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ATP Depletion and Inactivation of an ATP-Sensitive Taurine Channel by Classic Ion Channel Blockers

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SUMMARY

Cell volume regulation in different cell types is mediated in part by plasma membrane channel(s) that allow taurine and other important intracellular organic osmolytes to efflux from the cell. Previous studies have demonstrated that intracellular ATP is required for activation of a volume-sensitive taurine-permeable channel. The present study examined the relation between cellular ATP and ADP concentrations and swelling-induced [^{14}C]taurine efflux and anion current (whole-cell patch-clamp) after exposure of isolated skate (*Raja erinacea*) hepatocytes to metabolic poisons and a series of ion channel blockers. When intracellular ATP content was lowered with gradually increasing concentrations of 2,4-dinitrophenol, a sigmoidal relation between ATP content and volume-activated [^{14}C]taurine efflux was observed. Taurine efflux was progressively inhibited over a relatively narrow range of intracellular ATP levels, indicating that physiologic alterations in cellular nucleotides may modulate the opening of the channel. Surprisingly, the inhibition of [^{14}C]taurine efflux by a number of ion channel blockers [glibenclamide, 5-nitro-2-(3-phenylpropylamino)benzoate, diphenylamine-2-carboxylate, ketoconazole, gossypol, niflumic acid, and quinine] was related to a decrease in cellular ATP concentrations and ATP/ADP ratios, rather than to a direct interaction with the channel. In contrast, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and pyridoxal-5-phosphate inhibited volume-activated anion channels but had no effect on cellular ATP levels. These findings suggest multiple sites for regulation of volume-sensitive osmolyte channels and indicate that some putative ion channel blockers may actually alter the activity of ATP-regulated transporters by depleting cellular ATP.

Several key membrane transport systems are regulated by intracellular ATP, including CFTR, P-glycoproteins, K^+-ATP channels, a K^+-Cl^- cotransporter, and primary active pumps (1–5). Recent studies indicate that volume-activated taurine transport also depends on the availability of intracellular ATP (6–9). Regulatory volume decrease and [^{14}C]taurine efflux from skate hepatocytes are immediately inhibited by metabolic poisons when these are administered either immediately before or at any time during the volume adjustment (6). Although the molecular structure of the taurine release mechanism has not yet been elucidated, kinetic and patch-clamp studies in a number of cell systems indicate that it is a channel rather than a carrier (6, 8–12). Channel activation requires intracellular ATP or nonhydrolyzable ATP analogs (8). Patch-clamp analysis of skate hepatocytes confirms the presence of swelling-activated, anion-selective conductance with a taurine/Cl^- permeability of 0.17, which is activated only in the presence of intracellular ATP or nonhydrolyzable ATP analogs in the patch pipet (9). To characterize the properties of this channel and to assess the role of intracellular nucleotides, we examined the effects of a series of selective ion channel inhibitors on volume-activated [^{14}C]taurine efflux from isolated skate hepatocytes, while simultaneously analyzing intracellular ATP and ADP concentrations. Whole-cell, patch-clamp analysis of volume-activated channel activity was used to determine the specificity of several of these ion channel blockers.

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Materials and Methods

Hepatocytes were isolated from male skates as previously described (13, 14) and were preloaded with [^{14}C]taurine by incubation with 0.2 mM [^{14}C]taurine for 1.5–2.0 hr at 15° in normal elasmo-

ABBREVIATIONS: HPLC, high performance liquid chromatography; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

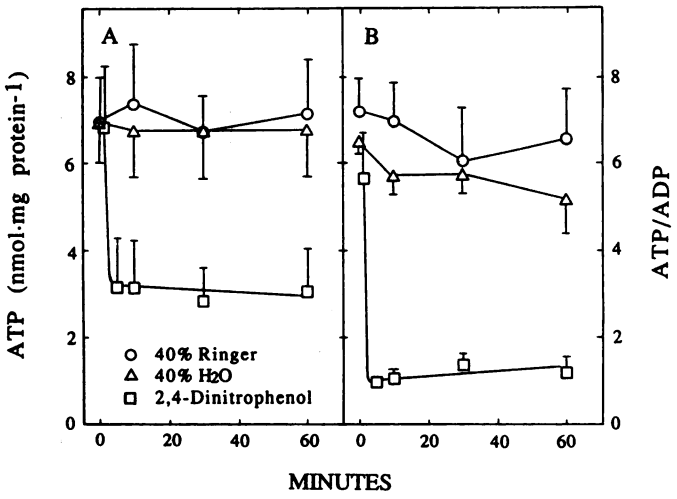


Fig. 1. Intracellular ATP content and ATP/ADP ratio in skate hepatocytes swollen in hyposmotic medium or treated with 0.5 mM 2,4-dinitrophenol. A, Intracelluar ATP content; B, ATP/ADP ratio. Cell suspension medium was diluted 40% with either elasmobranch Ringer solution or water at time 0. 2,4-Dinitrophenol was also added at time 0, under isosmotic conditions. Values are mean ± standard error of four experiments.

branch Ringer solution (270 mM NaCl, 4 mM KCl, 3 mM $MgCl_2$, 0.5 mM Na_2SO_4, 1 mM KH_2PO_4, 8 mM $NaHCO_3$, 350 mM urea). Cells were then centrifuged at 100 × *g* for 2 min and resuspended at a density of ~50 mg wet weight/ml of Ringer solution. Within 5–10 min of the final resuspension, inhibitors were added and cells were incubated for 30 min. Aliquots of the cell suspension were removed for ATP and ADP analysis and assessment of ^{14}C content of the cells, hypotonicity was induced by 40% dilutions of the remaining cell suspensions with either water or Ringer solution (control), and cellular ^{14}C content was measured 30 min later. Cells were separated by rapid centrifugation as previously described (14), and radioactivity was determined by liquid scintillation counting. ATP and ADP content was assessed by the HPLC method of Hill *et al.* (15). A 0.3-ml aliquot of cell suspension was added to an ice-cold microfuge tube containing 37.5 μl of 45% perchloric acid/9 mM EDTA. The sample was mixed and centrifuged to separate the acid-insoluble pellet, and 200 μl of the supernatant were mixed with 85 μl of 1.5 M $KHCO_3$ at 4°. The potassium perchlorate was allowed to settle to the bottom of the tube, the supernatant was filtered through a syringe filter (0.45 μm; Gelman), and 5–20 μl of this solution were injected onto the HPLC column. The HPLC system consisted of a Varian model 5000 liquid chromatograph equipped with a Bakerbond NP octadecyl (C_{18}) column (4.5 × 250 mm, 5 μm; J.T. Baker Research Products, Phillipsburg, NJ), a Vari-chrom adjustable-wavelength spectrophotometric detector (Varian Associates, Sunnyvale, CA), and an HP 3394A integrator (Hewlett-Packard, Palo Alto, CA). Adenine nucleotides were isocratically eluted at a flow rate of 1 ml/min, using a mobile phase of 0.1 M sodium phosphate, pH 6, containing 4% (v/v) methanol, detected at 259 nm, and quantitated using external standards. The acid-insoluble pellet was solubilized in 1 M NaOH for subsequent protein determination by the method of Lowry *et al.* (16).

Whole-cell patch recordings were performed as previously described (8, 12), on skate hepatocytes attached to poly-L-lysine-coated coverslips. Patch electrodes were pulled from borosilicate glass microhematocrit tubes (1.5-mm o.d.; Fisher Scientific, St. Louis, MO) that had been silanized with dimethyldichlorosilane (Sigma Chemical Co., St. Louis, MO). The electrodes were not fire polished before use and had DC resistances of 15–20 MΩ when filled with a solution containing 20 or 50 mM CsCl. Cells were used only if the series resistance was <30 MΩ.

An Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) was used to voltage-clamp cells following gigaseal formation and attainment of whole-cell access. Command voltage generation, data digitization, and data analysis were carried out on a Dell Optiplex 4100/MX, 486DX4–100, IBM-compatible computer (Dell Computer Corp., Austin, TX), using a DigiData 1200 AD/DA interface with pClamp software (Axon Instruments). Electrical connections to the amplifier were made using Ag/AgCl pellets and 3 M KCl/agar bridges.

The patch pipet solution contained 20 or 50 mM CsCl, 5 mM $MgSO_4$, 20 mM HEPES, 1 mM EGTA, 410 or 450 mM sucrose, 350 mM urea, 4 mM ATP, and 0.5 mM GTP (880 mOsM) and was brought to pH 7.2 with CsOH. The isosmotic bath perfusion contained 150 mM CsCl, 5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 3 mM HEPES, 2 mM Tris, 320 mM

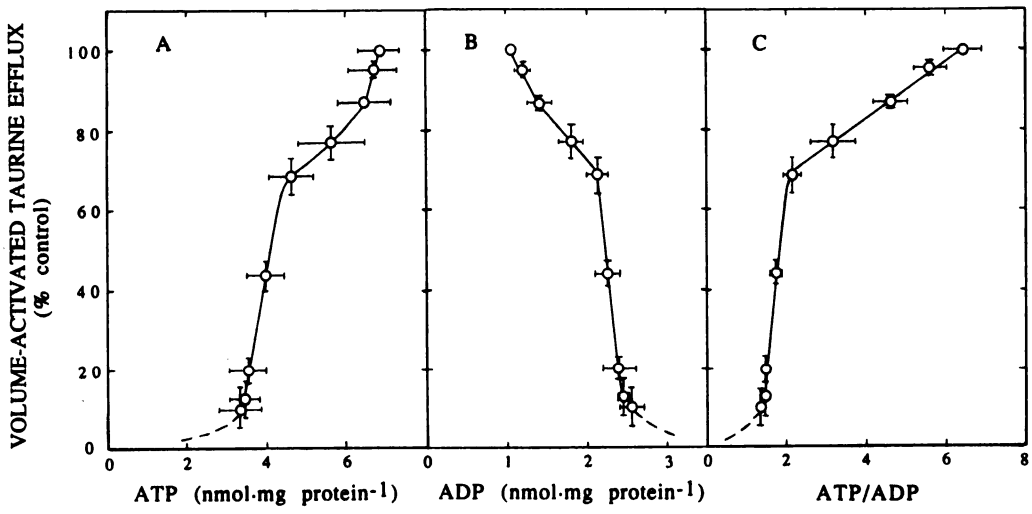
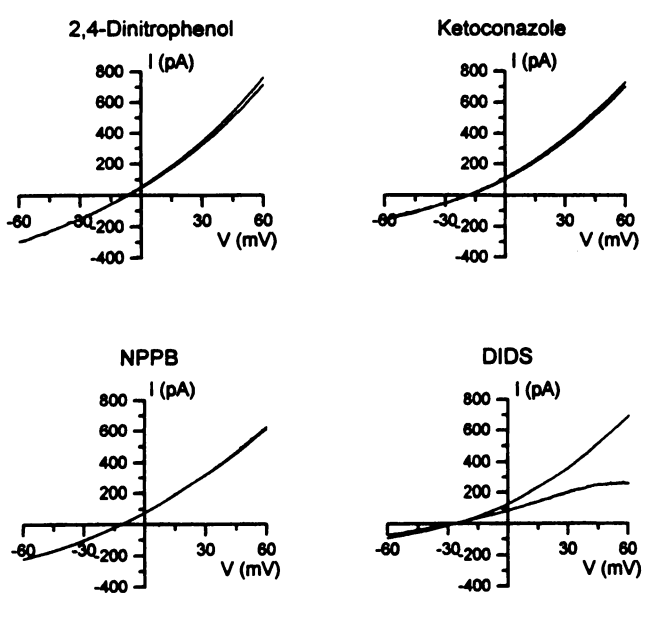


Fig. 2. Relation between volume-activated [^{14}C]taurine efflux and ATP content, ADP content, and ATP/ADP ratio in isolated skate hepatocytes exposed to increasing concentrations of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. A, ATP content; B, ADP content; C, ATP/ADP ratio. Hepatocytes preloaded with [^{14}C]taurine were incubated for 30 min with 2,4-dinitrophenol at concentrations of 0, 5, 10, 25, 50, 100, 200, 500, and 700 μM, and aliquots of the cell suspension were removed for ATP and ADP analysis and assessment of the ^{14}C content of the cells. Hypotonicity was then induced by diluting the remaining cell suspensions by 40% with either water or Ringer solution (control), and cellular ^{14}C content was measured 30 min later. Values are mean ± standard error of four to six experiments at each concentration of 2,4-dinitrophenol.



B

Drug (μM)	% Inhibition (n)	
2,4-Dinitrophenol (500)	6±1	(4)
Ketoconazole (100)	3±1	(3)
NPPB (100)	3±4	(5)
Pyridoxal 5-phosphate (2000)	92±4	(3)
DIDS (500)	62±9	(3)

Fig. 3. Effects of 2,4-dinitrophenol, ketoconazole, NPPB, and DIDS on volume-activated anion current and extent of inhibition of volume-activated anion current in skate hepatocytes by 2,4-dinitrophenol, ketoconazole, NPPB, pyridoxal-5-phosphate, and DIDS. A, Current changes in response to voltage ramps recorded from skate hepatocytes in the whole-cell mode. After swelling-induced activation of anion channel current, drugs were superfused into the bath. Traces are shown immediately before and 1 min after application of 500 μM 2,4-dinitrophenol, 100 μM ketoconazole, 100 μM NPPB, and 500 μM DIDS. Hyposmotic bath solution was identical to isosmotic solution except for a decrease in sucrose concentration to yield 650 mOsM. After whole-cell access was obtained, cells were swollen by perfusion with hyposmotic solution. Current levels at +60 mV were measured and used to calculate percent inhibition. For pyridoxal-5-phosphate, cells were preincubated for 15–30 min before whole-cell recording began. Current levels were also measured at +60 mV but at 3 min after swelling and compared with levels in control cells 3 min after swelling. B, Extent of inhibition of volume-activated anion current in skate hepatocytes by 2,4-dinitrophenol, ketoconazole, NPPB, pyridoxal-5-phosphate, and DIDS.

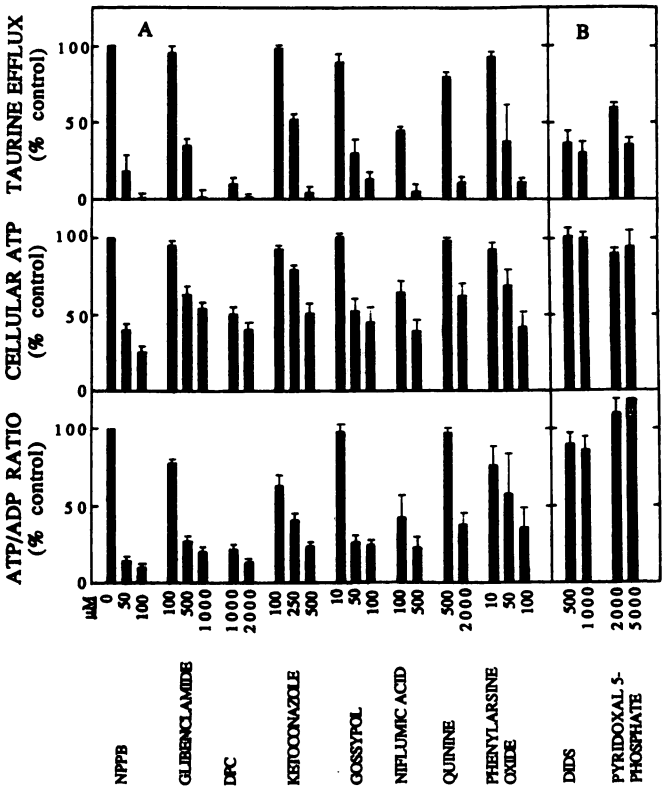


Fig. 4. Effects of ion channel blockers and other transport inhibitors on volume-activated [^{14}C]taurine efflux, cellular ATP content, and ATP/ADP ratios. Skate hepatocytes were loaded with [^{14}C]taurine and then treated for 30 min with the indicated concentrations of inhibitors at 15°. Control cells received an equal volume of vehicle (dimethylsulfoxide or elasmobranch Ringer solution). Aliquots of the cell suspensions were then removed for ATP and ADP analysis and assessment of the ^{14}C content of the cells, and hypotonicity was induced by 40% dilution of the remaining cell suspensions with either water or Ringer solution (control). Cellular ^{14}C content was measured 30 min after dilution. Inhibitors were not removed before dilution but remained (40% diluted) throughout the efflux period. Values are mean ± standard error of three to six cell preparations, each assayed in duplicate. *DPC*, diphenylamine-2-carboxylate.

sucrose, and 350 mM urea (980 mOsM) and was brought to pH 7.2 with CsOH. Hyposmotic bath solutions were identical to isosmotic solutions except for a decrease in sucrose concentration to yield 650 mOsM. After whole-cell access was obtained, cells were swollen by reduction of bath osmolality. When volume-activated anion current had stabilized, test drugs dissolved in hyposmotic medium were perfused into the bath. Current levels at +60 mV were measured and used to calculate percent inhibition. For experiments with pyridoxal-5-phosphate, cells were preincubated for 15–30 min in isosmotic solution containing the drug at a concentration of 2 mM. Cells were then patch-clamped and swollen in hyposmotic medium containing 2 mM pyridoxal-5-phosphate. Current levels were measured at +60 mV 3 min after swelling and compared with levels in untreated cells.

Results and Discussion

To examine the relation between intracellular ATP content and volume-activated [^{14}C]taurine efflux, skate hepatocytes were treated with increasing concentrations of 2,4-dinitrophenol before exposure to hyposmotic medium. 2,4-Dinitrophenol produced a rapid decrease in ATP content and ATP/ADP ratio to a new base-line level that was maintained for at least 1 hr (Fig. 1). Taurine efflux from the hepatocytes was initiated by a 40% dilution of the medium with water, a maneuver that by itself had no effect on cellular ATP content or ATP/ADP ratio (Fig. 1).

The relation between cellular ATP content and volume-activated [^{14}C]taurine efflux in skate hepatocytes exposed to increasing concentrations of 2,4-dinitrophenol (0–700 μM) is illustrated in Fig. 2A. [^{14}C]Taurine efflux exhibited a roughly sigmoidal relationship with cellular ATP levels. Efflux was inhibited by 50% when cellular ATP declined from approximately 7 nmol/mg of protein (~2 mM) to 4 nmol/mg of protein, with essentially complete inhibition when ATP levels reached 3 nmol/mg of protein. The magnitude of the cell volume increase after dilution with 40% water was similar in the presence and absence of metabolic inhibitors (6). The relation between cellular ADP levels and volume-activated taurine efflux was a mirror image of that observed between

ATP levels and volume-activated taurine efflux (Fig. 2B). When taurine efflux was plotted against cellular ATP/ADP ratios (Fig. 2C), a sigmoidal relation was also observed. There was a gradual inhibition of taurine efflux until the ATP/ADP ratio reached ~2, at which point there was a precipitous decrease in swelling-activated efflux (Fig. 2C). Whole-cell patch recording of volume-activated anion current in skate hepatocytes demonstrates that these effects of 2, 4-dinitrophenol are not directly related to an interaction of this compound with the channel (Fig. 3A).

To characterize this volume-activated channel, we used a series of ion channel blockers and transport inhibitors that have been used extensively to identify and characterize such proteins in other tissues. The effects of these compounds on volume-activated [^{14}C]taurine efflux and cellular ATP and ADP content are illustrated in Fig. 4. NPPB, glibenclamide, diphenylamine-2-carboxylate, ketoconazole, gossypol, niflumic acid, quinine, and phenylarsine oxide inhibited taurine efflux in a concentration-dependent manner; however, these compounds also decreased cellular ATP concentrations and ATP/ADP ratios (Fig. 4A). In contrast, DIDS and pyridoxal-5-phosphate inhibited taurine efflux but had no effect on cellular ATP levels or ATP/ADP ratios (Fig. 4B). In the case of NPPB, a commonly used Cl^- channel blocker, inhibition was most pronounced, decreasing taurine efflux to 18% of control at a concentration of only 50 μM. At this concentration, however, it was an equally powerful metabolic poison, decreasing ATP content and the ATP/ADP ratio to 40% and 14% of control, respectively (Fig. 4). At 100 μM, NPPB completely inhibited taurine efflux and decreased ATP levels to 25% of control. NPPB has previously been demonstrated to inhibit mitochondrial respiration in murine peritoneal macrophages (17) and T_{84} cells (18). Glibenclamide, diphenylamine-2-carboxylate, ketoconazole, gossypol, niflumic acid, quinine, and phenylarsine oxide also produced concentration-dependent ATP depletion and inhibition of taurine efflux (Fig. 4A). Phenylarsine oxide is a trivalent arsenical that inhibits endocytosis but has also been shown to inhibit mitochondrial respiration and deplete ATP (19, 20). The mechanism by which the other compounds deplete ATP in not known. It is important to note that these experiments were done at a relatively high cell density (5%) and that larger effects of the same concentrations of inhibitors were noted at lower cell densities. For example, 10 μM gossypol had no effect on any of the parameters measured when cell density was 50 mg wet weight/ml (Fig. 4). However, the same concentration of gossypol decreased cellular ATP levels by approximately 50% when the cell density was lowered to 20 mg wet weight/ml. Because gossypol and most of the compounds used are lipophilic and partition into cells, the amount of drug taken up by each cell at a given drug concentration is roughly inversely proportional to cell density.

To examine whether the inhibition of taurine efflux was also related to a direct interaction with the channel or could be attributed largely to the ability of these agents to deplete ATP, we performed two additional analyses. First, we replotted the data from Fig. 4A to examine the relation between the extent of ATP depletion and the degree of inhibition of taurine efflux (Fig. 5). These results were compared with the effects of 2,4-dinitrophenol on these same parameters. To facilitate this comparison, the lines from Fig. 2 (effects of 2,4-dinitrophenol) are reproduced in Fig. 5 (*dashed lines*). The effects of the transport inhibitors on ATP and ADP levels and taurine efflux closely follow the relationship defined by these lines, indicating that the inhibitory effects of these compounds may be explained largely by their ability to deplete ATP and/or increase ADP. This conclusion was further supported by electrophysiologic experiments using NPPB and ketoconazole, demonstrating that these compounds do not inhibit volume-activated anion current (Fig. 3A) and thus do not directly block the channel.

These findings are in contrast to the effects of DIDS and pyridoxal-5-phosphate. Both of these compounds inhibited [^{14}C]taurine efflux without altering cellular ATP levels (Fig. 4B). In addition, DIDS inhibited whole-cell anion current (Fig. 3). The inhibition was rapid (<60 sec) and voltage

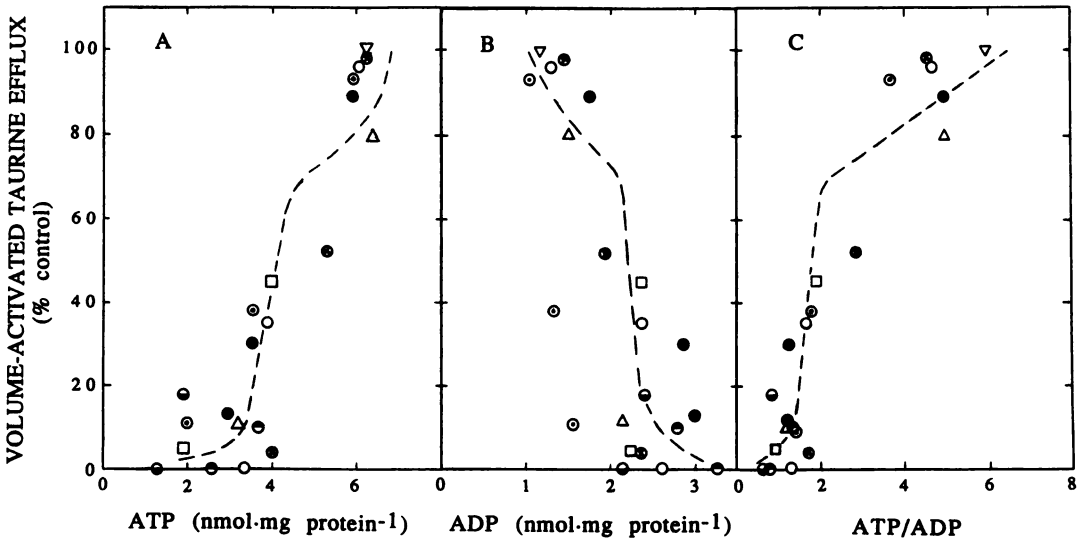


Fig. 5. Data from Fig. 4A replotted to illustrate the relation between volume-activated [^{14}C]taurine efflux and ATP content, ADP content, and ATP/ADP ratio in control (▽) skate hepatocytes and those treated with NPPB (◒), glibenclamide (○), diphenylamine-2-carboxylate (◓), ketoconazole (⊗), gossypol (●), niflumic acid (□), quinine (△), and phenylarsine oxide (⊙). A, ATP content; B, ADP content; C, ATP/ADP ratio. Concentrations of inhibitors are provided in Fig. 4A. *Dashed lines*, replotted from Fig. 2 to facilitate comparison.

dependent, suggesting that DIDS interacts directly with the channel protein. Pyridoxal-5-phosphate also inhibited whole-cell anion current, albeit in a different manner than DIDS. When swollen cells were exposed directly to the drug, whole-cell anion current dropped very slowly or was largely unaffected (data not shown). However, if the cells were pretreated with the drug for 15–30 min and then swollen, activation of whole-cell current was dramatically inhibited. Anion current that was measured 3 min after induction of swelling in cells pretreated with pyridoxal-5-phosphate was inhibited by 92 ± 4% (three experiments), compared with untreated control cells. The slow inhibition of whole-cell current by pyridoxal-5-phosphate suggests that the drug may be acting from an intracellular site. Studies of ion-transporting ATPases have shown that pyridoxal-5-phosphate covalently modifies ATP binding sites (21, 22). Although the mechanism by which pyridoxal-5-phosphate inhibits anion current and taurine efflux is not known, it may be through an interaction with an ATP binding site on the channel itself or associated regulatory proteins.

These findings have additional significant implications. First, this volume-activated channel in skate hepatocytes, which may be comparable to channels conducting organic osmolytes in other cell systems (6, 9–12), requires intracellular ATP. Taurine release by this mechanism is progressively inhibited over a narrow range of ATP and ADP concentrations, indicating that alterations in these cellular nucleotides can modulate the opening of the channel within a range that should not disrupt many other ATP-dependent cellular functions (8). Second, DIDS is known to inhibit a large number of membrane transport systems, including volume-activated anion channels. The present findings suggest that DIDS interacts directly with the channel. Third, although selective inhibitors of membrane transport proteins are often invaluable tools for their identification and characterization and may be useful as drugs in the treatment of a number of disorders (23), many of these chemicals, particularly the ion channel blockers, exhibit relatively low selectivity and frequently interact with multiple transport proteins (24). Our findings demonstrate that some of these compounds have an additional major metabolic effect, namely the depletion of ATP. In the case of the volume-activated taurine channel and perhaps other ATP-dependent transport systems, ATP depletion appears to be the mechanism of action of the putative transport inhibitors. This possibility must be considered in the evaluation of the effects of these inhibitors when whole-cell preparations are used.

Acknowledgments

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