperscript{2+}-gated K\textsuperscript{+} channel by endothelins in isolated coronary artery smooth muscle cells (23). In mouse erythrocytes, we observed that ET-1 increased both the Gardos channel activity and, by twofold, the affinity for internal Ca\textsuperscript{2+}, indicating a positive modulation of this channel by this peptide. The threefold increase in the $V_{\text{max}}$ of the Gardos channel by endothelins suggests that the active peptides either increase the number of active channels by recruiting quiescent units or, alternatively, increase the open time of the active units.

The displacement of 125I-ET-1 by unlabeled ET-1 at 4°C demonstrates the presence of endothelin receptors in mouse erythrocytes (Fig. 6). Saturation of the receptor by labeled ET-1 was obtained in <1 h at 4°C. A Scatchard analysis of radiolabeled ET-1 binding indicated a binding site with a $K_d$ of 156 ± 23 pM and 390 ± 35 sites/cell (Fig. 7A). These results are in agreement with the $K_d$ and binding kinetics described for endothelin receptors in other cell types (41, 48). However, the effects of ET-1 on the K\textsuperscript{+} channels are seen at much higher concentrations, suggesting the involvement of a receptor with lower affinity. Competition of unlabeled higher concentrations, suggesting the involvement of a receptor by labeled ET-1 was maximally displaced at 1.5 µM ET-1. Analysis of Fig. 7B using a modified version of the Scatchard plot as shown by Bylund (10), demonstrates the presence of at least two other sites, one with a $K_d$ of 167 ± 51 nM and the other with a $K_d$ of 787 ± 143 nM, with maximal binding values of 289 ± 38 and 1,598 ± 211 sites/cell, respectively. These data suggest that the effect of ET-1 on the channel might be mediated by these two low-affinity ET-1 receptors.

Unlabeled ET-1 and endothelin receptor antagonists (BQ-788 and BQ-123) displaced 125I-ET-1 with an order of potency of BQ-788 > ET-1 > BQ-123. The strong inhibition of 125I-ET-1 binding by BQ-788 may suggest that ET\textsubscript{B} receptors are mainly present in mouse erythrocytes. BQ-788 also blocks the transport effect of ET-1 (Fig. 9). A concentration of IRL 1620 (ET\textsubscript{B}-selective agonist) equivalent to that of ET-1 can induce similar ChTX-sensitive K\textsuperscript{+} fluxes in mouse erythrocytes, which are inhibited by BQ-788. It is not clear why BQ-788 significantly reduces the K\textsuperscript{+} influx below control values in the presence of the ET\textsubscript{B} agonist IRL 1620 (Fig. 9). We can speculate that when there is complete blockade of the ET\textsubscript{B} receptor by 1 µM BQ-788, IRL 1620 might be interfering with another receptor that significantly inhibits the Gardos channels or possibly that the presence of IRL 1620 and BQ-788 in the cell suspension blocks the channel directly (Fig. 9).

The intracellular signaling mechanisms that mediate ET-1 actions in nonerythroid cells include phospholipase C, diacylglycerol, and PKC (44). In single-channel studies, it has been observed that the sensitivity of the Gardos channel to Ca\textsuperscript{2+} is dependent on the phosphorylation state of the protein (25). The ET-1 effect on the mouse erythrocyte Gardos channel can be blocked by calphostin C (Fig. 10). This is consistent with a specific effect on the channel’s activity and suggests that the channel or an associated regulatory protein or proteins are required to be phosphorylated to be active. Although phosphorylation events are required for ET-1-induced Gardos activity, phosphorylation by PKC seems not to be essential for channel activation (Fig. 10). Furthermore, we found that the activity of PKC significantly increased by 84% in the presence of ET-1 and that this increase was inhibited by calphostin C. Recent studies indicated that human erythrocytes express only two isoforms of PKC, $\xi$ and $\alpha$, and that the Gardos channel is modulated by PKC$\alpha$ under low-oxygen conditions in human sickle cells (15). Furthermore, elevation of intracellular Ca\textsuperscript{2+} by A-23187 increased the translocation of PKC$\alpha$ to the cell membrane (15). Therefore, the effect of ET-1 on the Gardos channel might be mediated by PKC$\alpha$.

We have also shown that cytokines and chemokines can activate the Gardos channels in mouse erythrocytes. These active peptides are well known to act on a variety of immune cells via receptor-ligand interactions (33). Recently, chemokine receptors in erythrocytes were described (22). Among the active peptides tested in mouse erythrocytes, IL-10 and RANTES significantly increased the Gardos channel $V_{\text{max}}$ (Table 1). In addition, IL-10 as well as IL-8 alters the affinity of the Gardos channel for intracellular Ca\textsuperscript{2+} (Table 1). Because some of these ligands specifically interact with the chemokine receptor, it is possible that the Gardos channel and chemokine receptors are functionally coupled in CD-1 mouse erythrocytes. In addition, under pathological conditions, these active peptides may positively or negatively regulate the Gardos channel, with possible changes in the hydration and deformability of the erythrocytes. Recently, Kumar et al. (26) reported that IL-8 can also promote adherence to the endothelium of sickle, but not normal, erythrocytes. The events leading to the overexpression of adhesion molecules such as integrins and glycoproteins in sickle cells are not completely understood. Elevation of intracellular Ca\textsuperscript{2+} and PKC activation causing phosphorylation of integrins have been postulated as possible physiological mechanisms for the enhancement of adhesion molecules (16, 45). It has been shown that integrin $\alpha_\text{IIb} \beta_3$ and glycoprotein IV are expressed on circulating reticulocytes from sickle cell patients (24). Because we found that normal erythrocytes express ET-1 receptors that can increase PKC activity, the relationship of ET-1 with integrin and glycoprotein expression on sickle reticulocytes and erythrocytes should also be investigated.

Our data suggest that erythrocyte dehydration may take place via activation of the Gardos channel by
vasoactive peptides, in the absence of deoxygenation. Thus the modulation of Gardos channel activity by ET-1 might play an important role in the dehydration of sickle erythrocytes. It is possible that, on ET-1 receptor activation, intracellular Ca\(^{2+}\) and activation of PKC induce the opening of the Gardos channel, resulting in K\(^+\) and water loss and possibly formation of denser erythrocytes. This is in agreement with the activation of the Gardos channel by ET-1 in the absence of A-23187 (Fig 3) and could suggest the coupling of the Gardos channel to the ET-1 receptor. In addition, sickle erythrocytes have been shown to interact with vascular endothelial cells, stimulating the release of active peptides and regulating the expression of the ET-1 gene and protein in cultured endothelial cells (34). This effect seems to be specific for sickle cells, because induction of other genes, such as those for actin and platelet-derived growth factor-

ENDOTHELINS ACTIVATE Ca\textsuperscript{2+}-GATED K\textsuperscript{+} CHANNEL


