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The effect of a hyposmotic shock and purinergic agonists on $K^+(Rb^+)$ efflux from cultured human breast cancer cells

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Abstract

The effect of a hyposmotic shock and extracellular ATP on the efflux of $K^+(Rb^+)$ from human breast cancer cell lines (MDA-MB-231 and MCF-7) has been examined. A hyposmotic shock increased the fractional efflux of $K^+(Rb^+)$ from MDA-MB-231 cells via a pathway which was unaffected by Cl^- replacement. Apamin, charybdotoxin or removing extracellular Ca^{2+} had no effect on volume-activated $K^+(Rb^+)$ efflux MDA-MB-231 cells. An osmotic shock also stimulated $K^+(Rb^+)$ efflux from MCF-7 cells but to a much lesser extent than found with MDA-MB-231 cells. ATP-stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells in a dose-dependent fashion but had little effect on $K^+(Rb^+)$ release from MCF-7 cells. ATP-stimulated $K^+(Rb^+)$ efflux was only inhibited slightly by replacing Cl^- with NO_3^-. Removal of external Ca^{2+} during treatment with ATP reduced the fractional efflux of $K^+(Rb^+)$ in a manner suggesting a role for cellular Ca^{2+} stores. Charybdotoxin, but neither apamin nor iberiotoxin, inhibited ATP-stimulated $K^+(Rb^+)$ release from MDA-MB-231 cells. Suramin inhibited the ATP-activated efflux of $K^+(Rb^+)$. UTP also stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells whereas ADP, AMP and adenosine were without effect. A combination of an osmotic shock and ATP increased the fractional efflux of $K^+(Rb^+)$ to a level greater than the sum of the individual treatments. It appears that the hyposmotically-activated and ATP-stimulated K^+ efflux pathways are separate entities. However, there may be a degree of 'crosstalk' between the two pathways.

Keywords: Hyposmotic shock; Purinergic agonist; Breast cancer cell

1. Introduction

Recently, there has been significant interest in K^+ transport in human breast cancer cells. Several types of K^+ channels have been identified at the functional and molecular level in human breast cancer cells including K_{ATP} [1], Kv1.3 [2], Kv1.1 [3], hEAG [4], KCNK9 [5] and intermediate- and large Ca^{2+}-activated K^+ channels [6,7]. The interest in K^+ transport stems from the strong possibility that alterations in ion transport may regulate tumour cell proliferation and apoptosis (for a review, see [8]). It is envisaged that a thorough understanding of ion transport pathways, and the factors which regulate them, could help develop new therapeutic strategies [8].

Despite the identification of specific K^+ channels, very little is known about the regulation of K^+ transport in cultured breast cancer cells. In this connection, cell swelling and extracellular ATP are potent modulators of K^+ transport in many cell types. Volume-activated K^+ transport allows cells to regulate their hydration state within relatively narrow limits. However, it is becoming apparent that K^+ transport mechanisms which are normally activated by cell swelling also play an important role in initiating both apoptosis and proliferation. For example, volume-activated K^+ channels are involved in the early stages of apoptosis in mouse one-cell embryos [9] whereas volume-activated (K+Cl) cotransport appears to be necessary for the invasion and proliferation of cervical cancer [10]. It has been known for some time that extracellular ATP is an important regulator of cell

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growth and function. It is beyond doubt that extracellular ATP exerts some of its actions by regulating K^+ transport [11]. In this regard, it is apparent that extracellular ATP is able to modulate the activity of volume-activated solute efflux pathways including swelling-induced K^+ efflux [12,13].

The purpose of the present study was to investigate the regulation of K^+ transport in human breast cancer cells by cell-swelling and extracellular ATP. We have studied the transport of K^+ by MDA-MB-231 and MCF-7 human breast cancer cells. MDA-MB-231 and MCF-7 cells, originally derived from pleural effusions are, respectively, estrogen receptor-negative and -positive. In particular, we have designed experiments to characterize swelling-activated and ATP-stimulated K^+ transport pathways and for any interaction between the two mechanisms. Thus, we have examined the effect of osmotically-induced cell-swelling and purinergic agonists on the efflux of K^+ from MDA-MB-231 and MCF-7 cells.

2. Materials and methods

2.1. Culture of MDA-MB-231 and MCF-7 cells

MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine (2 mM), heat-inactivated fetal bovine serum (10%), penicillin (50 IU/ml) and streptomycin (50 μg/ml). Cells were cultured at 37 °C in a gas phase of air with 5% CO_2. Cells were seeded in 35 mm culture wells containing 2 ml of culture medium at a density of $0.3-1.0\times10^6$ cells per well. The cells were cultured for 1–3 days and were used at 60–90% confluency. Each experiment was conducted on a separate day using cells from either the same or a different passage.

2.2. Measurement of K^+ (Rb^+) efflux from MDA-MB-231 and MCF-7 cells

Solute efflux from cultured human breast cancer cells was measured according to the method described by Shennan et al. [14]. K^+ efflux was measured using an isotope of rubidium ($^{86}Rb^+$) as tracer. The culture medium was aspirated and the cells were washed (x3) with 2 ml of a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. The cells were loaded with $^{86}Rb^+$ by a 60- to 90-min incubation in a medium similar to that just described except that it also contained 7–10 μCi/ml $^{86}Rb^+$. Following the loading incubation the cells were washed three times in rapid succession (<1 min) with a radioactive-free buffer similar in composition to that described above. The efflux of $^{86}Rb^+$ was measured by the sequential addition and removal of 2 ml of buffer (see text and figure legends for precise details of composition) at 1-min intervals. The first sample was discarded because of the possibility that it contained a significant amount of isotope derived from the extracellular compartment. At the end of the wash-out period, cells were incubated in 2 ml of distilled water for 3 h in order to release the radioactivity remaining in the cells. Samples were prepared for counting by adding 10 ml of UltimaGold liquid scintillation cocktail.

The fractional efflux of K^+($^{86}Rb^+$) for each of the collection periods was calculated according to Eq (1):

$$\text{Fractional efflux}\left(\text{min}^{-1}\right) = \Delta X/(X_t.\Delta t) \quad (1)$$

where ΔX is the amount of radiolabelled rubidium released from the cells in the time interval Δt, and X_t. is the amount of isotope in the cells at the start of each collection period. K^+(Rb^+) efflux time courses were also analysed by plotting $\log_e(Rb_i/Rb_0)$as a function of time where Rb_0 is the amount of radioactivity associated with the cells at the beginning of the efflux time course and Rb_i is the amount of radioactivity remaining in the cells at time t. The negative slopes of these graphs provided a measure of the unidirectional efflux rate constants.

2.3. Measurement of taurine efflux

Taurine efflux was measured from MDA-MB-231 and MCF-7 cells using [^{3}H]-taurine as tracer. Cells were loaded with radiolabelled taurine by incubating in a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1.0 μCi/ml of [^{3}H]-taurine for 60–90 min at 37 °C. Taurine efflux was measured at 37 °C in a manner similar to that described above for $^{86}Rb^+$.

2.4. Loading of Cells with fura-2

Cells were washed three times with Tyrode to remove tissue-culture medium, and loaded with fura-2 by incubating the cells with 5 μM fura-2 acetoxymethyl ester (Sigma) in Tyrode, together with 0.025% pluronic F127 (Molecular Probes) for 60 min at room temperature. Cells were then washed again three times with Tyrode to remove extracellular dye, and then scraped off the plates into normal Tyrode (2 ml). Unloaded cells for background measurements were prepared in exactly the same way, except no fura-2 was added.

2.5. Microspectrofluorimetry

Measurements were made at room temperature using a 35 mm glass-bottomed culture dish (WPI); cells were placed into the chamber in 1 ml of Tyrode, and allowed to equilibrate for 20–30 min prior to beginning the experiment. Single cells, or groups of cells were selected, and the surrounding area masked out by an aperture. The cells were washed with 1 ml Tyrode, or 1 ml Ca-free Tyrode 5 min before the addition of ATP, which was added directly

to the chamber in 1 ml of Tyrode/Ca-free Tyrode as appropriate, to give a final concentration of 100 μM. A Cairn spectrophotometer system (Cairn Research, Sittingbourne, UK) was used to record changes in $[Ca^{2+}]$. Cells were stimulated by light at 360 or 380 nm at a frequency of 6.67 Hz, and emitted light (greater than 475 nm) was recorded by sampling and averaging the output signal at a rate of 40 Hz. Background readings for each wavelength at the same aperture settings were taken from unloaded cells, and these values subtracted from the raw data before calculation of the ratio of light emitted following stimulation at 360/380 nm.

2.6. Solutions

The efflux of $K^+(Rb^+)$ was measured under both isosmotic and hyposmotic conditions. The standard isosmotic buffer (303 mOsmol/kg water; range 291–311) contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. The hyposmotic buffer (185 mOsmol/kg water; range 180–192) was similar in composition except that the NaCl concentration was reduced to 75 mM. Nitrate was used to replace Cl^- when Cl^- free buffers were required. The osmolality of the buffers was measured using a MicroOsmometer (Vitech Scientific Ltd., UK). For the calcium measurements, the normal Tyrode solution contained: NaCl, 134; KCl, 6; HEPES, 10; $CaCl_2$, 1; $MgCl_2$, 1; and glucose, 10. Tyrode pH was adjusted to 7.4 at 20 °C with NaOH. Purinergic agonists were dissolved directly in the isosmotic and hyposmotic buffers no more than 60 min prior to use.

2.7. Statistics

Results are presented as mean values SEM. Differences were assessed using Student's paired or unpaired *t*-test as appropriate and were considered significant when $P<0.05$. Changes in efflux or between efflux profiles were also determined by 2-way ANOVA. For dose–response curves, fitted curves were calculated using a four-parameter fit (GraphPad Prism, version 4.00 for Windows). EC_{50} values (i.e., the dose of agonist required to give 50% of the maximum response) were calculated using the same software.

3. Results

Preliminary experiments revealed that $K^+(Rb^+)$ efflux from MDA-MB-231 and MCF-7 cells, measured under isosmotic conditions followed first order kinetics (results not shown).The unidirectional efflux rate constants calculated from semi-log plots of $\log_e$ (fraction of isotope remaining in cells) as a function of time were, respectively 0.0291 ± 0.0031 min^{-1} (n=4) and 0.0380±0.0019 min^{-1} (n=4) for MDA-MB-231 and MCF-7 cells.

3.1. The effect of a hyposmotic challenge on $K^+(Rb^+)$ and taurine efflux

The effect of reducing the osmolality of the incubation buffer from 303 to 185 mOsmol/kg water on $K^+(Rb^+)$ efflux from MDA-MB-231 and MCF-7 cells was examined. Fig. 1a shows that a hyposmotic shock markedly increased the fractional release of rubidium from MDA-MB-231 cells: the fractional efflux was increased (basal-to-peak) from 0.023±0.002 min^{-1} to 0.077±0.013 min^{-1}. Bumetanide (1 mM), a high affinity inhibitor of (Na^+–K^+–Cl^-) cotransport [15], affected neither the basal efflux of $K^+(Rb^+)$, nor the hyposmotically-stimulated moiety of efflux (Fig. 1a).

Amino acid efflux, especially that of taurine, is stimulated following cell swelling [16,17].Therefore, for comparison, we examined the effect of a similar hyposmotic challenge on taurine efflux from MDA-MB-231 cells. Reducing the osmolality of the incubation medium increased the fractional release of taurine (basal-to-peak)

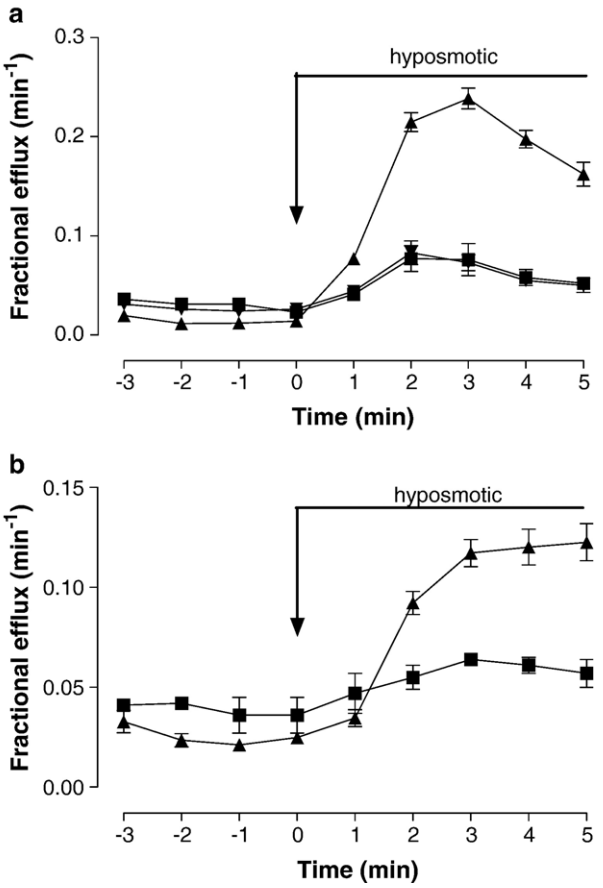

Fig. 1. The effect of a hyposmotic shock on $K^+(Rb^+)$ (■) and taurine (▲) efflux from (a) MDA-MB-231 and (b) MCF-7 cells. The isosmotic incubation medium contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. The hyposmotic buffer was similar in composition except that the NaCl concentration was reduced to 75 mM. The data denoted (▼) in a shows the effect of a hyposmotic challenge in the presence of 1 mM bumetanide Data shown are means±S.E. of 4 or 5 experiments.

from 0.014±0.001 min^{-1} to 0.239±0.010 min^{-1} (±S.E., n=4, P<0.001).

The efflux of $K^+(Rb^+)$ from MCF-7 cells was also stimulated by a hyposmotic shock, albeit to a lesser extent than that found with MDA-MB-231 cells (234% v 78%) (Fig. 1b). The fractional release of $K^+(Rb^+)$ was increased (basal-to-peak) from 0.036±0.009 min^{-1} to 0.064±0.003 min^{-1}. A hyposmotic shock also increased the fractional efflux of taurine from MCF-7 cells from a basal value of 0.025±0.002 min^{-1} to a peak value of 0.123±0.009 min^{-1} (±S.E., n=4, P<0.01) (Fig. 1b).

The increase in $K^+(Rb^+)$ efflux from mammary tumour cells elicited by a hyposmotic shock could be due to $K^+(Rb^+)$ efflux via channels and/or via (K^+–Cl^-) cotransport. To distinguish between the two possibilities, we examined the effect of replacing Cl^- (with NO_3^-) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells. Fig. 2 shows that replacing Cl^- did not affect $K^+(Rb^+)$ release under hyposmotic conditions suggesting that volume-activated $K^+(Rb^+)$ efflux is not mediated by (K^+–Cl^-) cotransport and could therefore be via K^+ channels. Furthermore, it is clear from Fig. 2 that substituting Cl^- with NO_3^- did not affect $K^+(Rb^+)$ efflux from MDA-MB-231 cells under isosmotic conditions.

We next examined the effect of removing extracellular calcium on $K^+(Rb^+)$ efflux from MDA-MB-231 cells. Removal of calcium from the medium at the start of the efflux wash-out period had no effect on both the basal and hyposmotically-sensitive portion of $K^+(Rb^+)$ efflux. (Fig. 3a). However, removing calcium from the loading buffer as well as the wash-out buffers increased $K^+(Rb^+)$ efflux under isosmotic conditions without affecting the peak response to a hyposmotic challenge (P<0.05; Fig. 3b).

Fig. 4 shows that neither apamin nor charybdotoxin (both tested at 100 nM) inhibited volume-activated $K^+(Rb^+)$ efflux from MDA-MB-231 cells, though the trend was that the

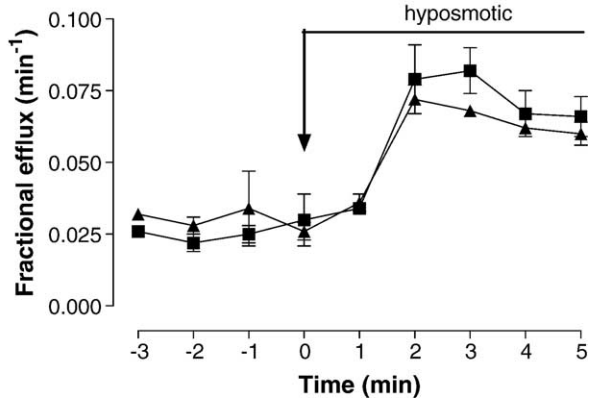

Fig. 2. $K^+(Rb^+)$ efflux from MDA-MB-231 cells incubated in the presence of extracellular Cl^- (■) or NO_3^- (▲). The Cl^- rich incubation media were the same as those described in Fig. 1. Cl^- was replaced with equimolar amounts of NO_3^- when required. Each point is the mean±S.E. of 4 experiments.

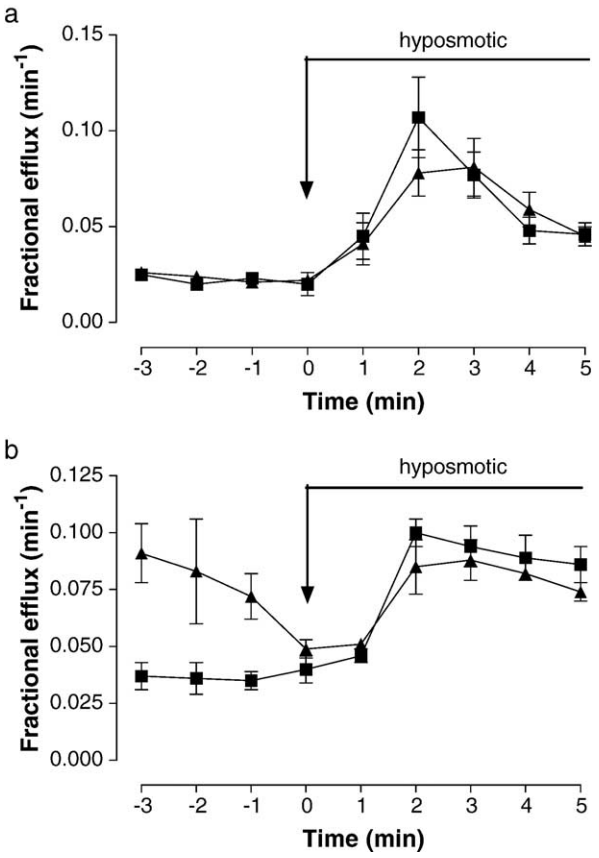

Fig. 3. The effect of (a) short-term (b) long-term removal (>60 min) of Ca^{2+} from the incubation buffers on $K^+(Rb^+)$ efflux from MDA-MB-231 cells measured under isosmotic and hyposmotic conditions. The normal-Ca^{2+} buffers were the same as those described in Fig. 1. The Ca^{2+}-free buffers were similar in composition except that they also contained 0.5 mM EGTA. The points denoted (■) and (▲), respectively, represent $K^+(Rb^+)$ efflux measured under normal-Ca^{2+}, and Ca^{2+}-free conditions. Each point is the mean±S.E. of 3–4 experiments.

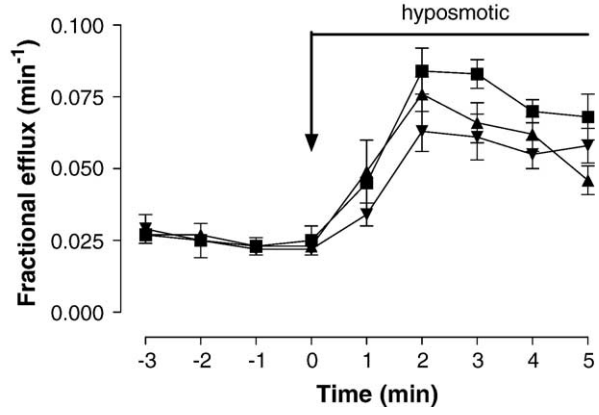

Fig. 4. The effect of charybdotoxin (▲) and apamin (▼) (both at 100 nM) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells measured under isosmotic and hyposmotic conditions. The points denoted (■) represent $K^+(Rb^+)$ efflux in the absence of drugs (i.e., control conditions). The composition of the buffers was the same as those described in Fig. 1. The drugs were present throughout the entire time course. Each point is the mean±S.E. of 3 experiments.

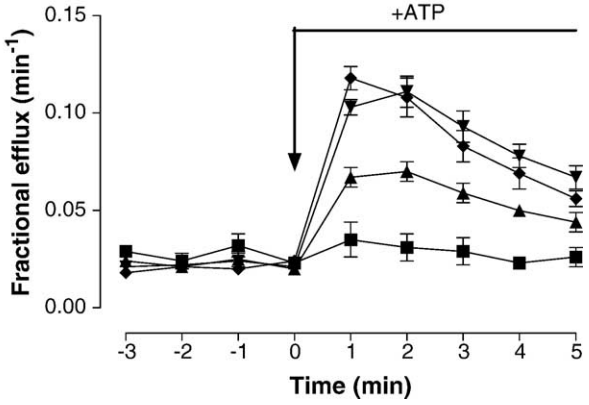

Fig. 5. The effect of extracellular ATP at 1 μM (■), 10 μM (▲), 100 μM (▼) and 1000 μM (♦) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells. The incubation medium contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 plus 0.001 to 1 ATP when required. Each point is the mean±S.E. of 4 experiments.

drug curves were lower than the control. Neither drug had any affect on $K^+(Rb^+)$ efflux from MDA-MB-231 cells under isosmotic conditions.

3.2. Effect of purinergic agonists on $K^+(Rb^+)$ efflux

Fig. 5 illustrates the effect of ATP (0.001–1 mM) on the fractional release of $K^+(Rb^+)$ from MDA-MB-231 cells. ATP increased $K^+(Rb^+)$ efflux in a dose dependent fashion, thus, ATP at a concentration of 10, 100 and 1000 μM, respectively increased $K^+(Rb^+)$ efflux (basal-to-peak) by 250, 428 and 392%. The concentration of ATP required to attain 50% of the maximal response (EC_{50}) was 20.7±6.4 μM. In contrast, ATP (100 μM) had no effect on taurine efflux from MDA-MB-231 cells (results not shown). UTP (0.001–1 mM) also stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells in a dose-dependent manner (Fig. 6) with an EC_{50} of 7.9±7.6 μM.

The Cl^- dependence of ATP-stimulated $K^+(Rb^+)$ release from MDA-MB-231 cells was examined to see if the increase in efflux was via K^+ channels and/or (K^+–Cl^-) cotransport (Fig. 7). ATP increased $K^+(Rb^+)$ efflux from MDA-MB-231 cells under Cl-free conditions, although replacing Cl^- slightly reduced the ATP-stimulated moiety of $K^+(Rb^+)$ efflux. Thus, the peak fractional efflux was reduced from 0.171±0.007 min^{-1} to 0.126±0.008 min^{-1} (±S.E., n=4; P<0.01). Consistent with the results shown in Fig. 2, replacing Cl^- had no effect on $K^+(Rb^+)$ efflux from MDA-MB-231 cells in the absence of exogenously added ATP.

We tested the effect of ATP, in the absence of extracellular calcium, on $K^+(Rb^+)$ efflux from MDA-MB-231 cells (Fig. 8). In agreement with the results shown in Fig. 3a, removing calcium from the incubation buffer at the beginning of the wash-out period had no effect on $K^+(Rb^+)$ efflux measured under basal conditions (Fig. 8a). However, calcium removal decreased (P<0.01) the ATP-sensitive moiety of $K^+(Rb^+)$ efflux from MDA-MB-231 cells (Fig. 8a). In particular, $K^+(Rb^+)$ efflux during the later part of the time-course was inhibited. There was a more pronounced change in the profile of $K^+(Rb^+)$ efflux from MDA-MB-231 cells when calcium was removed from the loading buffer as well as the buffers used during the wash-out period. Thus, under these conditions, basal $K^+(Rb^+)$ efflux was increased (P<0.05), whereas the ATP-activated portion of the efflux was markedly inhibited (P<0.01) (Fig. 8b).

The effect of the K^+-channel blockers charybdotoxin, iberiotoxin and apamin (all at 100 nM) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells is shown in Fig. 9. Charybdotoxin markedly inhibited the ATP-dependent moiety of $K^+(Rb^+)$ release whereas iberiotoxin and apamin had no effect. Thus, charybdotoxin reduced the peak fractional efflux from 0.133±0.009 min^{-1} to 0.042±0.001 min^{-1} (±S.E., n=3; P<0.0005). None of the drugs had any effect on $K^+(Rb^+)$ efflux in the absence of extracellular ATP.

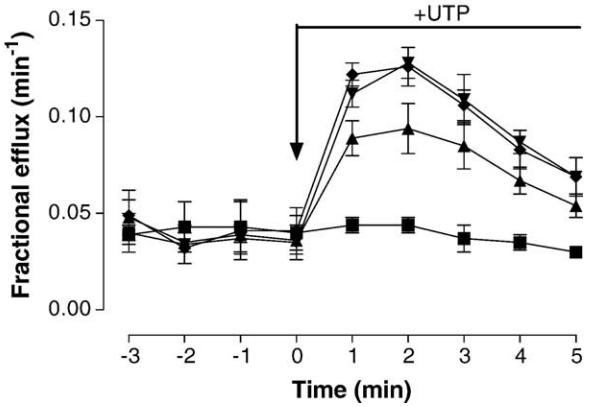

Fig. 6. The effect of UTP at 1 μM (■), 10 μM (▲), 100 μM (▼) and 1000 μM (♦) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells. The incubation medium contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 plus 0.001–1 UTP when required. Each point is the mean±S.E. of 3–4 experiments.

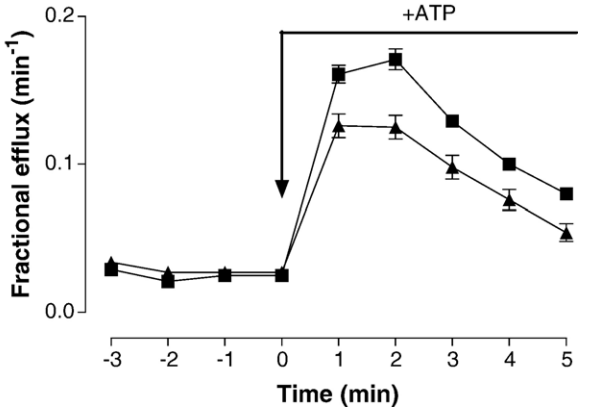

Fig. 7. ATP-stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells incubated in the presence of extracellular Cl^- (■) or NO_3^- (▲). The incubation buffer contained (mM) 135 NaCl or $NaNO_3$, 5 KCl or KNO_3, 2 $CaCl_2$ or $Ca(NO_3)_2$, 10 glucose and 10 Tris–MOPS, pH 7.4±0.1 ATP. Each point is the mean±S.E. of 4 experiments.

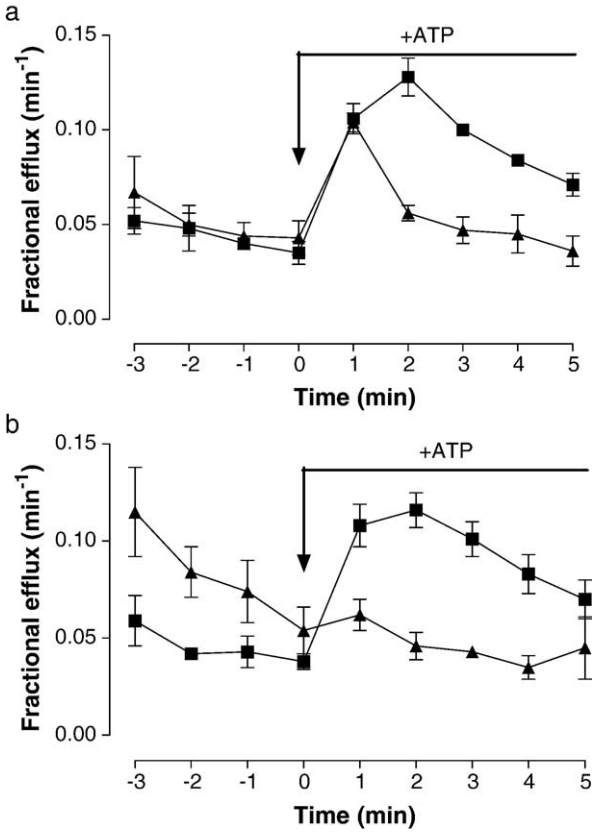

Fig. 8. The effect of (a) short-term and (b) long-term (>60 min) Ca^{2+} removal on ATP-stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells. The incubation medium contained (mM) 135 NaCl, 5 KCl, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4±2 $CaCl_2$±0.1 ATP. The Ca^{2+}-free buffers also contained 0.5 mM EGTA. The points denoted (■) and (▲), respectively, represent $K^+(Rb^+)$ efflux measured under normal-Ca^{2+} and Ca^{2+}-free conditions. Each point is the mean±S.E. of 3 experiments.

Adenosine, AMP and ADP did not significantly alter $K^+(Rb^+)$ efflux from MDA-MB-231 cells when tested at a final concentration of 0.1 mM (Fig. 10a). Moreover, ATP at a concentration of 0.01, 0.1 and 1 mM only had a weak transient effect ($P<0.05$) on $K^+(Rb^+)$ efflux from MCF-7 cells whereas ATP at 0.001 mM was without effect (Fig. 10b).

3.3. Effect of suramin and ATP-gamma-thio on $K^+(Rb^+)$ efflux

The ATP agonist ATP-gamma-thio (0.1 mM) stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells to the same extent as ATP (Fig. 11). The effect of ATP-gamma-thio on $K^+(^{86}Rb^+)$ efflux was inhibited by 0.5 mM suramin: the peak fractional release being reduced from 0.105±0.005 min^{-1} to 0.048±0.006 min^{-1} (±S.E., $n=3$; $P<0.001$).

3.4. Combined effects of ATP and a hyposmotic shock on $K^+(Rb^+)$ release

Fig. 12 illustrates the effect of ATP (0.1 mM), a hyposmotic shock and the two combined on $K^+(^{86}Rb^+)$ efflux from MDA-MB-231 cells. Consistent with results shown above, ATP and a hyposmotic shock both stimulated the fractional release of $K^+(Rb^+)$. A combination of ATP and a hyposmotic challenge appeared to have a synergistic effect ($P<0.001$)on the peak response of $K^+(Rb^+)$ efflux.

3.5. The effect of ATP on intracellular Ca^{2+}

Extracellular ATP (100 μM) induced a rapid increase in intracellular Ca^{2+} in MDA-MB-231 cells as indicated by the change in the fura 2 fluorescence ratio (Fig. 13a). The time taken to reach the peak value following the application of ATP was 19.0±6.2 s (±S.E., $n=3$). Thereafter, the $[Ca^{2+}]$ declined and returned to a basal level in 7.2±0.1 min (±S.E., $n=3$). Application of extracellular ATP to MDA-MB-231 cells which had been incubated in the absence of extracellular Ca^{2+} for 5 min prior to the addition of ATP and for the duration of the subsequent experiment, also induced a rapid increase in the intracellular $[Ca^{2+}]$ followed by a return to a basal level (Fig. 13b). Thus, the time taken to reach the peak response and return to a basal level was, respectively, 18.2 ±

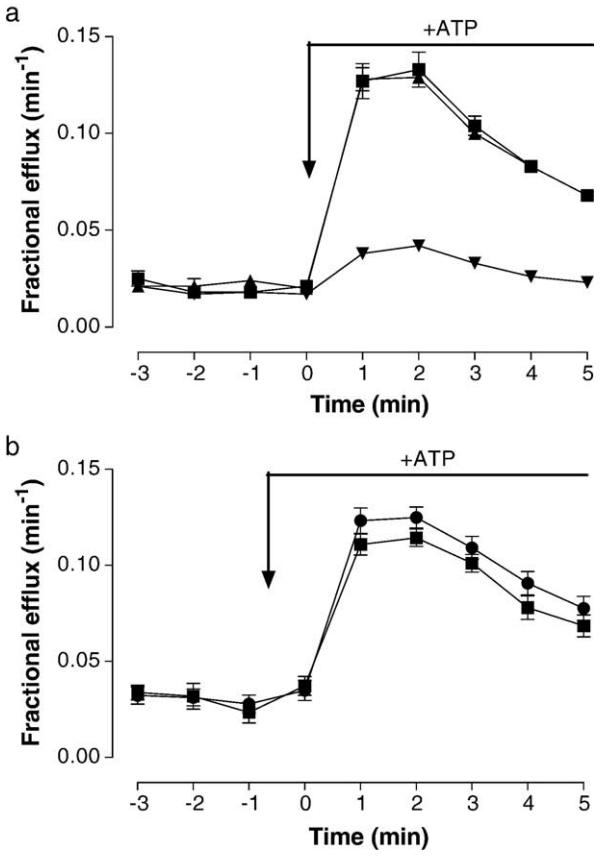

Fig. 9. (a) The effect of charybdotoxin (▼) and apamin (▲) and (b) iberiotoxin (●) on ATP-stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells. In each figure, the points denoted (■) represent $K^+(Rb^+)$ efflux with no drugs present (i.e., control conditions) The incubation medium contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$ and 10 Tris–Mops, pH 7.4±0.1 ATP. The drugs were present throughout the entire time course at a concentration of 100 nM. Each point is the mean±S.E. of 3 experiments.

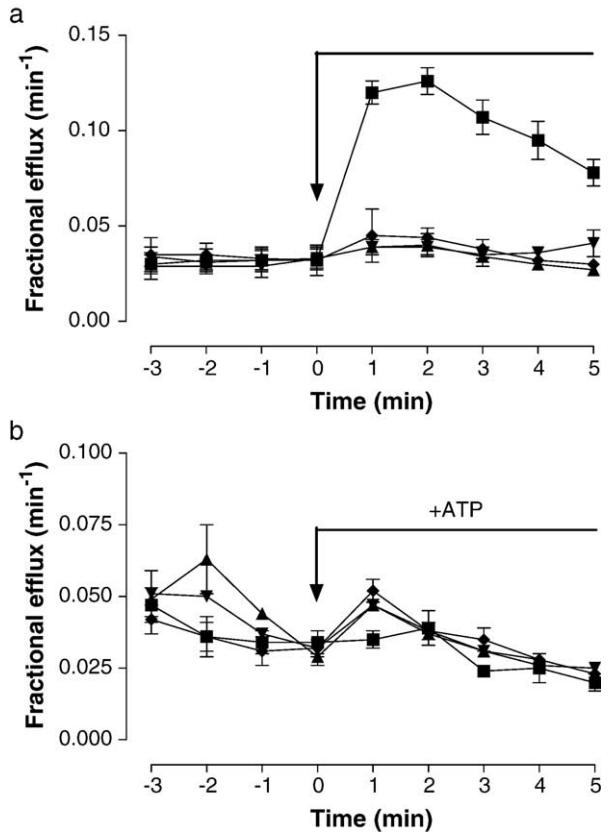

Fig. 10. (a) The effect of ATP (■), ADP (▲), AMP (▼) and adenosine (♦) (all at 0.1 mM) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells. The incubation media contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 plus ATP, ADP, AMP and adenosine at 0.1 mM as required. Each point is the mean±S.E. of 4 experiments. (b) The effect of ATP on $K^+(Rb^+)$ efflux from MCF-7 cells. The incubation media were the same as described for (a) except that they contained 1 μM (■), 10 μM (▲), 100 μM (▼) or 1000 μM (♦) ATP as required. Each point is the mean±S.E. of 4 experiments.

0.2 s and 3.9±0.3 min (±S.E., $n=3$). Extracellular ATP also induced a transient increase in the $[Ca^{2+}]_i$ in MCF-7 cells incubated in a calcium rich buffer (Fig. 13c). The peak

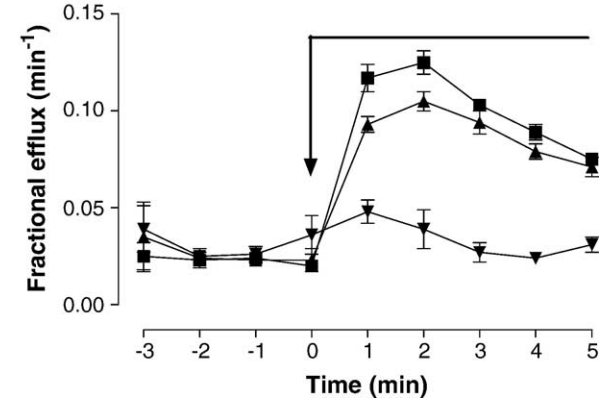

Fig. 11. The effect of ATP (■), ATP-gamma-thio (▲) and ATP-gamma-thio with suramin (▼) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells. The incubation media contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4±0.1 ATP, 0.1 ATP-gamma-thio and 0.5 suramin. Each point is the mean±S.E. of 3 experiments.

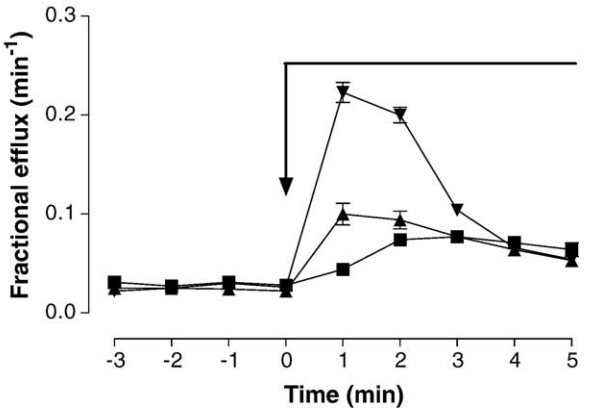

Fig. 12. The effect of ATP (▲), a hyposmotic shock (■) and their combination (▼) on $K^+(Rb^+)$ efflux from MDA-MB-231 cells. The incubation media were the same as those described in Figs. 1 and 4. Each point is the mean±S.E. of 3–4 experiments.

increase was attained 23.2±7.6 s (±S.E., $n=3$) and the intracellular $[Ca^{2+}]$ returned to a basal level 8.5±2.3 min (±S.E., $n=3$) after the application of ATP.

4. Discussion

4.1. The effect of a hyposmotic shock

Cells release K^+, Cl^- and organic solutes in response to cell swelling [18]. The efflux of solutes together with osmotically obliged water allow cells to return towards a normal volume: this process is termed a regulatory volume decrease (RVD). In accordance with this, the present results show that MDA-MB-231 cells release K^+ in response to a hyposmotic shock. There are at least two pathways which could provide a route for volume-sensitive K^+ efflux from MDA-MB-231 cells: (1) volume-activated K^+ channels and 2) (K^++Cl^-) cotransport. The lack of a dependence on Cl^- appears to rule out a role for (K^++Cl^-) cotransport and therefore suggests that K^+ channels provide a route for volume-activated K^+ efflux from MDA-MB-231 cells. In addition, the lack of a dependence on Cl^- together with the finding that bumetanide had no effect on $K^+(Rb^+)$ efflux suggests that the (Na^+–K^+–Cl^-) cotransporter does not mediate K^+ efflux under isosmotic and hyposmotic conditions. MCF-7 cells also released $K^+(Rb^+)$ in response to a hyposmotic shock, however, the increase in efflux was less pronounced than that found with MDA-MB-231 cells. This could be a reflection of the number of volume-activated K^+ channels expressed in each cell type. Moreover, the results suggest that MDA-MB-231 cells may be more adept at regulating their volume than MCF-7 cells. If true, this could confer an advantage upon MDA-MB-231 cells under conditions which cause cellular stress.

An osmotic challenge also stimulated taurine efflux from MDA-MB-231 and MCF-7 cells suggesting that both K^+ and taurine release could contribute to a RVD in MDA-MB-231

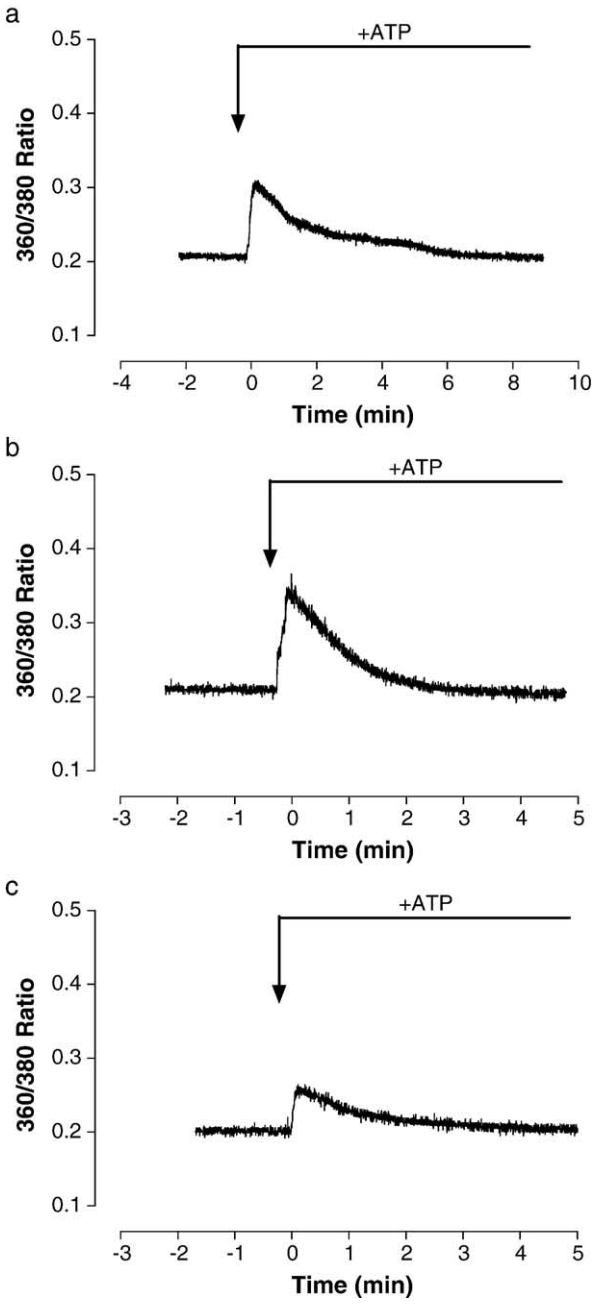

Fig. 13. The effect of extracellular ATP (100 μM) on the intracellular [Ca^{2+}] as judged by the change in fluorescence ratio in MDA-MB-231 (a,b) and MCF-7 cells (c). Cells were incubated in a buffer containing (mM) 134 NaCl, 6 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES–NaOH, pH 7.4. The Ca^{2+}-free buffer also contained 0.5 mM EGTA. In each case, the results are representative of 3 similar experiments.

and MCF-7 cells. In this connection, it has been suggested that volume-activated K^+ and taurine efflux may utilize a common pathway [19,20]. This may hold for MDA-MB-231 and MCF-7 cells given that $K^+(Rb^+)$ and taurine efflux are activated in parallel. However, in the absence of further evidence, this suggestion must remain speculative.

Swelling-activated K^+ efflux from a variety of cells is dependent upon Ca^{2+} mobilisation [21]. However, volume-dependent $K^+(Rb^+)$ efflux from MDA-MB-231 cells was not dependent on external calcium. Moreover, $K^+(Rb^+)$ release from MDA-MB-231 cells was not inhibited by Ca^{2+}-activated K^+ channel blockers. In this connection, Morales-Mulia et al. [22] have shown that volume-activated K^+ efflux from cerebellar granule neurones is not dependent upon external Ca^{2+} in spite of the fact that swelling increases intracellular Ca^{2+}. Similarly, swelling-induced efflux of K^+ from lactating rat mammary tissue is not dependent on Ca^{2+}[23]. We did find, however, that a prolonged exposure of the cells to a calcium-free buffer increased the basal efflux of $K^+(Rb^+)$ from MDA-MB-231 cells. This is a situation similar to that found with K^+ efflux from lactating rat mammary cells [23]. If volume-activated K^+ channels in breast cancer cells could be activated under isosmotic conditions they could possibly lead to cell shrinkage. According to the hypothesis of Maeno et al. [24], this would be a way of inducing apoptosis. Therefore, future work relating to the regulation of volume-activated K^+ channels in human breast cancer cells is merited.

4.2. The effect of purinergic agonists

Extracellular ATP is an important regulator of cell function. For example, ATP is able to increase K^+ and Cl^- efflux in a variety of cell types [11]. We now show that ATP markedly stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells in a dose-dependent manner. The findings that (1) UTP was as equipotent as ATP in stimulating $K^+(Rb^+)$ efflux, (2) ATP-stimulated $K^+(Rb^+)$ efflux was sensitive to suramin and (3) ADP, AMP and adenosine were ineffective in stimulating $K^+(Rb^+)$ efflux are consistent with the suggestion that MDA-MB-231 cells express P2Y2 receptors. In this connection, it is notable that other mammary cells such as MCF-7 [25] or 31EG4 [26] possess purinergic P2 receptors, and in the case of 31EG4 cells, specifically the P2Y2 subtype.

Nitrate ions were able to support ATP-stimulated $K^+(Rb^+)$ efflux from MDA-MB-231 cells suggesting that ATP is eliciting K^+ efflux predominantly via K^+ channels rather than (K^+–Cl^-) cotransport. However, it is notable that a small portion of the ATP-stimulated $K^+(Rb^+)$ efflux was dependent upon Cl^-. This suggests that (K^+–Cl^-) cotransport may play a minor role in ATP-stimulated K^+ release. However, it is possible that replacing Cl^- with NO_3^- affected intracellular Ca^{2+} and consequently $K^+(Rb^+)$ efflux. More experiments are required to resolve this issue.

Extracellular ATP increased the intracellular [Ca^{2+}] in MDA-MB-231 cells. This taken together with the finding that removal of extracellular Ca^{2+} from the incubation medium reduced ATP-stimulated $K^+(Rb^+)$ efflux suggests that MDA-MB-231 cells express Ca^{2+}-activated K^+ channels. ATP-stimulated $K^+(Rb^+)$ efflux was inhibited by charybdotoxin but not by iberiotoxin and apamin suggesting that intermediate-conductance channels may be involved. In contrast, Roger et al. [6] have recently reported the presence of iberiotoxin-sensitive channels in

MDA-MB-231 cells. The reason for this discrepancy is not yet clear.

The effect of ATP on $K^+(Rb^+)$ efflux from MDA-MB-231 cells under calcium-free conditions was, however, dependent on how long the cells were exposed to a calcium-free buffer. A prolonged exposure markedly inhibited ATP-stimulated $K^+(Rb^+)$ efflux throughout the entire time-course, whereas a short exposure had a more pronounced effect on the later part of the time-course. In relation to this, we found that extracellular ATP still increased intracellular calcium in MDA-MB-231 cells which had been exposed to a calcium-free buffer for a relatively short period (5 min) prior to the application of ATP. However, intracellular Ca^{2+} returned to a basal level more rapidly when cells were incubated in a calcium-free buffer (3.8 v 7.2 min). The most plausible explanation is that ATP initially stimulates the release of Ca^{2+} from intracellular stores, and this is followed by influx of extracellular Ca^{2+}. The physiological significance of ATP-stimulated $K^+(Rb^+)$ efflux MDA-MB-231 human breast cancer cells is unclear. However, it is notable that Roger et al. [6] reported that extracellular ATP increased the proliferation of MDA-MB-231 cells.

ATP had no significant effect on $K^+(Rb^+)$ efflux from MCF-7 cells. This result was surprising since MCF-7 cells possess P2 receptors which respond to a challenge with ATP or UTP by showing an increase in cytosolic-free calcium ($[Ca^{2+}]_i$) and Cl^- efflux as measured by ^{125}I transport [25]. In this study, we have confirmed that extracellular ATP is able to increase the $[Ca^{2+}]$ in MCF-7 cells, though without the associated efflux of $K^+(Rb^+)$. A Ca^{2+}-activated K^+ channel also exists in MCF-7 cells [27], so it would appear that the failure of ATP to stimulate K^+ efflux in these cells is not due to the lack of a potential route of efflux.

4.3. Relationship between volume-sensitive and ATP-stimulated $K^+(Rb^+)$ efflux pathways

Our results show that MDA-MB-231 cells already stimulated with ATP still respond to an osmotic shock by increasing the fractional efflux of $K^+(Rb^+)$. This suggests that volume-activated and ATP-stimulated $K^+(Rb^+)$ efflux occurs via separate pathways. In support of this notion is the differential effect of removing extracellular Ca^{2+} on volume-activated and ATP-stimulated $K^+(Rb^+)$ efflux. Moreover, the finding that charybdotoxin inhibited ATP-stimulated $K^+(Rb^+)$ efflux but not the hyposmotically-sensitive moiety is indicative of separate pathways. However, there could be a form of 'cross-talk' between the two routes given that the effects of ATP and an osmotic shock were more than additive. Such an interaction is seen between ATP and volume-activated amino acid efflux in astrocytes [28]. Given the increasing awareness of the role of K^+ in apoptosis, and the importance of regulating programmed cell death in mammary cells, this is clearly an area worth of further study.

Acknowledgements

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