Cholinergic stimulation of the Na^+/K^+ adenosine triphosphatase as revealed by microphysiometry

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ABSTRACT The activation of a wide range of cellular receptors has been detected previously using a novel instrument, the microphysiometer. In this study microphysiometry was used to monitor the basal and cholinergic-stimulated activity of the Na^+/K^+ adenosine triphosphatase (ATPase) (the Na^+/K^+ pump) in the human rhabdomyosarcoma cell line TE671. Manipulations of Na^+/K^+ ATPase activity with ouabain or removal of extracellular K^+ revealed that this ion pump was responsible for 8.8 ± 0.7% of the total cellular energy utilization by those cells as monitored by the production of acid metabolites. Activation of the pump after a period of inhibition transiently increased the acidification rate above baseline, corresponding to increases in intracellular $[Na^+]$ ($[Na^+]_i$) occurring while the pump was off. The amplitude of this transient was a function of the total $[Na^+]_i$ excursion in the absence of pump activity, which in turn depended on the duration of pump inhibition and the Na^+ influx rate. Manipulations of the mode of energy metabolism in these cells by changes of the carbon substrate and use of metabolic inhibitors revealed that, unlike some other cells studied, the Na^+/K^+ ATPase in TE671 cells does not depend on any one mode of metabolism for its adenosine triphosphate source. Stimulation of cholinergic receptors in these cells with carbachol activated the Na^+/K^+ ATPase via an increase in $[Na^+]_i$ rather than a direct activation of the ATPase.

INTRODUCTION

We recently have found that the activation of a wide variety of cellular receptors promptly leads to a significant increase in the rate at which cultured cells acidify their environment (1–4). The amplitude and kinetics of the effect vary with the receptor and cell type but typically involve a 10–100% increase in acidification rate within seconds to minutes after the application of agonist. To measure extracellular acidification rates, we have used the microphysiometer, an instrument that applies contemporary silicon technology to quantitative cell biology. The instrument uses a potentiometric semiconductor-based sensor to detect pH changes adjacent to cultured cells in a microvolume flow chamber.

At present, our understanding of the receptor pharmacology of the phenomena is greater than our understanding of the intracellular mechanisms that couple receptor activation to proton flux. The primary motivation for the present study was to gain such mechanistic information. A secondary goal was to determine whether microphysiometry could be a useful tool for cell-biology studies involving ion pumps, since it has the attractive properties of being noninvasive and providing data in real time.

What is known about how receptor activation changes the rate of extracellular acidification? In some cases, receptor activation increases the activity of the Na^+/H^+ antiporter system, causing a transient increase in extracellular acidification rate and concomitant increase in intracellular pH (5, 6). However, the most generally important source of sustained changes in extracellular acidification rate is changes in the rate of energy metabolism. The synthesis and subsequent hydrolysis of adenosine triphosphate (ATP) produce acid, typically in the form of lactic acid and CO_2. This statement is true for the carbon sources most commonly used by mammalian cells, and it holds for both glycolysis and oxidative metabolism. For a review, see Owicki and Parce (7).

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The rate of energy metabolism might sometimes be increased on receptor activation by a direct modification of the activity of key regulatory enzymes involved in energy metabolism. There is evidence for this (6, 8). The rate also might be raised by increases in the rates of processes that consume ATP. In this article we examine one such process, the maintenance of the Na^+ and K^+ gradients across the plasma membrane by the Na^+/K^+ ATPase, also called the Na^+/K^+ pump. This pump, which ejects three Na^+ from the cell and imports two K^+ at the cost of one ATP hydrolyzed, is a major sink of free energy in many cell types (see the review by Clausen et al. [9]).

Using a muscle tumor cell line (the rhabdomyosarcoma TE671) as a model system, we first show that it is possible to use the microphysiometer to detect the activity of the Na^+/K^+ pump. This involves examining the coupling, through ATP turnover, between the intracellular sodium-ion concentration ($[Na^+]_i$) and extracellular acidification. Because of reports that in some cells the Na^+/K^+ pump is preferentially fueled either by glycolysis or oxidative respiration, we then investigate the effects of changes in the mode of energy metabolism on pump activity. Next, we demonstrate that part of the increase in extracellular acidification rate on activation of cholinergic receptors in TE671 cells is due to increased activity of the Na^+/K^+ pump. Finally, we present evidence that the increased pump activity results from a receptor-mediated increase in $[Na^+]_i$ rather than some more direct regulation of the pump activity.

MATERIALS AND METHODS

Materials

Culture medium was obtained from Irvine Scientific (Santa Ana, CA). The Na^+ indicator sodium-binding benzofuran isophthalate (SBFI) acetoxymethyl ester, as well as Pluronic F-127, were from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma (St. Louis, MO).

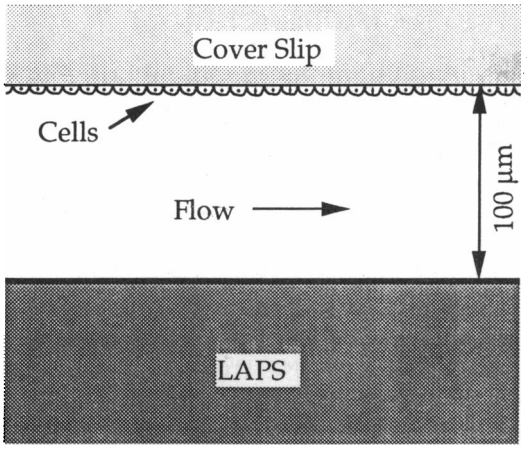

FIGURE 1 Cross-sectional view of the microphysiometer chamber. See text in Materials and Methods.

Cell culture

Cells of the adherent human rhabdomyosarcoma cell line TE671 were obtained from the American Type Culture Collection (Rockville, MD; line CRL8805). Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10 mM glucose, 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and buffered with bicarbonate. Cells were maintained at 37°C in 95% air/5% CO_2. Cultures were plated routinely at densities of 200/mm² in T-75 flasks, or onto either 12-mm-diameter cover slips coated with indium-tin oxide or uncoated 25-mm-diameter cover slips, in 12- or 6-well tissue-culture trays, respectively. The oxide-coated cover slips were used in the microphysiometer, and there were no apparent differences between cells grown on uncoated or oxide-covered cover slips. Cells were used at 90–100% confluence.

Solutions

Unless otherwise noted, all experiments were performed in the following medium, comprising balanced salts plus glucose (mM): NaCl, 138; KCl, 5; $MgCl_2$, 0.5; Na_2HPO_4, 0.81; NaH_2PO_4, 0.11; $CaCl_2$, 1.3; and glucose, 10, pH 7.4. The phosphate concentration in this solution was low to make the buffer capacity low and thus facilitate measurement of acidification rates. In experiments using pyruvate salts, the glucose was replaced by 10 mM pyruvate and 2 or 5 mM 2-deoxyglucose (2-DG).

The microphysiometer

As shown in Fig. 1 and described in more detail by Parce et al. (10) and McConnell et al. (4), the microphysiometer is based on a microflow chamber in which cells are in diffusive contact with a semiconductor-based pH sensor, the light-addressable potentiometric sensor (LAPS) (11). In this version of the instrument, the ceiling of the flow chamber was a glass cover slip that had been rendered conductive with a coating of indium-tin oxide, to which cells adhered. The sensor formed the floor of the chamber. The dimensions of the fluid channel were 100 μm high by 3.5 mm wide by 12 mm long, or 4 μl in volume. Other versions of the microphysiometer, not used in this study, use flow chambers in which the cells are retained between two microporous polycarbonate membranes rather than attached to cover slips.

The LAPS essentially reports a surface potential at the electrolyte/sensor interface that depends on pH in a Nernstian fashion (61 mV per pH unit change at 37°C). There are three electrical connections: to the base of the LAPS, to a control electrode (the conducting surface of the cover slip), and to a reference electrode downstream from the chamber. There is negligible direct current in the system. The surface potential is determined only at regions of the sensor illuminated by a light source that is intensity modulated at 5 kHz, and so the sensor signal is carried in the amplitude of a weak 5 kHz photocurrent. Since the illuminated region is only ~3 mm², data were obtained from a 300-nl portion of the flow chamber.

The flow of culture medium, as well as selection of fluid stream and data acquisition, was controlled by a personal computer. Extracellular acidification rates were determined as the rate of change of sensor output during periodic interruptions of fluid flow, which cause transient acidifications of <0.1 pH unit. Generally fluid flow alternated off and on (100 μl/min) for equal 15-s periods. One microvolt per second corresponds closely to an acidification rate of $-1 \cdot 10^{-3}$ pH/min. Results are reported in microvolts per second or, in the case where different chambers are directly compared, as normalized acidification rate. The latter takes into account variations in acidification rates among

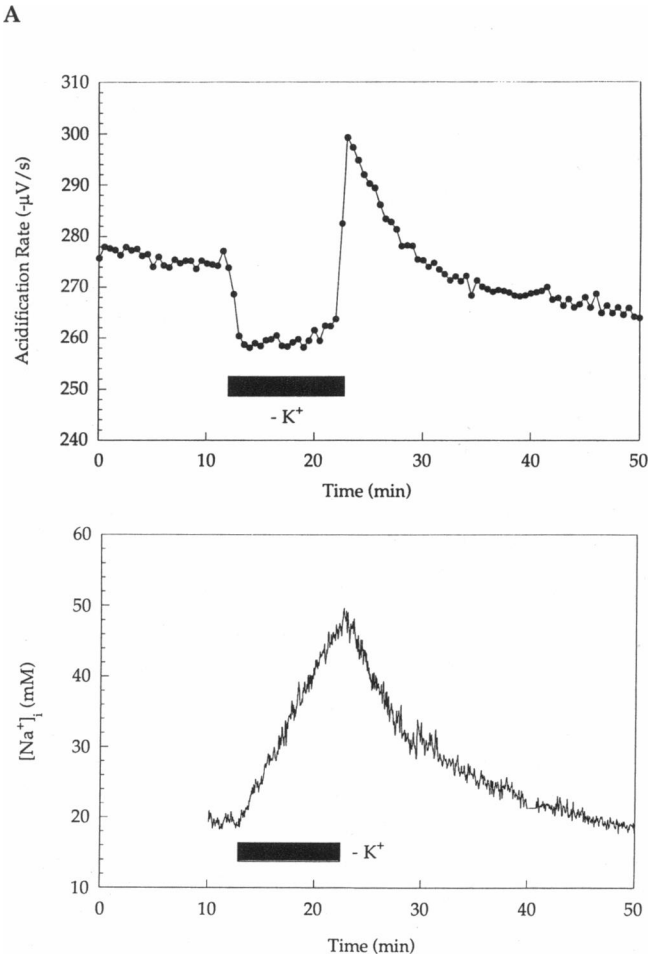

FIGURE 2 Inhibiting the Na^+/K^+ ATPase by removing extracellular K^+ changed the extracellular acidification rate. (*A*) Removal of extracellular K^+ decreased the acidification rate, and replacement of the K^+ after 10 min caused an overshoot that decayed to the original basal acidification rate. (*B*) Measurement of $[Na^+]_i$ verified that inhibiting the Na^+/K^+ ATPase caused the accumulation of Na_i^+. The kinetics of decay to basal $[Na^+]_i$ after extracellular K^+ was replenished were similar to those for the return to basal acidification rate in *A*.

chambers resulting from differing absolute numbers of cells in those chambers.

Intracellular Na^+

TE671 cells growing on 25-mm cover slips were incubated for 1 h at 37°C in medium augmented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, containing 4 μM SBFI mixed beforehand in a 1:1 suspension with a 25% wt/vol solution of Pluronic F-127/dimethyl sulfoxide. The cover slip was then transferred to a petri dish containing fresh medium and incubated for another 30 min to allow complete de-esterification of the dye.

The cover slip was then transferred to a thermostatically controlled (37°C) closed perfusion chamber with a fluid flow path 100 μm in height by 3.4 mm across, and perfused at a rate of 50 μl/min. A switching valve placed before the chamber allowed changeover of solution in the chamber within 30 s. The geometry of the chamber, the mean flow rate, and the perfusate solutions were essentially identical to those of the microphysiometer chamber. The chamber was placed on the stage of an inverted microscope (Diaphot; Nikon Inc., Melville, NY) for viewing and fluorescence measurements.

Fluorescence was measured in a Photon Technology Inc. (model 4000; Deltascan, South Brunswick, NJ) dual-excitation spectrofluorometer. The excitation monochromators were set at 350 and 385 nm (bandwidth of 1 nm), and the fluorescence was measured at 510 nm (bandwidth 20 nm) as the ratio of the intensities resulting from excitation at 350 and 385 nm. Calibrations were usually performed at the end of each experiment, except where dye loss made calibration impossible. Calibrations were performed using the high-K^+ technique of Negulescu et al. (12), with calibration salts of 0, 20, 30, 50, and occasionally 100 mM Na^+ containing 10 μM gramicidin D. We have made no corrections for nonideality or for buffering of $[Na^+]_i$ by the fluorophore; neither should be an important effect, and so Na^+ levels are reported as concentrations. Backgrounds were initially determined by fluorescence measurements of unloaded cells, and in later experiments simply by removal of the experimental cells within the chamber via fast perfusion with distilled water (there was no significant cell autofluorescence).

The field measured typically contained ~100 cells. Thus, the signal measured was an average response of all the cells in the field, as for the microphysiometer measurements.

RESULTS AND DISCUSSION

Effects of K-free solutions and ouabain

TE671 cells were placed in the microphysiometer and perfused with medium. When the solution bathing the cells was switched to K^+-free medium to inhibit the Na^+/K^+ adenosine triphosphatase (ATPase), the acidification rates of the cells dropped quickly (<30 s) by 8.8 ± 0.7% (mean ± SEM, $n = 83$) (Fig. 2 *A*). The magnitude of the drop corresponds to the steady-state activity of the Na^+/K^+ ATPase in the balanced salt solution, which most likely is rate limited by the intrinsic "leakiness" of the cells to Na^+. This "leakiness" is the sum of all inward fluxes of Na^+, including Na^+-coupled transport, Na^+ channels, and passive membrane permeabilities. Upon restoration of extracellular K^+, the acidification rates rose quickly to values higher than those obtained before removal of K^+. The rates then decayed to the original values with an approximately exponential time course ($t_{1/2} = 272 \pm 6.5$ s, $n = 66$).

One possible explanation for this transient elevation of acidification rate is that $[Na^+]_i$ may have increased during the period in which the Na^+/K^+ pump was inhibited. Restoration of extracellular K^+ would then have resulted in increased ATP hydrolysis and corresponding increased extracellular acidification, until the normal $[Na^+]_i$ was restored. To test this hypothesis, $[Na^+]_i$ was monitored with SBFI in TE671 cells, while K^+ was transiently removed from the bathing medium. Fig. 2 *B* shows that a steady rise in $[Na^+]_i$ (~3 mM/min) occurred in the absence of extracellular K^+. Replacement of K^+ caused the $[Na^+]_i$ to return to its original value, presumably due to increased activity of the Na^+/K^+ pump. The excess $[Na^+]$ in the cell decayed with an approximately exponential time course ($t_{1/2} = 287 \pm 28$ s, $n = 16$).

The similarity of the kinetics of the return to baseline of the two processes upon replacement of K^+ indicates close coupling between acidification-rate changes and ATP-flux changes in the cells resulting from changes in the Na^+/K^+ ATPase activity. These results are quite consistent with those of other workers who have studied the effects of ouabain or K^+ removal on voltage-clamped currents (13–18). In those studies the authors looked at the current resulting from the electrogenic Na^+/K^+ pump as a fraction of the total current, analogous to our looking at the fraction of the total cellular acidification rate corresponding to the Na^+/K^+ pump activity. The metabolic analogue of the holding voltage is the cellular maintenance of a steady-state ATP level.

Although the removal of K^+ from the bathing salts is expected to inhibit the Na^+/K^+ ATPase, it may have other effects, such as a membrane hyperpolarization, that could cause changes in metabolism and thus affect acidification rates of the cells. Another way to block Na^+/K^+ ATPase activity is to use the inhibitor ouabain. As shown in Fig. 3 *A*, ouabain caused a drop in acidification rate (8%) similar to that caused by K^+ removal. Removal of ouabain resulted in a slow recovery of acidification rate, consistent with the slow rate of dissociation of ouabain from the enzyme (18). Clearly the dominant effect of removal of K^+ from the medium on the acidification rate was due to inhibition of the Na^+/K^+ ATPase. If in fact the overshoot in acidification rate upon K^+ replacement were due to enhanced Na^+/K^+ ATPase activity, then this overshoot should be inhibited by ouabain. Fig. 3 *B* shows that if ouabain was present while K^+ was reapplied to the salts after its removal for 10 min, the reactivation of acidification was indeed inhibited. Thus, both the decrease in acidification rates after removal of extracellular K^+ and the overshoot upon its replacement appear to provide a real-time measure of the Na^+/K^+ ATPase activity.

Effects of changes in $[Na^+]_i$ and Na^+ permeability, p_{Na}

Another approach to demonstrate the tight coupling between the extracellular acidification rate and Na^+/K^+ pump activity is to modulate the enzyme's activity by

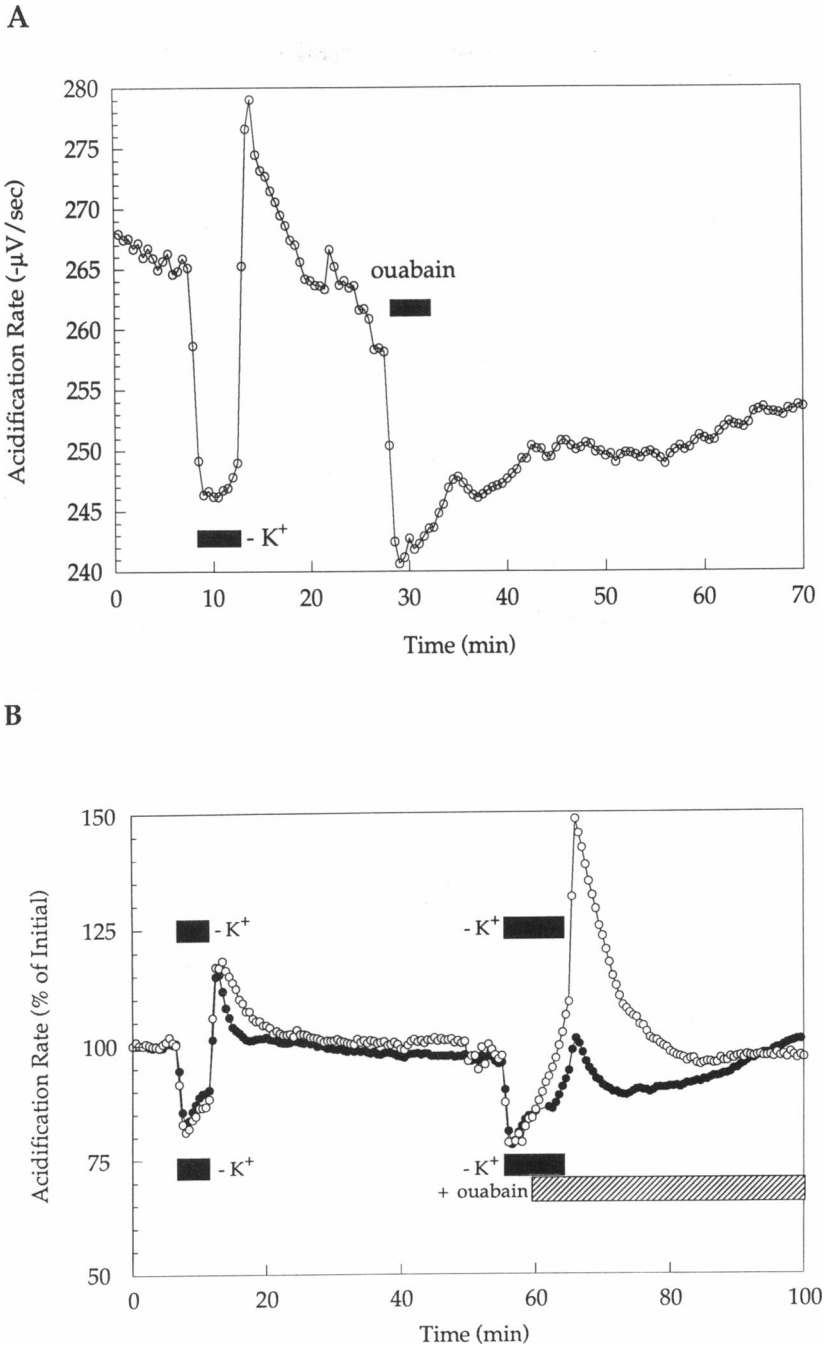

FIGURE 3 Ouabain and the removal of extracellular K^+ decreased acidification rates to similar extents, and ouabain blocked the acidification overshoot when K^+ was restored. (*A*) Extracellular K^+ was removed for 5 min, causing the acidification rate to decrease and then overshoot, as in Fig. 2 *A*. Subsequent application of 100 μM ouabain for 5 min caused a similar decrease that recovered slowly after ouabain removal, presumably due to the slow unbinding kinetics of ouabain. (*B*) In one chamber (*open circles*), extracellular K^+ was removed for first 5 min and then 10 min. In a second chamber (*closed circles*), K^+ was removed in the same fashion, but 100 μM ouabain was introduced midway through the second K^+ deprivation and remained present through the end of the experiment. Ouabain inhibited the overshoot in acidification rate, which suggests that the overshoot represents the energetic consequences of Na^+/K^+ pump activity rather than some other effect of K^+ deprivation. The rise in acidification rates during long periods of K^+ deprivation may be related to increases in $[Na^+]_i$.

changing $[Na^+]_i$. Fig. 2 *B* demonstrates that $[Na^+]_i$ can be varied by controlling the time cells are exposed to K^+-free medium. Therefore, the overshoot in acidification rates upon K^+ restoration should vary with the amount of time K^+ was withheld from the cells.

Fig. 4 *A* shows acidification rates in four chambers run in parallel with cells treated identically. The responses to increasing periods of exposure to K^+-free medium were quite similar among the four chambers: the amplitude of the decrease in acidification rate due to K^+ removal re-

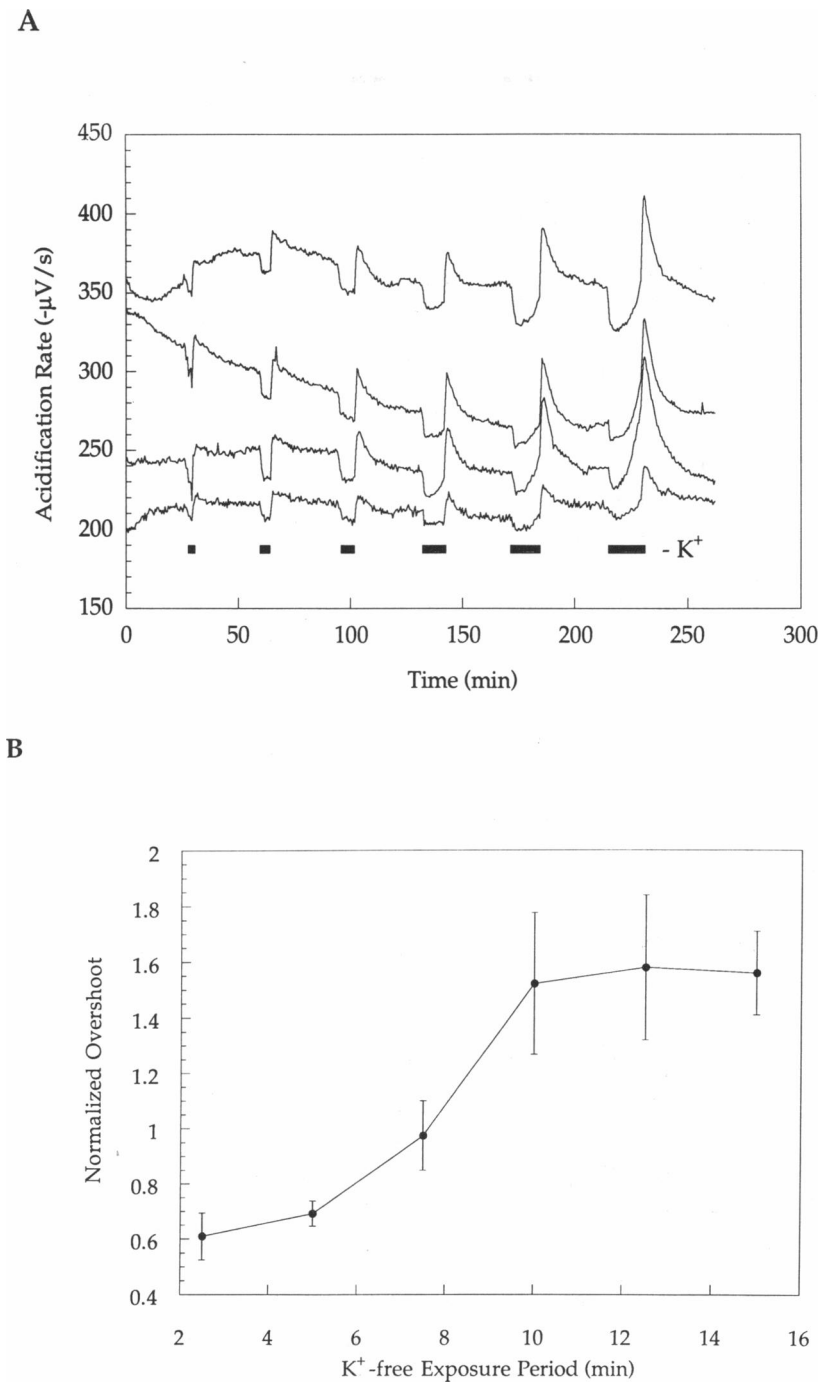

FIGURE 4 Increasing the time of K^+ deprivation increased the amplitude of the acidification overshoot. (*A*) Cells in four chambers were deprived of extracellular K^+ for increasing periods (from 2.5 up to 15 min). In each chamber the amplitude of the replenishment overshoot increased with deprivation time, as would be expected if the overshoot represents the extra activity of the Na^+/K^+ ATPase as it restores basal $[Na^+]_i$. There is some variation among the chambers in the amplitudes of the effects of K^+ deprivation. This presumably is due to differences in leakiness of the cells and, secondarily, in Na^+/K^+ pump activity. (*B*) The amplitudes of the overshoots in *A* rise monotonically with deprivation time and appear to saturate. Overshoots were measured as the amplitude of the peak upon K^+ restoration minus the difference between the basal rate just prior to K^+ removal and the value during the period of K^+ removal. The data were normalized by the decrease in acidification rate during K^+ deprivation immediately preceding the overshoot to correct for the variability in absolute response discussed above.

mained constant for a given chamber, whereas the amplitude of the overshoot increased with increasing lengths of time the Na^+/K^+ pump was off. For the longest K^+-free incubation periods, there was some increase in the acidification rate while the Na^+/K^+ pump was inhibited, probably reflecting the response of a variety of metabolic consequences of altering significantly the ionic composition of the cytoplasm. This effect is also observed during exposure of cells to ouabain with K^+ present in the medium (Fig. 3 *B*).

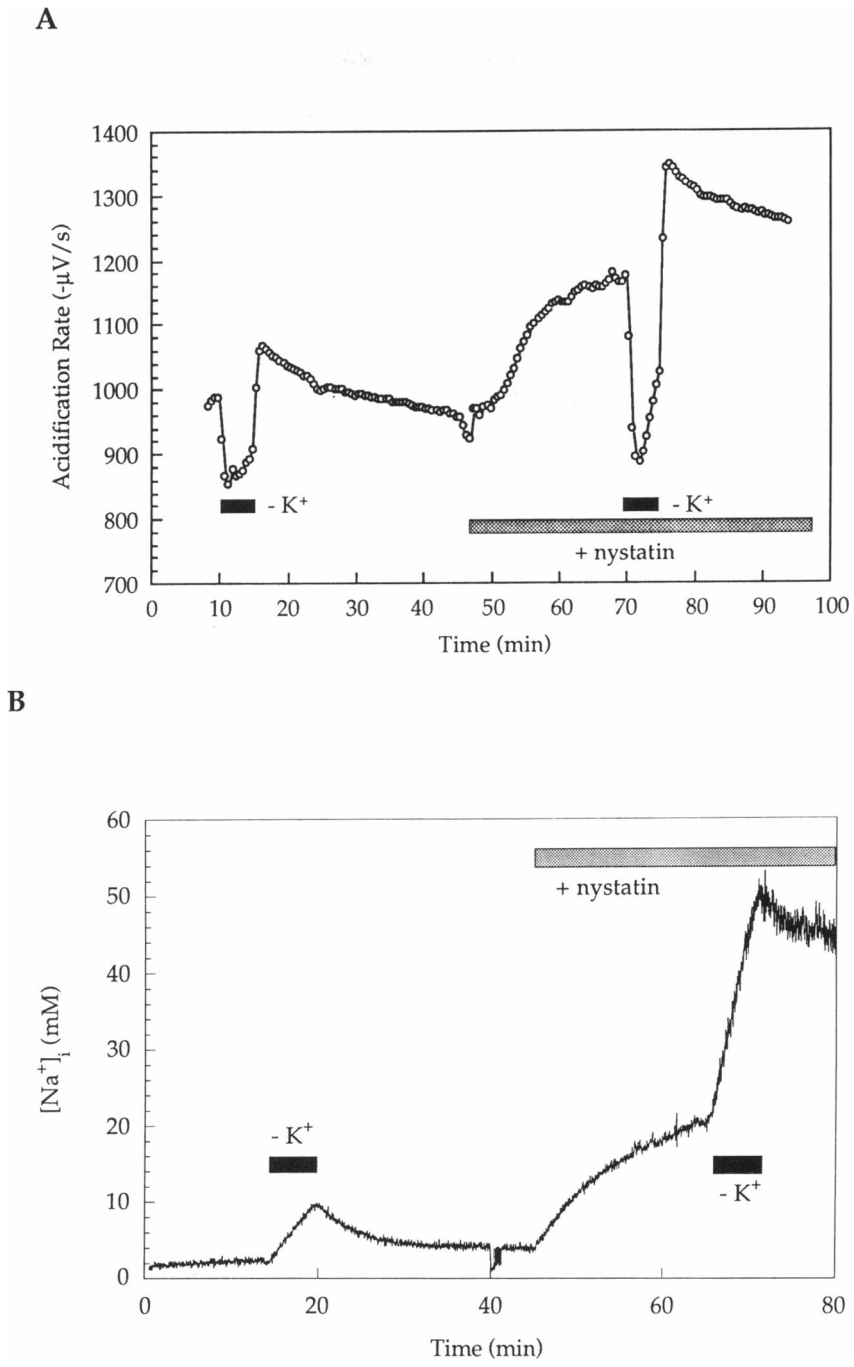

FIGURE 5 The amplitude of the acidification overshoot correlates with membrane permeability to Na^+. The permeability of the plasma membrane for Na^+ was increased by exposing the cells to 25 μg/ml of the ionophore nystatin. (*A*) Nystatin increased the acidification rate when the Na^+/K^+ ATPase was active but had little effect on the acidification rate during periods when the Na^+/K^+ ATPase was inhibited by removal of extracellular K^+. Nystatin increased the overshoot upon replenishment of K^+. These effects of nystatin are consistent with the hypothesis that the effects of K^+ deprivation on acidification rate reflect the activity of the Na^+/K^+ pump. (*B*) Intracellular Na^+ measurements confirm that nystatin increased $[Na^+]_i$ as well as the rate of increase in $[Na^+]_i$ when extracellular K^+ was removed.

Analysis of the data in Fig. 4 *A* demonstrates a correlation between the period of Na^+/K^+ pump inhibition and amplitude of the overshoot (Fig. 4 *B*). This effect appears to level off for inhibition times greater than 10 min, possibly reflecting saturation of the Na^+/K^+ pump with Na_i^+. Similar results were obtained with another adherent cell line (CHO-K1), indicating that these results are not unique to the TE671 cell line (data not shown).

Ionophores provide a second method for manipulating $[Na^+]_i$. Addition of nystatin, a monovalent cationophore, to cells in the microphysiometer caused the acidi-

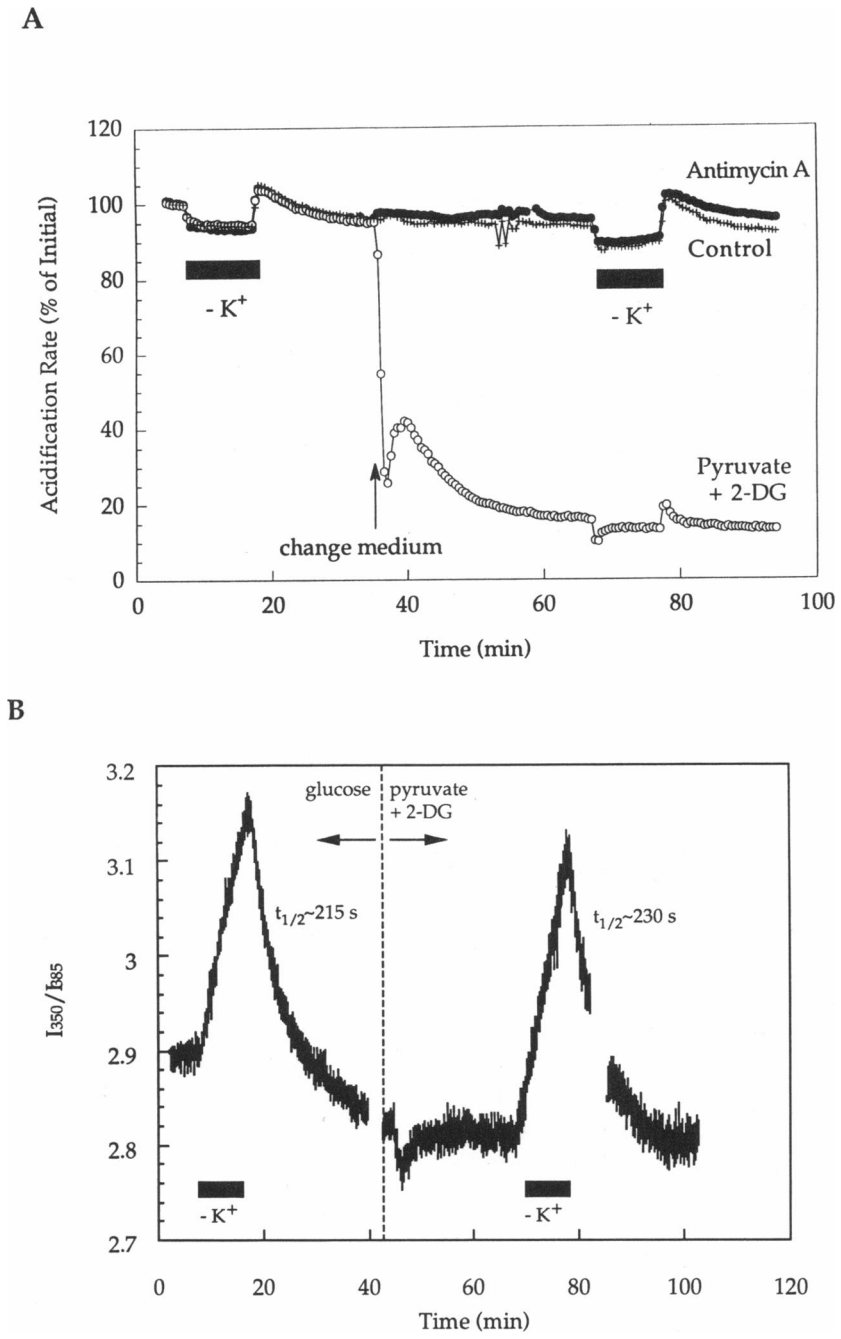

FIGURE 6 The steady-state activity of the Na^+/K^+ ATPase is independent of whether the energy metabolism of the cells is glycolytic or oxidative. (*A*) Acidification rates in three chambers responded essentially identically to a 10-min deprivation of K^+. One chamber (*solid circles*) was then forced to be completely glycolytic by exposure to 5 μM antimycin A. The second (*open circles*) was forced into purely oxidative metabolism by removal of glucose and substitution with 10 mM pyruvate and 2 mM 2-DG. The third chamber (*plus signs*) remained on glucose-containing salts as a control. Subsequent removal of extracellular K^+ for 10 min decreased the acidification rates by the same percent of the new steady-state rate for all three treatments. A representative experiment is shown. (*B*) Measurements of $[Na^+]_i$ were made on cells in a single chamber sequentially under control (predominantly glycolytic) and oxidative conditions as above. The $[Na^+]_i$ basal level did not change between the two conditions. The kinetics of recovery from 10-min deprivation of K^+, a measure of Na^+/K^+ ATPase activity, were quite similar under the two conditions. The break in the SBFI data resulted when the memory buffer on the computer became full and it was necessary to restart data accumulation (see also Fig. 7 *B*).

fication rate to rise to an elevated steady-state value (Fig. 5 *A*). Subsequent removal of K^+ resulted in an acidification rate similar to that measured in the absence of K^+ before addition of nystatin. These data indicate that the elevation in acidification rate caused by nystatin is primarily due to an increase in activity of the Na^+/K^+ pump, probably due to the increased flux of Na^+ into the cells. Analogous results were obtained in studies of monensin effects on the ouabain-suppressible clamp current in chick myocytes (18). Also consistent with a nystatin-

mediated increase in Na^+ flux into the cells is the increased acidification overshoot upon replacement of K^+. As expected, direct measurement of $[Na^+]_i$ revealed an increased steady-state concentration due to nystatin addition as well as a very rapid rise in concentration upon K^+ removal (Fig. 5 *B*). Similar results were obtained with monensin and gramicidin D (data not shown).

Thus, both the amplitude of the undershoot of acidification rates occurring during K^+ deprivation and the overshoot resulting from its replacement are indicators of changes in p_{Na} and $[Na^+]_i$. The overshoot, which reflects the time integral of Na^+ influx during K^+ removal, is the more sensitive indicator of such changes. These observations, coupled with the ouabain sensitivity of the overshoot and the similarity of the kinetics of the return of the acidification rate transient to baseline and the SBFI measurements of the kinetics of the return of $[Na^+]_i$ to baseline, argue very strongly that the changes in acidification rates seen with these manipulations do indeed track the activity of the Na^+/K^+ pump in these cells.

Dependence of the Na^+/Ka^+ pump on mode of energy metabolism

Having established that the activity of the Na^+/K^+ pump can be monitored with the microphysiometer, we next examined whether the pump activity in TE671 cells selectively depended on glycolysis or respiration as a source of ATP. These studies were motivated by reports demonstrating that different cellular processes may sometimes be fueled preferentially from one or the other of these major pathways of energy metabolism, independent of the overall balance between the two pathways in the cell. Brain and kidney are the two organs that expend the most energy on the Na^+/K^+ pump (19). Oxidative metabolism accounts for nearly all the ATP used by the pump in brain, and only part of that used in renal tubules (20), Rous-transformed hamster cells and Ehrlich ascites cells (21), or the renal cell line MDCK (22, 23). In vascular smooth muscle, Paul and collaborators (24) found that ATP derived from aerobic glycolysis was primarily used for transport functions, whereas ATP originating from oxidative metabolism was used to drive contractile processes. Similar findings were reported for the rabbit heart by Weiss and Hiltbrand (25). The conclusions above were based on experiments in which the Na^+/K^+ pump activity was manipulated and the resultant changes in O_2 consumption and/or lactate production were examined or, conversely, in which the mode of energy metabolism was changed and the effects on Na^+/K^+ pump activity were determined.

We chose the latter approach and forced the cells into one mode of energy metabolism or the other by using inhibitors of glycolysis or oxidative metabolism and by manipulating carbon source. Addition of antimycin A, a site 2 blocker of the electron transport chain and a nearly universal activator of glycolysis, caused a very slight increase in metabolic rate, as can be seen in Fig. 6 *A*. An increase is expected since completely glycolytic cells should produce six times the number of protons as cells depending completely on oxidative metabolism of glucose and assuming a constant ATP turnover rate (7). The minor increase shown here (~3%) indicates that these cells are normally almost entirely glycolytic. This is not surprising given their tumor origin and that fact that cells in culture (as opposed to in vivo) commonly are primarily glycolytic (22, 26–29).

Substitution of glucose with pyruvate and the glycolytic inhibitor 2-DG decreased the basal acid production rate by ~85% compared with that of the control cells. Since the theoretical decrease in acidification rate for a shift from glycolysis to oxidative metabolism of pyruvate at constant ATP turnover rate is 87% (7), these data are evidence that the ATP turnover rate is independent of the mode of energy metabolism.

Upon removal of K^+ from the medium, the acidification rates dropped by ~9%, regardless of whether the cells were in glycolytic ($8.1 \pm 0.9\%$; mean ± SEM, $n = 8$), oxidative ($10.0 \pm 0.8\%$, $n = 10$), or control energy-metabolism mode ($8.8 \pm 0.7\%$, $n = 83$). Assuming that the manipulations with carbon source and inhibitors did not change $[Na^+]_i$, the fact that the drop in acidification rate due to K^+ removal was nearly the same percentage in all cases implies that the ATP for the Na^+/K^+ pump is equally provided for by either glycolysis or oxidative metabolism. Fig. 6 *B* shows that the $[Na^+]_i$ indeed did not change when the cells were shifted to oxidative metabolism from a (largely) glycolytic state. Furthermore, the kinetics of the changes in $[Na^+]_i$ due to removal and replacement of extracellular K^+ were essentially identical for the two metabolic conditions. Thus, the activity of the Na^+/K^+ pump was invariant with respect to mode of energy metabolism even under conditions with slight Na^+ loading. The insensitivity of the Na^+/K^+ ATPase to the source of ATP is also demonstrated by the fact that the overshoot of acidification rate in response to K^+ replacement also scales with the basal acidification rate, independent of mode of cellular energy metabolism. This observation is also indicative of the tight coupling of ATP synthesis and hydrolysis in these cells. Since the same amount of ATP was hydrolyzed regardless of metabolic mode to remove the excess Na^+ accumulating during the period of K^+ deprivation, the same number of excess protons resulting from that hydrolysis should have been produced. These protons should have appeared outside the cells due to the cellular pH_i homeostasis mechanisms. That they did not most likely was a result of near simultaneous uptake of those protons via ATP synthesis.

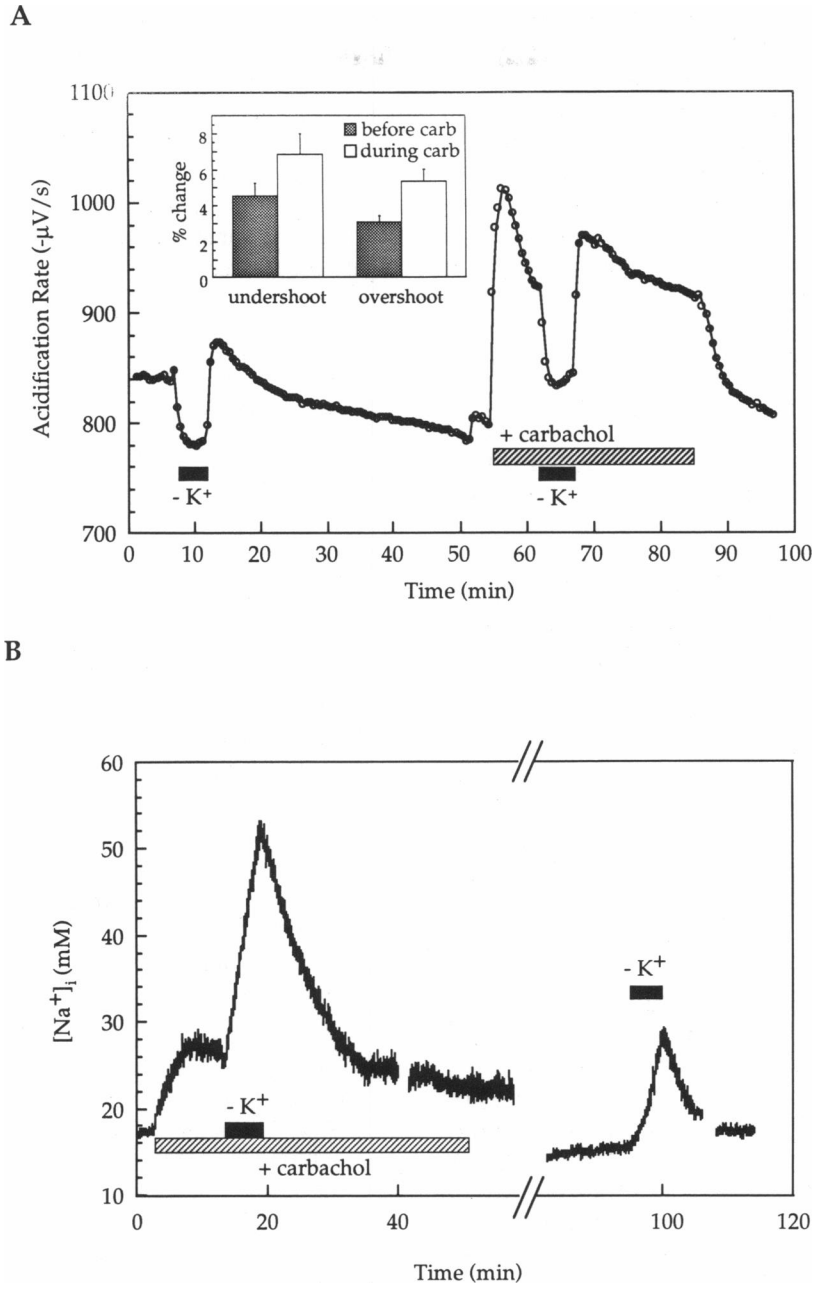

FIGURE 7 Stimulation of muscarinic receptors in TE671 cells increased the activity of the Na^+/K^+ ATPase via increase in $[Na^+]_i$. (*A*) The response of acidification rate to stimulation with 1 mM carbachol was an increase that peaked and decayed to a higher plateau while the agonist remained present. Deprivation of extracellular K^+ for 5 min during carbachol stimulation causes a larger decrease in acidification rate than deprivation before stimulation. The inset summarizes the results of six such measurements on independent chambers. (*B*) Treatment with 1 mM carbachol increased $[Na^+]_i$ and increased the rate of accumulation of Na_i^+ during deprivation of extracellular K^+ but did not alter the kinetics of return to basal $[Na^+]_i$ after the restoration of K^+.

Increase in NaK^+/K^+ ATPase activity by cholinergic stimulation

TE671 cells express both nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors (30–33). The nAChR expressed has properties of skeletal muscle nAChR, and, based on binding studies, Bencherif and Lukas (32) have assigned the mAChR to the M_3 subclass. We have demonstrated previously that triggering acetylcholine receptors on TE671 cells with the agonist carbamyl choline (carbachol) results in immediate and significant increases in extracellular acidification rates (33). These responses are completely attributable to stimulation of the muscarinic acetylcholine receptors based on the ability of atropine to block the response. Increased acidification rates may be in part due to increased activity of the Na^+/K^+ ATPase in response to

receptor triggering. Such increases could be caused either indirectly by a rise $[Na^+]_i$ or directly by activation of the Na^+/K^+ pump. The activity of the Na^+/K^+ pump has been found to be modulated in a number of systems (34, see also 35–43), usually as a result of changes in $[Ca^{++}]_i$ and/or protein kinase C activation. Since activation of the mAChR in TE671 cells results in an increase in inositol trisphosphate concentration (32) and an increase in $[Ca^{++}]_i$ (Miller, D. L., unpublished observations), it seemed reasonable to suspect there might be some Na^+/K^+ pump modulation in these cells as well.

To determine the role of the Na^+/K^+ pump in the acidification response to receptor triggering, we tested the response of the cells to exposure to K^+-free medium before and during exposure to 1 mM carbachol (Fig. 7 *A*). The response of the cells to carbachol was robust, with the acidification rate increasing by 27.5 ± 3.0% (n = 7). Removal of K^+ from the medium resulted in a larger decrease in acidification rate in the presence of carbachol (6.8 ± 1.2% versus 4.5 ± 0.7% before carb, n = 6), indicating enhanced Na^+/K^+ pump activity due to receptor triggering. Replacement of K^+ resulted in a larger acidification-rate overshoot, suggesting that carbachol-enhanced pump activity is due to an increased flux of Na^+ into the cells (3.1 ± 0.4% before carbachol versus 5.4 ± 0.7% after, n = 6).

To confirm this hypothesis, we examined changes in $[Na^+]_i$ directly with SBFI during the carbachol response (Fig. 7 *B*). As expected, carbachol increased $[Na^+]_i$ as well as the rate of Na_i^+ accumulation upon removal of K^+. The presence of carbachol did not alter the kinetics of the removal of Na_i^+ in response to K^+ replacement ($t_{1/2}$ = 245 s, n = 2). The $[Na^+]_i$ remained elevated all the while the carbachol was present, and decayed slowly to its original value after carbachol removal (not shown). Thus, the permeability of the cells to Na^+ due to receptor triggering is not transiently enhanced but rather remains elevated throughout the period of exposure of the cells to agonist. The mechanism by which Na^+ permeability is enhanced by muscarinic receptor activation is currently under investigation. In other systems Na^+ influx increases due to receptor activation have been attributed to activation of the Na^+/H^+ antiporter; however, preliminary experiments have indicated that is not the case in these cells (unpublished observations).

CONCLUSIONS

We have determined that the activity of the Na^+/K^+ ATPase is closely coupled to the rate of production of acidic metabolites by the human rhabdomyosarcoma cell line TE671. Manipulation of the activity of the ATPase caused changes in the metabolic flux in these cells that were measurable noninvasively in real time with a microphysiometer. Parallel measurements of changes in $[Na^+]_i$ with a ratiometric fluorescence technique corroborated the results from the microphysiometer. Use of both techniques indicated that the pool of ATP used by the pump is derived nonpreferentially from either glycolysis or oxidative metabolism. Furthermore, we were able to demonstrate that the Na^+/K^+ ATPase is activated when carbachol stimulates cholinergic receptors in these cells. The activation of the ATPase is secondary to an increase in $[Na^+]_i$ that is caused by increased Na^+ influx during receptor stimulation. We are continuing to investigate the source of the increase in Na^+ influx.

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