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Dual role of ATP in supporting volume-regulated chloride channels in mouse fibroblasts

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Abstract

The effects of inhibitors of protein tyrosine kinases (PTKs) on the Cl^- current ($I_{Cl(vol)}$) through volume-regulated anion/chloride (VRAC) channels whilst manipulating cellular ATP have been studied in mouse fibroblasts using the whole-cell patch clamp technique. Removal of ATP from the pipette-filling solution prevented activation of the current during osmotic cell swelling and when the volume of patched cells was increased by the application of positive pressure through the patch pipette to achieve rates exceeding 100%/min. Equimolar substitution of ATP in the pipette solution with its non-hydrolyzable analogs, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) or adenylyl-(β,γ-methylene)-diphosphonate (AMP-PCP), not only supported activation of the current but also maintained its amplitude. The PTK inhibitors, tyrphostins A25, B46, 3-amino-2,4-dicyano-5-(4-hydroxyphenyl)penta-2,4-dienonitrile and genistein (all at 100 μM), inhibited $I_{Cl(vol)}$ in a time-dependent manner. Tyrphostin A1, which does not inhibit PTK activity, did not affect the current amplitude. The PTK inhibitors also inhibited $I_{Cl(vol)}$ under conditions where ATP in the pipette was substituted with ATPγS or AMP-PCP. We conclude that in mouse fibroblasts ATP has a dual role in the regulation of the current: it is required for protein phosphorylation to keep VRAC channels operational and, through non-hydrolytic binding, determines the magnitude of $I_{Cl(vol)}$. We also suggest that tyrosine-specific protein kinases and phosphatases exhibit an interdependent involvement in the regulation of VRAC channels.

Keywords: Volume-regulated anion channel; Protein tyrosine kinase; Protein tyrosine phosphatase; ATP; Non-hydrolyzable ATP analog; Fibroblast

1. Introduction

Volume-regulated anion/chloride channels (VRACs) are widely distributed in mammalian cells and are believed to be involved in a multitude of cellular functions including the regulation of cell volume, cell proliferation, intracellular pH and electrical processes in the cell membrane. The mechanisms of their activation are not presently known but the definitive stimulus is an expansion in cell volume, which experimentally is achieved either by osmotic cell swelling or by cell inflation through the measuring pipette in the whole-cell patch clamp mode. It is generally accepted that the same VRACs are activated by either method [1].

One of the distinct features of the current flowing

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through VRAC channels ($I_{Cl(vol)}$) is its reliance on the presence of ATP. In whole-cell patch clamp experiments the current is significantly suppressed when the pipette-filling solutions do not contain millimolar ATP (reviewed in [2]). ATP is known to modulate the function of many ion transporters and channels through a variety of mechanisms that may or may not require ATP hydrolysis [3]. One of the major regulatory processes that rely on ATP hydrolysis is protein phosphorylation. There is ample evidence, primarily pharmacological in nature, that protein phosphorylation, both serine/threonine- and tyrosine-specific, is involved in the activation and/or regulation of $I_{Cl(vol)}$ in various cell types (reviewed in [1]). Thus, the proposed involvement of tyrosine protein phosphorylation is supported by observations that $I_{Cl(vol)}$ in dog cardiac myocytes [4], rat astrocytes [5] and bovine endothelial cells [6] could be suppressed by inhibitors of protein tyrosine kinases (PTK) or, in bovine chromaffin cells [7] and mouse fibroblasts [8] by inhibitors of protein tyrosine phosphatases (PTP). These electrophysiological findings are supported by biochemical observations, albeit limited. An immediate increase in PTK activity following cell swelling has been detected in cardiac myocytes [9] and cardiac fibroblasts [10], astrocytes [5] and C6 glioma cells [11]. In the most conclusive experiments to date, a Cl^- current identical to $I_{Cl(vol)}$ has been induced in lymphocytes in the absence of an increase in cell volume by the intracellular introduction of the purified tyrosine kinase $p56^{lck}$ [12]. Examples of the serine/threonine-specific protein phosphorylation involvement are also abundant. Protein kinase A has been implicated in the regulation of $I_{Cl(vol)}$ in chick heart cells [13], whereas the involvement of protein kinase C in canine colonic smooth muscle [14], rat brain endothelial cells [15] and guinea-pig cardiac myocytes [16] was recently described. Yet another kinase, Rho kinase, has been shown to modulate the current in bovine endothelial cells [17].

On the other hand, several studies raised the possibility that non-hydrolytic ATP binding alone may be sufficient for the activation of $I_{Cl(vol)}$, without any need for ATP hydrolysis. This suggestion is based upon the observation that the current could be induced under conditions where ATP in the pipette solution was substituted with its non-hydrolyzable analogs and so it appears that activation of VRAC channels does not require phosphorylative events ([18–21]; reviewed in [1,2]). Furthermore, it has recently been suggested that the ATP dependence of $I_{Cl(vol)}$ is conditional on the rate of cell volume expansion, so that current activation becomes independent of ATP if the rate exceeds $\sim$65%/min [18].

The relationship between the two modes of ATP involvement is not clear at present. The studies supporting either of the hypotheses were carried out on different cell types which makes direct comparison difficult. We attempted to address these issues by investigating the effects of PTK inhibitors on $I_{Cl(vol)}$ in mouse fibroblasts whilst manipulating cellular ATP. Our findings support the model in which both (tyrosine) protein phosphorylation and non-hydrolytic ATP binding are necessary for the activation of VRAC channels.

2. Materials and methods

The experiments were carried out on mouse fibroblasts (LM TK-, ATCC No. CCL-1.3). Cell monolayers were cultured at 37°C in DMEM/F12 medium mixture supplemented with 10% fetal bovine serum and antibiotics. For electrical and optical recordings, cells were plated on 12-mm glass coverslips and cultured for 24–48 h. The coverslips were placed in a recording chamber mounted on the stage of an inverted microscope (Zeiss Axiovert 100). The $\sim$500-μl chamber was continuously perfused with the control bath solution using a conventional gravity-fed flow system ($\sim$1 ml/min). Whole-cell patch clamp recordings were performed using the Axopatch 200A amplifier. Digidata 1200 interface board and pClamp 6 software (Axon Instruments) were used to generate voltage-clamp command voltages and acquire data (50 ms sampling interval). Patch pipettes were made from thin-walled borosilicate glass and had resistances between 2 and 5 MΩ when filled with the pipette solution; no series resistance compensation was employed. The membrane conductance, defined as the slope of the I–V characteristics at the reversal potential, was measured every 30 s by ramping the membrane voltage from −60 to +60 mV (over 2.5 s) relative to a chosen holding potential [22]. As each cell served as its own control,

no attempts were made to calculate the current or conductance densities. The membrane voltage values were corrected for liquid junction potentials arising between the pipette or the bath electrode and the various extracellular solutions. Junction potentials were measured separately against a 3 M KCl-filled reference pipette [23].

The cells were normally bathed in a solution containing (mM): 130 NaCl, 5 $CaCl_2$, 2 $MgCl_2$, 2.8 KCl and 10 HEPES, with the pH adjusted to 7.4 with Tris–OH and the osmolality adjusted to 300 mOsm/l with mannitol. The hyposmotic solution (150 mOsm/l) used in the regulatory volume decrease (RVD) experiments, was a 1:1 mixture of the control bath solution and water. The pipette solution contained (mM): 127 K-aspartate, 16 KCl, 2 $MgSO_4$, 2 ATP, 1 EGTA, 20 HEPES, pH 7.2 with Tris–OH (285 mOsm/l). The hyperosmotic pipette solution contained (mM): 140 CsCl, 2 $MgSO_4$, 2 ATP, 1 EGTA, 20 HEPES, pH 7.2 with Tris–OH and mannitol to obtain an osmolality of 315 mOsm/l. The osmolality of the solutions was measured with a Micro-Osmometer model 3MO (Advanced Instruments, Needham Heights, MA, USA).

The PTK inhibitors used in this study were: 3-ADHD (3-amino-2,4-dicyano-5-(4-hydroxyphenyl)-penta-2,4-dienonitrile) from Tocris, tyrphostin A1 (AG9, α-cyano-(4-methoxy)cinnamonitrile), tyrphostin A25 (AG82, α-cyano-(3,4,5-trihydroxy)cinnamonitrile), tyrphostin B46 (AG555, α-cyano-(3,4-dihydroxy)-*N*-(3-phenylpropyl)cinnamide) and genistein (4′,5,7-trihydroxyisoflavone), all from Calbiochem. Stock solutions of these substances (in DMSO, the 3-ADHD was dissolved in ethanol) were kept at −20°C and diluted to the required concentrations immediately prior to their use. The final concentration of DMSO or ethanol was 0.1–0.5%. The PTK inhibitors were applied extracellularly using an application pipette (~50 μm tip) whose tip was positioned ~250 μm from the patched cell. ATP (adenosine 5-triphosphate, disodium salt) and ATPγS (adenosine 5′-*O*-(3-thiotriphosphate), tetralithium salt) were from Sigma, AMP-PCP (adenylyl-(β,γ-methylene)-diphosphonate, tetralithium salt) was from Boehringer Mannheim.

An increase in the volume of patched cells was induced by short (1–3 s) pulses of positive pressure (1.3–2.6 kPa) applied through the patch pipette, under continuous visual control or, in other experiments, by cell swelling during dialysis with the hyperosmotic pipette solution. The cell volume was measured using a video imaging system consisting of a CCD video camera attached to the microscope equipped with differential interference contrast optics and a 40× (NA 0.65) objective lens. Cell images were collected every 30 s during the 8 min-long recording periods and analyzed off-line using the UTHSCSA ImageTool v. 1.28 program (developed at the University of Texas Health Science Center at San Antonio, TX, USA, and available from the Internet by anonymous FTP from maxrad6.uthscsa.edu). In the cell inflation experiments the cell images were first videotaped and later captured off-line into the computer memory, one per second for 10–20 s before and after inflation. Cell images were displayed on a computer monitor and the cross-sectional area (CSA) of the soma of single cells was measured by manually tracing the cell borders. The relative cell volume changes (V/V_0) were calculated using the equation $V/V_0 = (\text{test CSA}/\text{control CSA})^{3/2}$ [24].

All experiments were conducted at room temperature (21–23°C). Unless otherwise indicated, results are presented as means ± S.E.M. Statistical comparisons were performed using Student's *t*-test (Prism 2.01, GraphPad Software, San Diego, CA, USA). A *P*-value of less than 0.05 was adopted for statistically significant differences.

3. Results

3.1. ATP dependence of $I_{Cl(vol)}$ in mouse fibroblasts

The suppression of $I_{Cl(vol)}$ in mouse fibroblasts by PTP inhibitors observed in our previous studies [8] strongly indicated that the functional state of VRAC channels is controlled by tyrosine-specific protein phosphorylation. In the present study, we also confirmed that, as in many other cell types (reviewed in [1,2]), the amplitude of $I_{Cl(vol)}$ in mouse fibroblasts decreased dramatically when ATP was removed from the patch pipette-filling solutions. It is important to emphasize, that this requirement for ATP was observed not only in osmotically-swollen cells but also in cells whose volume was increased rapidly by inflation through the patch pipette. This finding contrasts

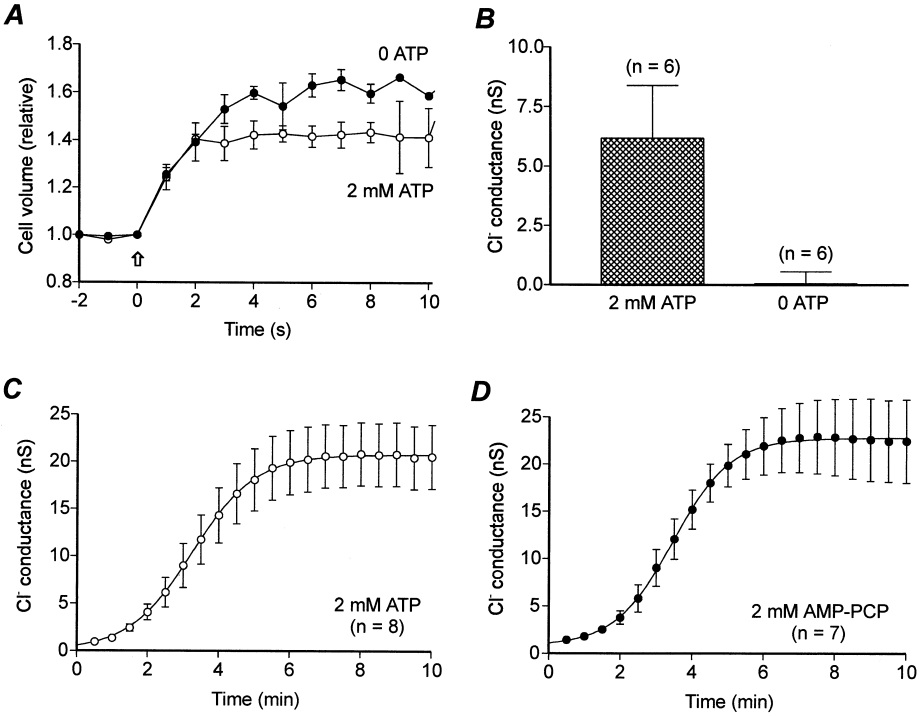

Fig. 1. ATP dependence of $I_{Cl(vol)}$ in mouse fibroblasts. (A) An increase in cell volume caused by a short pressure pulse (arrow) applied through the measuring pipette in cells dialyzed with 2 mM ATP-containing solution (open circles) or with ATP-free solution (closed circles). The volume of four cells was measured for each condition. (B) The amplitude of volume-regulated Cl^- conductance in cells dialyzed with 2 mM ATP (left bar) or without ATP (right bar). The pre-inflation values of the membrane conductance have been subtracted from those measured after cell inflation. (C, D) Comparison of the Cl^- conductance induced by cell swelling during dialysis with the hyperosmotic (315 mOsm/l) solutions containing either 2 mM ATP (C) or 2 mM AMP-PCP (D).

with the recently described observation [18], where the activation of VRAC channels in N1E115 neuroblastoma cells became independent of ATP when the rates of cell volume expansion by cell swelling exceeded ~65%/min. Cell inflation can yield much higher rates of cell volume expansion (up to 1000%/min, e.g. [22]) than those achievable during cell swelling (less than 100%/min, e.g. [18]). In our experiments, the amplitude of the cell inflation-induced $I_{Cl(vol)}$ was compared in a number of cells from the same coverslip that were intracellularly dialyzed with 2 mM ATP-containing solution (ATP-cells) or with ATP-free solution (non-ATP-cells) in alternate order. The magnitude of cell inflation was controlled so as to keep the rates of cell volume expansion at moderate levels. In both cell groups the rates were similar, the pooled data averaged 120 ± 3%/min ($n = 8$), i.e. twice the rate of the fastest cell swelling reported by Bond et al. [18]. The increase in cell volume averaged 40% in the ATP-cells and 60% in the non-ATP-cells (Fig. 1A). The difference in the amplitude of $I_{Cl(vol)}$ in these two cell groups was very distinct and significant ($P < 0.001$): on average, the volume-regulated Cl^- conductance was 6.2 ± 2.2 nS ($n = 6$) in the ATP-cells and 0.1 ± 0.5 nS ($n = 6$) in the non-ATP-cells (Fig. 1B). Thus, in contrast to the N1E115 neuroblastoma cells [18], ATP dependence of $I_{Cl(vol)}$ in mouse fibroblasts was not compromised by increasing the rate of cell volume expansion.

We also tested whether $I_{Cl(vol)}$ could be activated when ATP in the pipette solution was replaced with its non-hydrolyzable analogs, ATPγS or AMP-PCP. In separate experiments, the average conductance of inflation-induced $I_{Cl(vol)}$ was 15.4 ± 1.5 nS ($n = 8$) when the pipette-filling solution contained 2 mM ATP, and 15.9 ± 1.3 nS ($n = 9$) when ATP in the pipette solution was replaced with 2 mM ATPγS. Similar results were obtained in pair-matched comparisons of $I_{Cl(vol)}$ in cells from the same coverslips swollen during intracellular dialysis with hypertonic (315 mOsm/l) solutions containing either 2 mM ATP

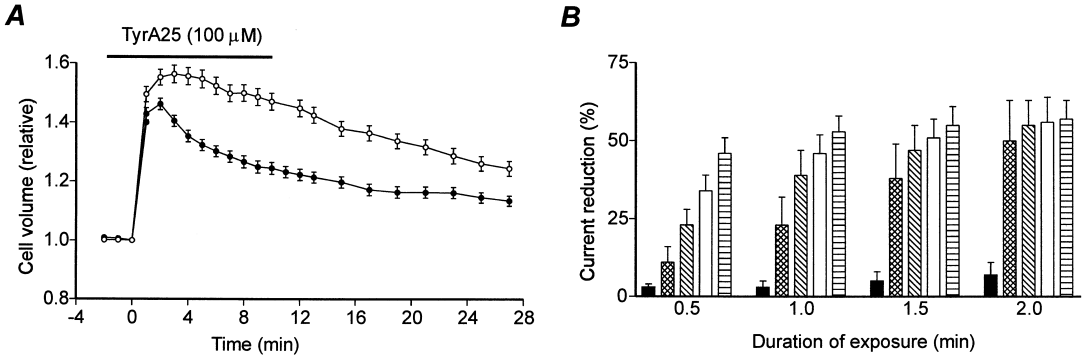

Fig. 2. Suppression of RVD and $I_{Cl(vol)}$ with PTK inhibitors. (A) The effect of tyrphostin A25 (100 μM) on cell swelling and subsequent shrinkage (RVD) caused by a decrease (at time zero) in osmolality of the bath solution from 300 to 150 mOsm/l. The drug was added 2 min before the bath change and persisted in the bath for 12 min (open circles, $n=26$). Middle curve (closed circles), RVD in control conditions (no drug, $n=41$). (B) The four groups of bars show the degree of reduction in the outward component of $I_{Cl(vol)}$ (measured at +25 mV) at 0.5, 1.0, 1.5 and 2 min of exposure to tyrphostin A1, 3-ADHD, genistein, tyrphostin B46 and tyrphostin A25, respectively. Each drug was used at 100 μM.

or 2 mM AMP-PCP, another non-hydrolyzable ATP analog. The amplitude of the volume-regulated Cl^- conductance was not significantly different ($P=0.738$) in these two groups of cells: 20.5 ± 3.4 nS ($n=8$) in the ATP-cells and 22.4 ± 4.4 nS ($n=7$) in the AMP-PCP-cells (Fig. 1C,D).

The above results show that the activation of $I_{Cl(vol)}$ in mouse fibroblasts requires the presence of cellular ATP and that under whole-cell conditions, ATP replacement with its non-hydrolyzable analogs does not significantly affect the current. In addition, the rate of cell volume expansion does not seem to be a factor in determining the ATP dependence of $I_{Cl(vol)}$.

3.2. Inhibition of $I_{Cl(vol)}$ by PTK inhibitors

Next we asked whether $I_{Cl(vol)}$ in mouse fibroblasts is affected by inhibitors of PTKs, as this has been shown in cardiac myocytes [4] and bovine endothelial cells [6]. This question is of particular interest since, in our earlier studies [8], we demonstrated that $I_{Cl(vol)}$ in mouse fibroblasts could be suppressed by PTP inhibitors. In order to reduce the possibility of non-specific effects, we tested several PTK inhibitors with different modes of action, those that act by binding to the substrate binding sites (tyrphostins) and those that act at the ATP binding site (genistein) of a PTK.

In the experiments on intact cells, tyrphostin A25 (100 μM) effectively suppressed regulatory volume decrease (Fig. 2A), similar to what we have earlier observed with PTP inhibitors [8]. The average rate of cell shrinkage in the presence of tyrphostin A25 was only $1.38\pm0.10\%$/min (3–10 min interval, $n=26$), compared to $4.0\pm0.5\%$/min (2–6 min interval, $n=41$) in the control cells. Tyrphostin A25 also inhibited, as did all other PTK inhibitors tested, the $I_{Cl(vol)}$ in whole-cell conditions. The drugs were applied extracellularly 3.5–4.5 min after the activating stimulus (cell inflation), when the current amplitude attained its maximal value. The inhibition seen was time-dependent. Immediately after the onset of drug application (within 15–20 s), inhibition was substantially smaller than at later times when it reached stationary levels. There was also some voltage dependence of the inhibitory effects, such that the inward currents were inhibited generally less than the outward ones although the difference was significant only with tyrphostin A25 (see below). Maximal suppression of the outward (at +25 mV) or the inward $I_{Cl(vol)}$ current (at −95 mV), measured 2 min after drug exposure, averaged $50\pm13\%$ versus $51\pm12\%$ ($n=8$) for 3-ADHD, $56\pm8\%$ versus $44\pm8\%$ ($n=8$) for tyrphostin B46 and $55\pm8\%$ versus $48\pm7\%$ ($n=11$) for genistein (Fig. 2B). Tyrphostin A1, which inhibits PTKs only at very high (millimolar) concentrations and therefore is commonly used as a negative control for tyrphostins, at 100 μM produced much smaller, statistically insignificant inhibition of $I_{Cl(vol)}$: $7\pm4\%$ for the outward and $4\pm8\%$ ($n=11$) for the inward component.

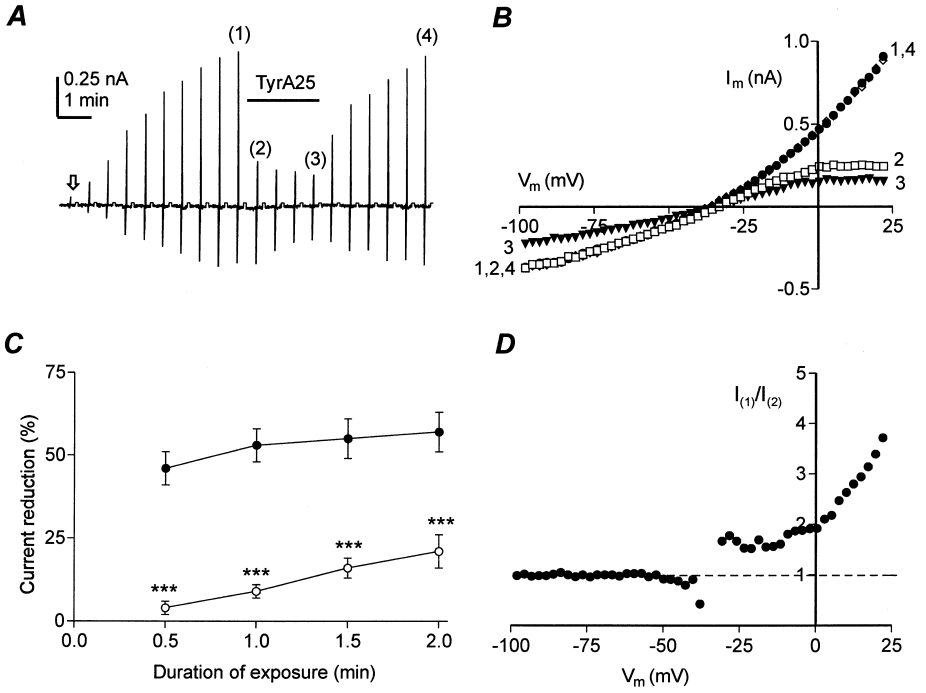

Fig. 3. Features in the inhibition of $I_{Cl(vol)}$ with tyrphostin A25. (A) Current recording during application of 100 μM tyrphostin A25 (horizontal bar). $I_{Cl(vol)}$ was induced by cell inflation (arrow). The current spikes were caused by symmetrical voltage ramps (±60 mV from a holding potential of −35 mV). (B) *I–V* relationships for $I_{Cl(vol)}$ recorded immediately prior to drug application (curve 1), after 15–20 s (curve 2) and 2 min (curve 3) of drug exposure and after drug washout (curve 4). The corresponding current spikes in (A) are marked with these numbers in brackets. (C) Comparison in the degree of suppression of the outward (closed circles) and inward (open circles) currents at various time points during drug application. Data points are means ± S.E.M. (n = 11–16). (D) Voltage dependence of the fast inhibition shown as the ratio of the *I–V* characteristics taken immediately before and 15–20 s after drug application (curves 1 and 2 in B).

3.3. *Tyrphostin A25 suppressed $I_{Cl(vol)}$ in a voltage-dependent manner*

As mentioned earlier, tyrphostin A25 was quite unlike the other PTK inhibitors tested in this study due to several definitive features in its block of $I_{Cl(vol)}$. A characteristic pattern in its inhibition of the volume-regulated Cl^- current was the rapid onset, so that most of the current decrease occurred in less than 30 s (the time interval between two successive voltage ramps) (Fig. 3A). This initial phase of current blockade exhibited pronounced voltage dependence: the inward current was not inhibited at all, whereas inhibition became progressively stronger with increasing depolarization once the current reversed its direction (Fig. 3B,D). In Fig. 3B two current–voltage relationships are compared, one taken just prior to tyrphostin application (curve 1, see also Fig. 3A) and the other taken immediately after application (curve 2). Fig. 3D presents this voltage dependence as the ratio of both the *I–V* relationships shown in Fig. 3B. The initial decrease in the amplitude of the outward current at +25 mV was 46 ± 5% (n = 18) compared with only 4 ± 2% (n = 18) for the inward current at −95 mV (Fig. 3C). The difference is highly significant, $P < 0.0001$ (Fig. 3C). With prolongation of tyrphostin exposure the difference between both the outward and inward current inhibition became less pronounced (curve 3, Fig. 3B) but remained significant even after 2 min (Fig. 3C): 57 ± 6% as compared to 21 ± 5% (n = 13). Inhibition was reversible upon removal of the drug from the extracellular medium (Fig. 3A, curve 4) and recovery of the current amplitude was accompanied by complete restoration of the original shape of the *I–V* curve (curves 1 and 4, Fig. 3B). These features seen in the inhibitory effect suggest a substantial contribution of direct inhibition of the VRAC channels by tyrphostin A25.

Despite the peculiarities of the tyrphostin A25 ef-

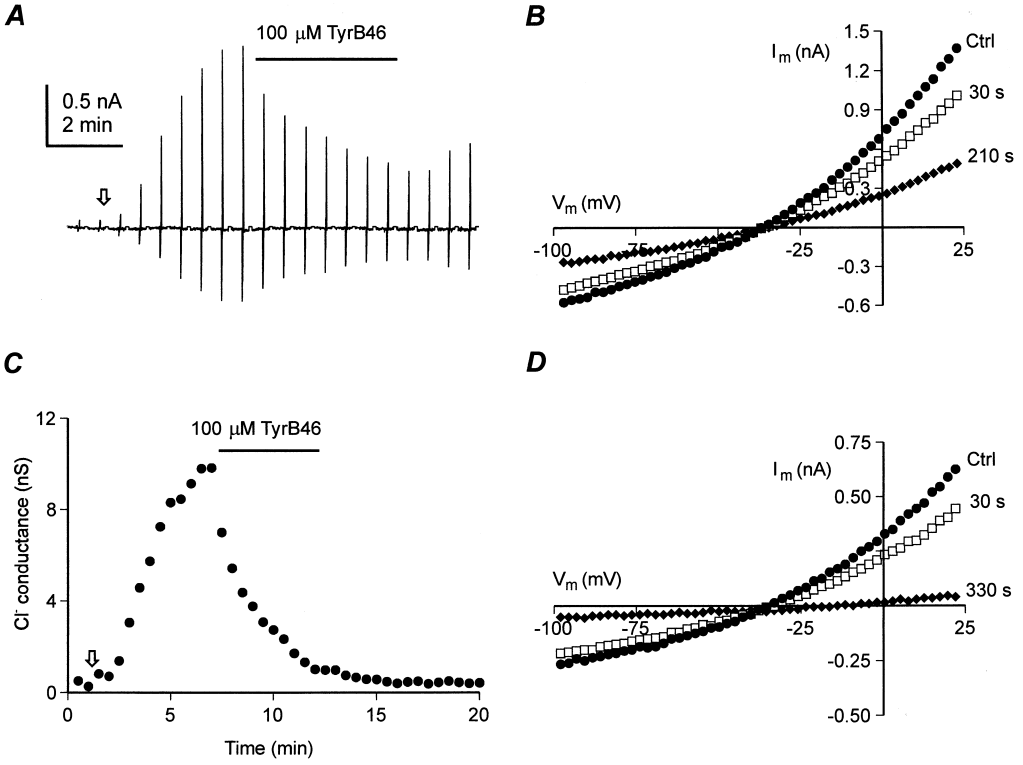

Fig. 4. Effects of the PTK inhibitors on $I_{Cl(vol)}$ in the presence of the non-hydrolyzable ATP analogs. (A) Current recording during application of 100 μM tyrphostin B46 (horizontal bar). The current was induced by inflation (arrow) of the cell dialyzed with 2 mM ATPγS-containing solution. The current spikes were caused by ±60 mV ramps applied every 30 s from a holding potential of −35 mV. (B) *I–V* relationships taken immediately prior (circles), 30 s (open squares) and 210 s (closed squares) after drug exposure. (C, D) A similar experiment carried out on another cell dialyzed with 2 mM AMP-PCP. In (C), the amplitude of volume-regulated Cl^- conductance is plotted before and during application of 100 μM tyrphostin B46 (horizontal bar). In (D), *I–V* relationships taken at different times after the onset of drug application are shown.

fects, suppression of $I_{Cl(vol)}$ in mouse fibroblasts by other PTK inhibitors appears to result from their expected effects on the activity of PTKs. This is supported by the lack of effect on $I_{Cl(vol)}$ by tyrphostin A1 which is known for its inability to suppress PTK activity at micromolar concentrations and also by the fact that tyrphostins B46 and 3-ADHD acted similarly to genistein, although these drugs inhibit tyrosine kinases by distinctly differing mechanisms. Hence, these results support the suggestion that PTKs and so tyrosine-specific protein phosphorylation play an important role in the activation of the current.

3.4. PTK inhibitors suppress $I_{Cl(vol)}$ activated in the presence of non-hydrolyzable ATP analogs

The suppression of $I_{Cl(vol)}$ with the PTK inhibitors described in Section 3.3 appears to contradict the earlier observation (see Fig. 1C,D) of no significant difference in the amplitude of the current when ATP in the measuring pipette was replaced with ATPγS or AMP-PCP, the ATP analogs which do not support protein phosphorylation. Therefore, we tested whether the PTK inhibitors would affect $I_{Cl(vol)}$ under conditions where ATP in the pipette solution is replaced with its non-hydrolyzable analogs. Surprisingly, they did. Fig. 4A shows a current recording from a representative experiment in which the 'ATPγS-supported' $I_{Cl(vol)}$ was suppressed with tyrphostin B46. Inhibition progressed throughout the application of the drug, was reversible upon drug removal and showed no significant voltage dependence (Fig. 4B). At 100 μM, tyrphostin B46 suppressed the outward current at +25 mV on average by 58 ± 8% ($n = 7$). Similarly, tyrphostins B46 and

A25 both suppressed $I_{Cl(vol)}$ activated in the presence of the truly non-hydrolyzable ATP analog AMP-PCP. However, this effect appeared to be irreversible as the decrease in the current in all cells tested continued even after the PTK inhibitor application was terminated (Fig. 4C). Whether this is due to the differences between the two ATP analogs used (ATPγS can support protein kinase activity, whereas AMP-PCP cannot, e.g. [25]) is not clear.

4. Discussion

The role of ATP in the activation of volume-regulated Cl^- channels has been the subject of numerous recent studies. Two major modes of ATP involvement have been described, namely, the non-hydrolytic ATP binding and the support of protein phosphorylation (reviewed in [1,2,26]). Whether the corresponding models for regulation of VRAC channels are exclusive or they complement each other is not clear, partly because the principal studies were carried out on different cell types. A further complication arises from a huge difference in the operational levels of ATP: where ATP binding requires millimolar ATP concentrations, a 1000-fold less ATP is sufficient for protein phosphorylation [3]. The present study was designed to clarify the relationship between the two models by testing some of their postulates under critical conditions using the same cell type, mouse fibroblasts. These cells possess VRAC channels which are characterized by moderate outward rectification [7,22], have a $I^- > Cl^-$ permeability sequence and do not inactivate at high positive membrane voltages [8].

The results obtained in this study provide for several important conclusions. First, they support the requirement for high levels of cytosolic ATP for the activation of $I_{Cl(vol)}$ (Fig. 1B). Most probably this role for ATP is related to its non-hydrolytic binding to VRAC channels or other regulatory proteins because equimolar substitution of ATP in the pipette solution with its non-hydrolyzable analogs did not affect the current amplitude (Fig. 1C,D). This requirement remained in force even with very high rates of cell volume expansion ($>100\%$/min) achieved by the inflation of cells through the measuring pipette, contrasting with the recently reported loss of ATP dependence of $I_{Cl(vol)}$ at high ($>65\%$/min) rates of osmotic cell swelling in N1E115 neuroblastoma cells [18]. The underlying differences still remain unclear: VRACs in different cell types and the various methods employed to increase cell volume, i.e. osmotic cell swelling versus cell inflation.

Secondly, our results confirmed that $I_{Cl(vol)}$ in mouse fibroblasts can be suppressed by PTK inhibitors (Fig. 2). The conclusion that the drugs used acted by inhibiting PTKs is supported by the observations that suppression of $I_{Cl(vol)}$ was seen with PTK inhibitors with different modes of action (tyrphostins versus genistein) but not with tyrphostin A1, the inactive tyrphostin homolog [5,27–29]. This conclusion is not compromised by the unusual effects of tyrphostin A25 (Fig. 3) whose action appears to involve direct inhibition of VRAC channels (possibly open channel block). The possibility of a non-PTK site of action for the PTK inhibitors seems much less likely, although it cannot be completely ruled out. Although similar effects of PTK inhibitors have previously been reported for other cell types (see Section 1 for references), it was an unexpected finding because in our previous study on these same cells we observed that the current was also suppressed by inhibitors of PTPs [8]. If tyrosine protein phosphorylation directly regulates VRAC channels, one would expect inhibitors of PTKs to cause changes in the current amplitude opposite to those seen with the inhibitors of PTPs (see, for example, [6]). Therefore, we suggest that not only tyrosine protein phosphorylation plays an important role in the activation of $I_{cl(vol)}$ in fibroblasts, but PTPs and PTKs are involved at multiple steps in the activation process or possibly regulate each others activities (e.g. [30]).

The finding that the PTK inhibitors remained effective when ATP in the pipette solutions was replaced with its non-hydrolyzable analogs (Fig. 4) suggests that protein phosphorylation must have taken place even under these conditions. Since the ATP analog AMP-PCP is truly non-hydrolyzable and cannot support protein phosphorylation, one has to conclude that the ATP substitution was not complete, i.e. some residual ATP remained in these dialyzed cells at levels sufficient to support PTK activity. This suggestion is reasonable from our estimation of the rate of ATP wash-out in the whole-cell experiments. For the patch pipettes used in this study

(average access resistance of 5–6 MΩ), dialysis with ATP-free solution would cause the cellular concentration of ATP to drop exponentially with a time constant of ~100 s (see [31]). This translates into a time period of more than 8 min (the recording time in our experiments) for the cellular ATP level to decrease from 2 mM to 10 μM. For comparison, the half-maximal activity of the cAMP-dependent protein kinase occurs at only 3.1 μM ATP [32]. Even metabolic cell poisoning, which is arguably the strongest maneuver to deplete cellular ATP, may not guarantee the removal of micromolar levels of ATP which is intrinsic to the living cell. It has been reported that after 1 h of incubation with 2 μM rotenone (inhibitor of respiration) in glucose-free medium the cellular level of ATP in EL-4 ascites tumor cells decreased to 4% of the initial level [33]. Similarly, a 90 min poisoning of nasal respiratory cells or the 16HBE14o$^-$ cells with the respiratory inhibitor antimycin A (10 μM) and the glycolytic inhibitor 2-deoxyglucose (10 mM) resulted in only a 93–97% decrease in ATP content [34]. Thus, the residual ATP may be as high as 4–7% of its normal level and be sufficient for various protein kinases. Indeed, the extent of phosphorylation of the sodium/hydrogen exchanger, NHE1, was not detectably altered by the acute ATP depletion which caused a profound inhibition of cation exchange in transfected fibroblasts [35]. This problem has to be recognized for a more cautious consideration of ATP dependence of $I_{Cl(vol)}$.

In summary, the present study suggests that cellular ATP affects $I_{Cl(vol)}$ in mouse fibroblasts in two ways: by supporting protein phosphorylation (at very low levels) and by non-hydrolytic binding (at higher levels). The relationship between these two processes is not clear at this time but ATP binding seems to be an important determinant of the current amplitude. Because significant changes in cellular ATP are expected to occur only in metabolic stress, it is reasonable to suggest that non-hydrolytic ATP binding only becomes operational in pathological conditions whereas (tyrosine) protein phosphorylation is involved in the physiological control of VRAC channels.

Acknowledgements

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