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BIOCHEMICAL CHARACTERIZATION OF THE $Na^+/K^+/Cl^-$ CO-TRANSPORT IN CHICK CARDIAC CELLS

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SUMMARY. Cultured chick cardiac cells possess a $Na^+/K^+/Cl^-$ co-transport system that is inhibited by the "loop diuretics" benzmetanide (IC_{50} = 0.3 μM), bumetanide (IC_{50} ≈ 0.6 μM), piretanide (IC_{50} = 1.5 μM) and furosemide (IC_{50} = 5 μM). The $K_{0.5}$ values for Cl^- and Na^+ activation of the bumetanide-sensitive $^{86}Rb^+$ uptake are 59 mM and 40 mM respectively. Bumetanide also inhibits a $^{22}Na^+$ uptake component that is suppressed when external Cl^- or K^+ are substituted by impermeant ions. The ratio of bumetanide-sensitive $^{86}Rb^+$ to $^{22}Na^+$ uptake is close to 1. The cardiac $Na^+/K^+/Cl^-$ cotransport is a major uptake pathway for Na^+ and K^+. It accounts for 50% of the initial rate of $^{86}Rb^+$ uptake and 17% of the initial rate of $^{22}Na^+$ uptake by chick cardiac cells. It is activated two-fold by an hyperosmotic shock produced with 200 mM mannitol.

INTRODUCTION. Several cation transporting systems have now been well characterized in the plasma membrane of cardiac cells. These are the voltage-dependent Na^+ channel (1) and Ca^{2+} channel (2), and electrogenic cation exchange systems such as the Na^+/Ca^{2+} exchange system (3) and the (Na^+,K^+)ATPase (4). Cardiac cells also have electroneutral cation transporting systems in their plasma membrane such as the (Ca^{2+})ATPase (5) and the Na^+/H^+ antiporter which is important in cardiac cells to control the intracellular Na^+ content (6) and to protect the cells against an intracellular acidosis (7). Evidence has been presented for the presence of another electroneutral cation transport system in chick cardiac cells : the $Na^+/K^+/Cl^-$ cotransport. This system is probably involved in volume regulation (8). In this paper we charaterize some of the biochemical and pharmacological properties of the cardiac $Na^+/K^+/Cl^-$ cotransport.

MATERIALS AND METHODS. Eagle's minimum essential medium and foetal calf serum were from GIBCO. $^{22}NaCl$ was from Amersham, $^{86}RbCl$ and ^{36}ClH were from the Commissariat à l'Energie Atomique (Saclay, France). Furosemide, ouabain and phorbol 12-myristate 13-acetate were from the Sigma Chemical Co. Bumetanide and benzmetanide were from Leo Pharmaceuticals, Ballrup, Denmark. Piretanide, metolazone and Hoechst 740B were kind gifts by Dr. J.C. Ellory. Forskolin was from Calbiochem. Cultures of embryonic chick cardiomyocytes were prepared as previously described (6).

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The basal solution used for flux experiments was 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Tris at pH 7.4. When the concentrations of Na^+ or K^+ were varied, N-methyl-D-glucamine chloride was used as a substituent to maintain a constant ionic strength. When external Cl^- was changed, it was replaced by isoosmotic amounts of methanesulfonate. Flux experiments were performed using $^{22}Na^+$ (5 μCi/ml) or $^{86}Rb^+$ (8 μCi/ml) as a tracer for K^+. At the end of the uptake period, cells were rinsed 3 times with 0.1 M $MgCl_2$ to remove extracellular radioactivity. Cells were then dissolved into 2 ml of 0.1 N NaOH and counted in a gamma counter. Initial rates of $^{22}Na^+$ or $^{86}Rb^+$ uptake were measured after one minute of incubation. Cell proteins were determined according to Hartree (9) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION. The relative contributions of the (Na^+,K^+)ATPase and of the $Na^+/K^+/Cl^-$ co-transport to total $^{86}Rb^+$ uptake by chick cardiac cells was assessed from the effect of two specific inhibitors of these pathways : ouabain and bumetanide. The inset A of Fig. 1 shows that ouabain, at a concentration of 0.2 mM, which is sufficient to completely block the (Na^+,K^+)ATPase (4), inhibited 37 ± 5 % of the initial rate of $^{86}Rb^+$ uptake. Bumetamide (0.1 mM) by itself produced a 50 ± 5 % inhibition of the initial rate of $^{86}Rb^+$ uptake. The effects of ouabain and bumetanide were additive since a mixture of the two molecules produced a mean 86 ± 6 % inhibition of the initial rate of $^{86}Rb^+$ uptake. The main panel of Fig. 1 presents the dose-response curve for bumetanide inhibition of the initial rate of $^{86}Rb^+$ uptake. The IC_{50} value for bumetanide action is observed at 0.6 μM, for an external Cl^- concentration of 140 mM. Reducing

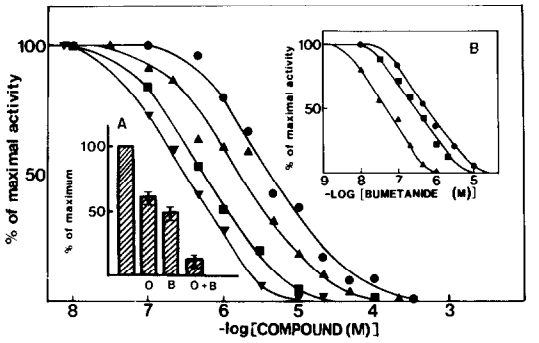

Figure 1 : Pharmacological properties of the $Na^+/K^+/Cl^-$ co-transport in chick cardiac cells.
Main panel : Dose-response curves for benzmetanide (▼), bumetanide (■), piretanide (▲) and furosemide (●) inhibition of the initial rate of $^{86}Rb^+$ uptake by ouabain (0.2 mM) - treated cardiac cells.
Inset A : Influence of ouabain (0, 0.2 mM) and bumetanide (B, 0.1 mM) on the initial rate of $^{86}Rb^+$ uptake by chick cardiac cells.
Inset B : Dose-response curves for bumetanide inhibition of the initial rate of $^{86}Rb^+$ uptake measured at different external Cl^- concentrations. Experiments were performed in the presence of 0.2 mM ouabain, 140 mM Na^+, 5 mM K^+, 145 mM Cl^- (●), 70 mM Cl^- (■) or 30 mM Cl^- (▲). Methanesulfonate was used as a substituent for Cl^- to maintain a constant ionic strength.

$[Cl^-]_o$ from 145 mM to 70 mM and 30 mM progressively decreased the IC_{50} value for bumetanide action from 0.6 μM to 200 nM and 50 nM (Fig. 1B). This observation suggests that bumetanide recognizes a Cl^- binding site on the $Na^+/K^+/Cl^-$ co-transport as previously described for duck red blood cells (10).

Three other potent "loop diuretics" benzmetanide, piretanide and furosemide were also found to inhibit $^{86}Rb^+$ uptake by chick cardiac cells (Fig. 1 main panel). The rank order of potency of the different molecules tested is benzmetanide (IC_{50} = 0.3 μM) > bumetanide (IC_{50} = 0.6 μM) > piretanide ($K_{0.5}$ = 1.5 μM) > furosemide ($K_{0.5}$ = 5 μM). In contrast to "loop" diuretics, the two thiazides, metolazone and Hoechst 740B as well as the K^+ sparing diuretics amiloride, benzamil and triamterene had no detectable inhibitory effect on $^{86}Rb^+$ uptake by chick cardiac cells. The pharmacological profile of the cardiac $Na^+/K^+/Cl^-$ cotransport is identical to that of the $Na^+/K^+/Cl^-$ cotransport described in other cell types (11-17).

Substitution of external Cl^- by the impermeant anion methanesulfonate at constant $[Na^+]_o$ and $[K^+]_o$ supressed the bumetanide-sensitive $^{86}Rb^+$ uptake component (Fig.2 inset). The main panel of Fig. 2 presents the $[Cl^-]_o$ dependence of the initial rate of bumetanide-sensitive $^{86}Rb^+$ uptake. The $K_{0.5}$ value for Cl^- activation of the bumetanide-sensitive $^{86}Rb^+$ uptake component was 56 mM. This value is close to that reported for the $Na^+/K^+/Cl^-$ cotransport of MDCK cells (18). Substitution of external Na^+ with N-methyl-D-glucamine (Fig. 3, inset A), choline or Li^+ resulted in a 86 % reduction of the initial rate of bumetanide-sensitive $^{86}Rb^+$ uptake. This suggests that the bumetanide-sensitive $^{86}Rb^+$ uptake component consists of two parts : a major (86 %) Na^+-dependent component and a minor (14 %) Na^+-independent component. The Na^+ independent ^{86}Rb uptake was suppressed when external Cl^- was substituted by methanesulfonate (Fig. 3, inset A) and was inhibited by furosemide and bumetanide. The $K_{0.5}$ value for bumetanide inhibition of this component was 0.6 μM (Fig. 3 inset B). The main panel of Fig. 3 shows the dose-response curves for $[Na^+]_o$ activation of the initial rate of $^{86}Rb^+$ uptake. The $K_{0.5}$ value for $[Na^+]_o$ activation was 40 mM, a value lower than the $K_{0.5}$ value found in MDCK cells (K_{Na} = 85 mM) (11) and 3T3 fibroblasts (K_{Na} = 69 mM) (12) but much higher than the corresponding value for human red blood cells and human foreskin fibroblasts (K_{Na} = 8-15 mM) (19-20).

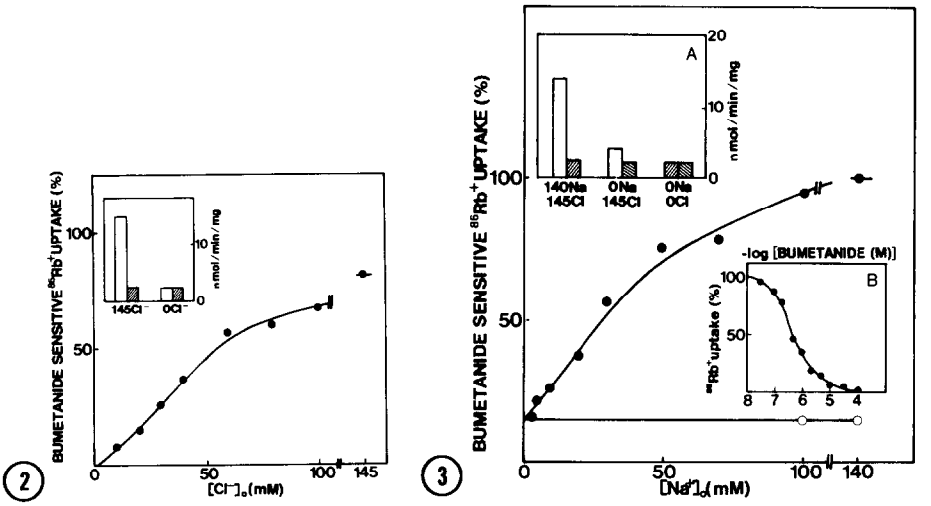

Figure 2 : The dependence on $[Cl^-]_o$ of the activity of the $Na^+/K^+/Cl^-$ co-transport in chick cardiac cells.
Main panel : Dose-response curves for Cl^- activation of the bumetanide-sensitive $^{86}Rb^+$ uptake component. Experimental conditions were 140 mM Na^+, 5 mM K^+, 0.2 mM ouabain.
Inset : Influence of external Cl^- removal on the initial rate of $^{86}Rb^+$ uptake by cardiac cells incubated in the absence (open bars) or the presence (shaded bars) of 0.1 mM bumetanide. Ouabain (0.2 mM) was present in all experiments.

Figure 3 : The dependence on $[Na^+]_o$ of the activity of the $Na^+/K^+/Cl^-$ co-transport in cardiac cells.
Main panel : Dose-response curve for Na^+ (●) or Li^+ (○) activation of the initial rate of bumetanide-sensitive $^{86}Rb^+$ uptake component. Experimental conditions were 5 mM K^+, 145 mM Cl^-, 0.2 mM ouabain. Na^+ and Li^+ were substituted with N-methyl-D-glucamine to maintain a constant ionic strength. The rate of bumetanide-insensitive $^{86}Rb^+$ uptake was subtracted from all experimental values.
Inset A: Influence of external Na^+ and Cl^- removal on the initial rate of $^{86}Rb^+$ uptake by cardiac cells incubated in the absence (open bars) or the presence (shaded bars) of 0.1 mM bumetanide. Ouabain (0.2 mM) was present in all experiments. Na^+ and Cl^- were replaced by isoosmotic amounts of N-methyl-D-glucamine and methanesulfonate respectively.
Inset B : Dose response curve for bumetanide inhibition of the initial rate of $^{86}Rb^+$ uptake by chick cardiac cells incubated in a Na^+ free medium.

Bumetanide also inhibits a $^{22}Na^+$ uptake component. The bumetanide sensitive $^{22}Na^+$ uptake component, which represents 17 % of the total $^{22}Na^+$ uptake component, was suppressed either when external K^+ was substituted by N-methyl-D-glucamine or when external Cl^- was substituted by methanesulfonate (Fig. 4). The ratio of the initial rates of bumetanide sensitive $^{22}Na^+$ to $^{86}Rb^+$ uptake component was 1.03 ± 0.20, indicating a transport stoichiometry close to $1Na^+/1K^+$.

In chick cardiac cells, a major Na^+ uptake pathway is the Na^+/H^+ exchange system (6). The relative contributions of the Na^+/H^+ antiport and of the $Na^+/K^+/Cl^-$ cotransport system was assessed from the relative effects of ethylisopropylamiloride, a specific

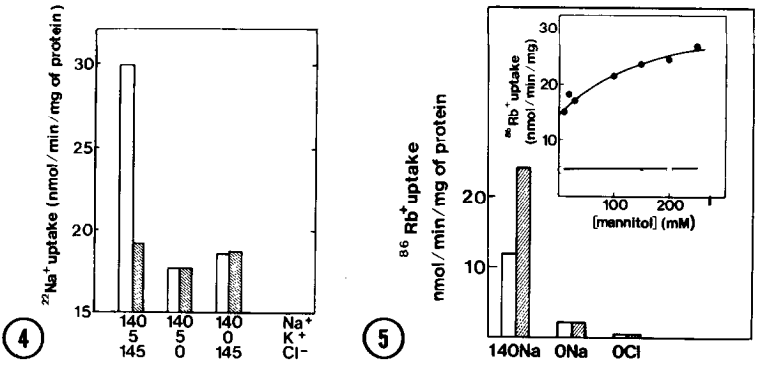

Figure 4 : $^{22}Na^+$ uptake by chick cardiac cells.
Influence of Cl^- and K^+ removal on the initial rate of $^{22}Na^+$ uptake by chick cardiac cells. Incubated in the absence (open bars) or the presence (shaded bars) of 0.1 mM bumetanide. All incubation media were supplemented with 0.1 mM ethylisopropylamiloride and 0.2 mM ouabain.

Figure 5 : Influence of an hyperosmotic shock on the activity of the $Na^+/K^+/Cl^-$ cotransport systems of chick cardiac cells.
Main panel : Influence of the addition of 200 mM mannitol on the initial rate of bumetanide-sensitive $^{86}Rb^+$ uptake. Experiments were performed using either a physiological medium (control), a Na^+-free or Cl^--free medium. Open bars : isoosmotic conditions, shaded bars : hyperosmotic condition produced with 200 mM mannitol.
Inset : Dose-response curve for mannitol activation of the initial rate of $^{86}Rb^+$ uptake by chick cardiac cells incubated in the absence (●) or in the presence (○) of 0.1 mM bumetanide.

inhibitor of the Na^+/H^+ antiport (21) on one hand and of bumetanide on the other. In 4 experiments, the activity of the $Na^+/K^+/Cl^-$ cotransport represented 69 % ± 15 % of the activity of the Na^+/H^+ antiport. A common feature of the $Na^+/K^+/Cl^-$ cotransport in various cell types is its capacity to be regulated. The system has been reported to be sensitive to phorbol esters in BALB/c 3T3 preadipose cells (22), to changes in intracellular Ca^{2+} and cAMP levels in human red blood cells (23, 24) shark rectal gland (25), vascular smooth muscle cells (19) and human fibroblasts (12). None of these regulations have been found in chick cardiac cells. Forskolin (10 μM), an activator of adenylate cyclase (26), phorbol 12-acetate 13-myristate (1 μM), a potent activator of protein kinase C (27), and the calcium ionophore A23187 were found to have no action on the rate of bumetanide-sensitive $^{86}Rb^+$ uptake by chick cardiac cells.

The cardiac $Na^+/K^+/Cl^-$ cotransport was found to be sensitive to changes in the osmolarity of the incubation medium. Fig. 5 shows that a 2-fold increase in the initial rate of bumetanide sensitive $^{86}Rb^+$ uptake is observed upon the addition of 200 mM mannitol to the incubation medium. The inset of Fig. 5 presents the dose response curve for mannitol activation of the $Na^+/K^+/Cl^-$ cotransport. Mannitol had no action on the rate of $^{86}Rb^+$ uptake under conditions in which the activity of the $Na^+/K^+/Cl^-$

cotransport was blocked by bumetanide (Fig. 5 inset) or in the absence of external Cl^- (Fig. 5). Fig. 5 also shows that mannitol had no action on the small, bumetanide sensitive and Cl^- dependent $^{86}Rb^+$ uptake component that is observed when external Na^+ was substituted with N-methyl-D-glucamine.

In conclusion, chick cardiac cells possess a Cl^- dependent cation transporting system that is inhibited by "loop" diuretics. It is a major uptake pathway for Na^+ in addition to the Na^+/H^+ antiport. Its activity is sensitive to changes in the osmolarity of the medium which supports the idea of a potential role for regulating the cardiac cell volume (8) as in other cell types (28).

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