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Net efflux of chloride from cell suspensions measured with a K^+ electrode

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Under appropriate conditions (presence of cation ionophores) net KCl efflux measured with a K^+ electrode can be used to estimate conductive Cl^- fluxes, a sensitive procedure that allows continuous recording. The procedure was tested in human red cells by demonstrating effects of ionophores and of an anion transport inhibitor, and in dissociated MDCK cells by demonstration of cAMP and volume-activated Cl^- fluxes.

Chloride permeates membranes by a variety of mechanisms including conductive channels [1], and electroneutral systems such as anion exchangers as exemplified by the well studied chloride-bicarbonate exchanger of the red blood cell [2], and cotransporters such as those for $Na^+/K^+/2Cl^-$, K^+/Cl^- or Na^+/Cl^- [3]. The conductive component can be directly assessed by electrophysiological measurements: short-circuit currents for intact epithelial layers, microelectrodes for individual cells, and patch clamp for whole cell or single-channel recording. In cell populations, however, the conductive component is more difficult to assess. Cl^--dependant changes in membrane potential have been noted using potential sensitive dyes [4], but the data are qualitative in nature. Isotopic flux determinations measure all pathways and because the conductive component is usually small compared to the others [5–7], especially exchange, its contribution is difficut to assess. The conductive component has been estimated by subtraction [7], using inhibitors or special conditions to minimize non-conductive flux pathways, but the procedure is awkward and subject to limitations such as inhibitor specificity and effectiveness, and co-ion (K^+) permeability. The extreme case is the red blood cell, in which the conductive flux is about four orders of magnitude smaller than the exchange (reviewed in Ref. 8). With this cell other technologies have evolved using cation ionophores to substantially increase conductive permeability to K^+, thereby, allowing escape of KCl (driven largely by the outward K^+ gradient) at a rate that is limited by conductive Cl^- permeability (reviewed in Ref. 8). The net KCl efflux has been measured in several ways including: ionophore-induced changes in K^+ efflux measured by isotope [9], or K^+ determinations using flame photometry [10–12]; associated cell volume changes, measured by changes in turbidity or absorbance of red cell suspensions [13,14]; or measurements of Cl^- or SO_4^{2-} efflux under conditions in which exchange is rigorously excluded [8]. A similar approach has been successfully applied to osmotically swollen lymphocytes [5], ascites [6] and MDCK cells [15], using Coulter Counter technology to measure ionophore-mediated volume changes. This procedure is rapid, easy and convenient, but is relatively insensitive and subject to imprecision due to dispersion of cell sizes and sensitivity to cell size [15]. Fluorescent, Cl^--sensitive dyes [16] and Cl^--sensitive electrodes [17] have been used successfully with vesicle systems, but, for numbers of reasons, not as yet with cells.

A potentially sensitive and simple technique to measure Cl^--limited net KCl efflux, explored in this paper, is the use of K^+ electrodes. They have often been used to measure K^+ in cell suspensions (see, for example, Refs. 13, 18 and 19). The modification described here involves the addition of ionophores, valinomycin or gramicidin to ensure that conductive Cl^- permeability is rate-limiting, similar in principle to the various procedures noted above. The procedure was tested in red blood cells and in dissociated Madin-Darby Canine Kidney (MDCK) cells, with the conductive Cl^- permeability modulated by hypotonic shock, cyclic adenosine monophosphate (cAMP) activation, and inhibitors. The method is simple, sensitive, rapid, and provides continuous monitoring.

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Recently expired Bank Blood obtained from the Hematology Department at the Hospital for Sick Children was centrifuged and the buffy coat discarded. The cells were washed three times in Hepes-buffered saline before experimental use. In some experiments the cells were pretreated with the transport inhibitor, DIDS (125 μM) for 10 min at 37°C. A selected clone of MDCK cells [4] was grown to confluence. The cells were dissociated by light trypsinization, washed and suspended in a non-growth medium [4,15]. Cell suspensions (3 ml) were placed in a small beaker into which the electrodes could be inserted. The suspension was lightly stirred. The K^+ electrode and double-junction reference electrode were from Orion, and the potentiometer from Beckman (model 71 pH meter). The system was calibrated against K^+ standards after each experiment.

Valinomycin and gramicidin were used to enhance conductive K^+ fluxes. The latter is considerably more effective (100-fold in the case of red cells [8]), but unlike valinomycin, it does not discriminate between Na^+ and K^+, so Na^+ of the medium was replaced by the impermeant cation, *N*-methylglucamine$^+$ (NMG) [15]. The sensitivity of the procedure can be adjusted over a wide range by manipulation of cell density and initial K^+ concentration of the medium. With limited numbers of cultured cells it was sometimes necessary to work with low external K^+, in the 10 to 100 μM range (the electrode is sensitive to < 1 μM). Low K^+ concentrations may influence the rate of K^+ efflux, but that should not disturb the measurements of ionophore-induced Cl^- efflux. At the end of each run the remaining cellular K^+ was released by addition of digitonin (40 μM), providing a reading that represents release of 100% of initial cell K^+. Data were normalized to that value.

The effects of valinomycin on loss of K^+ from red cells is illustrated in Fig. 1A. With increasing concentrations of ionophore the rate of loss was increased, but

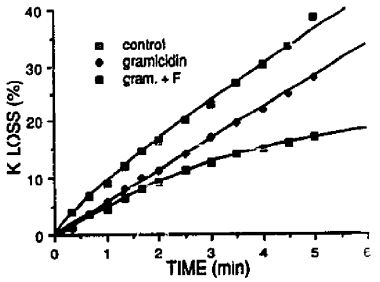

Fig. 2. Effect of Forskolin on K^+ efflux from control and gramicidin-treated MDCK cells. Suspensions contained $1.3 \cdot 10^5$ cells per ml. Gramicidin was 2 μM. Forskolin was 50 μM. Initial K^+ was 10 μM. Temperature was 22°C.

the initial rate (estimated from the 2.5-min value) approaches a maximum value. A similar set of data were obtained in Na^+ free medium indicating no effects of NMG^+ substitution, required in gramicidin experiments as noted above. The response pattern found with gramicidin (Fig. 1B) was similar to that found with valinomycin, but the maximal rate was about three times as high (about 9% of cell K^+ lost per minute). These data support the suggestion that gramicidin is superior to valinomycin in attaining Cl^- limitation of net KCl efflux [8].

To further test that the assumption of anion limitation, red cells were pretreated with 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), an agent that does not inhibit K^+ permeation but is a potent, highly specific inhibitor of anion transport [2]. The gramicidin-induced flux was reduced significantly (estimated at 45% during the first 2.5 min, see inset of Fig. 1B). This result is consistant with prior studies, in which DIDS and related compounds have been reported to block conductive Cl^- fluxes to the extent of 50 to 60% [8,10,12,14] It is worth noting that the ionophore concentration required to achieve 'maximal' KCl efflux was found to be

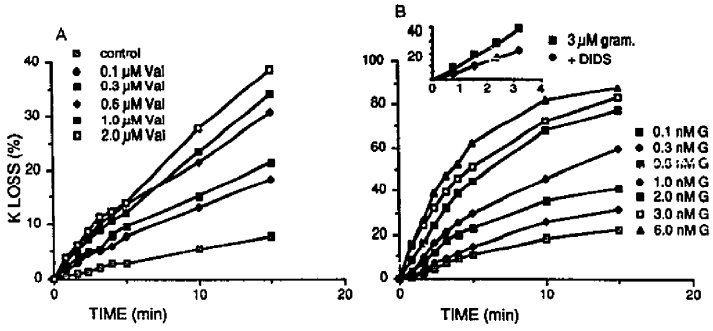

Fig. 1. K^+ loss from human red blood cells induced by valinomycin (A) or gramicidin (B), and inhibitory effect of DIDS (inset in B). The valinomycin and gramicidin concentrations are indicated. Suspensions contained 10^7 cells per ml. External K^+ was 25 μM for valinomycin and 100 μM for gramicidin. Temperature was 22°C. DIDS was 125 μM and gramicidin, 3 μM.

dependent on red cell density, so that comparisons of ionophore effects must take cell density into account.

Monolayers of MDCK, like many other epithelial cells are known to transport K^+ and Cl^- via channels that are modulated by cyclic adenosine monophosphate (cAMP) (see literature cited in Ref. 4). The cAMP activation was recently demonstrated in dissociated MDCK cells by monitoring changes in membrane potential using fluorescent dyes [4]. The effects could also be demonstrated with the K^+ electrode (Fig. 2). In control cells the efflux slowed down appreciably soon after initiation of the experiment. Gramicidin had little effect for one or two minutes, but thereafter, efflux was maintained at a considerably higher rate than in the control. With gramicidin plus forskolin (an activator of cAMP-activated Cl^- pathways (see Ref. 4), an immediate and sustained increase was evident and the rates were maintained at a level above those of gramicidin alone. The components of the system were further assessed by manipulating the experimental conditions. For example, no delay in the gramicidin effect occurred if the ionophore was added 3 to 10 min after initiation of the experiment. An interpretation is that K^+ permeability is initially high (Cl^- is rate-limiting), so gramicidin does not immediately increase the efflux of KCl. After 2 to 3 min, however, the rate of K^+ efflux decreases and becomes rate-limiting, so that gramacidin, induces an immediate increase in KCl efflux. Preliminary evidence suggests that the observed spontaneous reduction in K^+ flux (in the control) is a response to the reduction in external K^+ to low levels (50 μM) at the beginning of each experiment, a manipulation that is necessary to achieve the desired sensitivity for the electrode procedure. The enhancement by forskolin is presumably due to cAMP activation of a Cl^- pathway, previously demonstrated by membrane depolarization [4]. It can also be produced by cAMP analogs such as 8-Br-cAMP (not shown). A small forskolin activation was sometimes seen in the absence of gramicidin, probably representing the reported cAMP stimulation of K^+ pathways [4]. Variability was noted in different cultures, in terms of control rates, as well as gramacidin and forskolin enhancement. These appear to be related to the degree of confluence of each culture [4,15]. Activation by forskolin in the presence of gramicidin (Cl^-) was found in 16 of 19 cases. Activation in the absence of gramicidin (K^+) was noted in 7 of 13 cases.

MDCK cells, when osmotically swollen will reshrink by loss of KCl [20,21], a process called Regulatory Volume Decrease or RVD. In lymphocytes [5] and in Ehrlich ascites cells [6] RVD involves the opening of conductive K^+ and Cl^- pathways. Recently the same underlying mechanism has been demonstrated in MDCK cells by volume measurements in the presence and absence of gramicidin [15]. RVD was also readily measureable with the K^+ electrode (Fig. 3). No increase

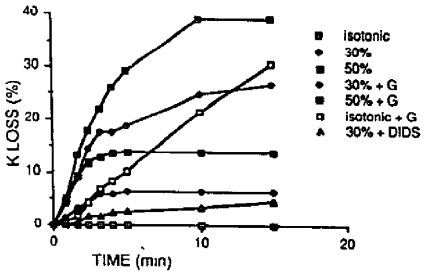

Fig. 3. K^+ efflux from osmotically swollen MDCK cells with and without gramicidin or DIDS. Suspensions contained $1.7 \cdot 10^5$ cells per ml. Gramicidin was 1.3 μM and DIDS, 200 μM (pretreatment for 10 min). Initial K^+ was 50 μM. Temperature was 37° C.

in K^+ release was evident with 10% dilution of the medium, but the flux was progressively increased with increasing hypotonicity above 20% (for clarity only the 30 and 50% data are displayed in Fig. 3). The effects were transient, lasting only a few minutes. In the presence of of gramicidin, the effects were larger and more prolonged. It should be noted, however, that the gramicidin control (isotonic) was unusually high is this experiment. The putative role of anion conductive pathways was further evaluated by the use of DIDS. This substance and its analogs, although best known as an inhibitors of anion exchange in red blood cells [2], are capable, as noted, of inhibiting conductive fluxes of red cells [8,10,12,14]. Recently they have also been found to block conductive Cl^- channels, especially in epithelial cells (see Refs. 22 and 23, and a number of additional references cited herein). In MDCK cells DIDS is reported to inhibit volume-activated Cl^- flux, with 50% inhibition at 2 μM. Incidently, in this study the Cl^--channel blocker, diphenylamine-2-carboxylate (DPC), was ineffective. The inhibitory action of DIDS was confirmed using the K^+ electrode technique as illustrated in Fig. 3. It can be noted that because the cell concentrations required in the electrode studies is considerably higher than in Coulter Counter studies, higher concentrations of DIDs are also required.

Clearly, the K^+ electrode procedure allows measurements of volume-activated conductive K^+ (without gramicidin) and Cl^- (with gramicidin) permeabilities in agreement with those obtained by direct volume measurements (with the Coulter Counter) [15], the latter being activated to a greater degree. In general, cell swelling activations (Fig. 3) were much larger than those induced by cAMP (Fig. 2). In fact, the latter were not readily detectable by cell volume measurements using the Coulter Counter (unpublished observations), a relatively insensitive procedure, but were easily measured with the more sensitive K^+ electrode technique. The results using the K^+ electrode, reported here, were clearly superior to those obtained by volume measurements using the Coulter Counter [15], with respect to

sensitivity, precision, time resolution and continuous monitoring. As noted, however, larger numbers of cells were required.

To briefly summarize, conductive Cl^- efflux from cell suspensions can be continuously measured by net KCl efflux in the presence of cation ionophores, using a simple K^+ electrode. Sensitivity can be manipulated by adjusting the cell density of the suspension and the external K^+ concentration. Inhibitory effects of DIDS, and activating effects of cell swelling and of cAMP were readily demonstrated. The procedure should be particularly useful for cell suspensions when Cl^- currents or conductances cannot be directly or readily measured.

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