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ACTIVATION OF MUSCARINIC CHOLINERGIC RECEPTORS ON HUMAN SH-SY5Y NEUROBLASTOMA CELLS ENHANCES BOTH THE INFLUX AND EFFLUX OF K⁺ UNDER CONDITIONS OF HYPOOSMOLARITY

Daniel J. Foster, Anne M. Heacock, Richard F. Keep and Stephen K. Fisher

Molecular and Behavioral Neuroscience Institute (SKF, AMH) and Departments
of Pharmacology (DJF, SKF), Neurosurgery and Molecular and Integrative
Physiology (RFK), University of Michigan, Ann Arbor, Michigan.

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Corresponding author: Dr. Stephen K. Fisher, University of Michigan, Molecular and Behavioral Neuroscience Institute, 5039 Biomedical Science Research Building, Ann Arbor, MI 48109-2200, e-mail: skfisher@umich.edu. Telephone: (734)-763-4376; Fax: (734)-936-2690

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Non-standard abbreviations: DMEM, Dulbecco's modified Eagle's medium; HEPES, N-[2 hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; GPCR, G-protein-coupled receptor; PAR, protease-activated receptor; mAChR, muscarinic cholinergic receptor; S1P, sphingosine 1-phosphate; PKC, protein kinase C; Oxo-M, oxotremorine-M; LPA, lysophosphatidic acid; ANOVA, analysis of variance; DIOA, R-(+)-[(2-n-butyl-6,7-dichloro-2-cyclopentenyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid; NKCC, Na⁺-K⁺-2Cl⁻ cotransporter; KCC, K⁺-Cl⁻ co-transporter.

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Abstract

The ability of receptor activation to regulate osmosensitive K^+ fluxes (monitored as $^{86}Rb^+$) in SH-SY5Y neuroblastoma has been examined. Incubation of SH-SY5Y cells in buffers rendered increasingly hypotonic by a reduction in NaCl concentration resulted in an enhanced basal efflux of Rb^+ (threshold of release, 200 mOsm), but had no effect on Rb^+ influx. Addition of the muscarinic cholinergic agonist, oxotremorine-M (Oxo-M), potently enhanced Rb^+ efflux ($EC_{50} = 0.45 \mu M$) and increased the threshold of release to 280 mOsm. Oxo-M elicited a similarly potent, but osmolarity-independent, enhancement of Rb^+ influx ($EC_{50} = 1.35 \mu M$). However, when incubated under hypotonic conditions in which osmolarity was varied by the addition of sucrose to a fixed concentration of NaCl, basal- and Oxo-M-stimulated Rb^+ influx and efflux were demonstrated to be dependent upon osmolarity. Basal- and Oxo-M-stimulated Rb^+ influx (but not Rb^+ efflux) were inhibited by inclusion of ouabain or furosemide. Both Rb^+ influx and efflux were inhibited by removal of intracellular Ca^{2+} and inhibition of protein kinase C activity. In addition to Oxo-M, agonists acting at other cell-surface receptors previously implicated in organic osmolyte release enhanced both Rb^+ efflux and influx under hypotonic conditions. Oxo-M had no effect on cellular K^+ concentration in SH-SY5Y cells under physiologically relevant reductions in osmolarity (0-15%) unless K^+ influx was blocked. Thus although receptor activation enhances the osmosensitive efflux of K^+ , it also stimulates K^+ influx and the latter permits retention of K^+ by the cells.

Introduction

Regulation of cell volume is of prime importance to the CNS due to the restricted volume of the skull (Pasantés-Morales et al., 2000, 2002). Even modest alterations in brain cell volume can have profound effects within the CNS as the spatial relationships between neurons, astrocytes and extracellular space are compromised. Brain cells swell either via changes in plasma osmolarity (hypoosmotic swelling) or intracellular ion and water distribution (isotonic swelling or cellular/cytotoxic edema). The most prevalent cause of hypoosmotic swelling is a condition known as hyponatremia, which is defined as a reduction in serum Na^+ concentration from a normal value of 145 mM to 136 mM or below. Hyponatremia, which may result from congestive heart failure, nephrotic syndrome, hepatic cirrhosis, inappropriate secretion of anti-diuretic hormone or psychotic polydipsia, occurs in 2.5% of hospitalized patients (Lien and Shapiro, 2007). It disproportionately affects the young and the elderly and causes predominantly neurological symptoms such as lethargy, confusion and coma.

Following hypoosmotic stress, cells swell in proportion to the reduction in osmolarity and then normalize their volume in a recovery process known as regulatory volume decrease in which osmolytes (K^+ , Cl^- and small organic molecules) are extruded and cell volume is normalized via the exit of obligated water (McManus et al., 1995). Inorganic osmolytes, such as K^+ and Cl^- , constitute the quantitatively major component of the osmolyte pool (60-70%), whereas organic osmolytes such as taurine, glutamate and inositol comprise the remainder (Pasantés-Morales et al., 2002). In most (but not all) tissues, the

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extrusion of Cl^- and organic osmolytes appears to occur via a common, volume-sensitive organic osmolyte and anion channel, which is primarily permeable to Cl^- , but impermeable to cations (Sanchez-Olea et al., 1996; Lang et al., 1998; Nilius and Droogmans, 2003; Abdullaev et al., 2006). Although less extensively studied, the efflux of K^+ has been reported to occur via a variety of different K^+ channels including those gated by voltage or activated by stretch, swelling or Ca^{2+} (Pasantés-Morales et al., 2006).

When monitored *in vitro*, the efflux of both inorganic and organic osmolytes is relatively insensitive to hypoosmotic stress, often requiring reductions in osmolarity (>30%) that are not typically encountered *in vivo*. However, recent studies from this and other laboratories have demonstrated that the volume-sensitive efflux of osmolytes from neural tissues can be enhanced following the activation of certain G-protein-coupled receptors (GPCRs), including the P_{2Y} purinergic (Mongin and Kimelberg, 2002, 2005), M3 muscarinic cholinergic (mAChR: Loveday et al., 2003; Heacock et al., 2004), lysophospholipid (Heacock et al., 2006) and the protease-activated-1 receptors (Cheema et al., 2005, 2007; Ramos-Mandujano et al., 2007). Receptor activation not only increases the extent of osmolyte release, but also lowers the threshold osmolarity ('set-point') at which osmolytes are released. The latter observation raises the possibility that tonic agonist activation of cell-surface receptors may permit neural cells to respond to more physiologically relevant reductions in osmolarity.

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Although inorganic osmolytes are released from cultured neural cells to the same or greater extent than is observed for organic osmolytes under both basal (swelling-activated) and receptor-stimulated conditions (Abdullaev et al., 2006; Cheema et al., 2007), chronic hyponatremia results in a disproportionately greater percentage loss of organic osmolytes than of inorganic osmolytes from the brain (Lien et al., 1991; Videen et al., 1995; Pasantes-Morales et al., 2002; Massieu et al., 2004). One potential explanation for this observation is that, under hypoosmotic conditions, the volume-dependent efflux of inorganic osmolytes is accompanied by a compensatory uptake phase, as previously proposed for K^+ (Mongin et al., 1994, 1996). However, the issue of whether receptor activation can promote the uptake of osmolytes under hypoosmotic conditions has not, to the best of our knowledge, been previously investigated. To address this question, in the present study we have examined the ability of mAChRs (and other GPCRs) to regulate K^+ homeostasis in human SH-SY5Y neuroblastoma cells under conditions of hypoosmotic stress. The results indicate that receptor activation facilitates **both** the efflux and influx of K^+ in an osmosensitive manner. Under conditions of either isotonicity or limited reductions in osmolarity (15%), the efflux of K^+ is effectively countered by an influx of K^+ , such that no net loss of cell K^+ occurs. Only under more pronounced reductions in osmolarity (30%) does the rate of K^+ efflux exceed that of influx and result in a net loss of K^+ . Thus, receptor activation serves to regulate both the release and uptake of osmolytes. A preliminary account of part of this work has appeared elsewhere (Foster et al., 2008).

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Methods

Materials. Rubidium Chloride ($^{86}\text{Rb}^+$ -labeled; 241 MBq/mg) was obtained from PerkinElmer Life and Analytical Sciences (Shelton, CT). 3-O-Methyl-D-[1- ^3H]glucose (148 GBq/mmol) was from GE Healthcare (Piscataway, NJ). Oxotremorine-M, sphingosine 1-phosphate, thrombin, bumetanide, DIOA, ouabain, furosemide, tetraethylammonium chloride, barium chloride dihydrate, atropine and 3-O-methyl-D-glucose were purchased from Sigma-Aldrich (St. Louis, MO). Iberiotoxin, apamin, and glibenclamide were obtained from Tocris Bioscience (Ellisville, MO). Chelerythrine, thapsigargin and phloretin were obtained from Calbiochem (San Diego, CA). Lysophosphatidic acid was purchased from Avanti (Alabaster, AL). Dulbecco's modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (Franklin Lakes, NJ). Universol was obtained from Valeant Pharmaceuticals (Costa Mesa, CA).

Cell culture conditions. Human SH-SY5Y neuroblastoma cells (passages 70-89) were grown in tissue culture flasks (75 cm²/250ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal calf serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330-340 mOsM. Cells were grown at 37°C in a humidified atmosphere containing 10% CO₂. The medium was aspirated and

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cells detached from the flask with a trypsin-versene mixture (Cambrex Bio Science, Walkersville, MD). Cells were then resuspended in DMEM/10% fetal calf serum with penicillin/streptomycin and subcultured into 35-mm, six-well culture plates at a density of 250 to 300,000 cells/well for 4 to 5 days. Cells that had reached 70-90% confluence were routinely used.

Measurement of K⁺ efflux. K⁺ efflux from SH-SY5Y neuroblastoma cells was determined using ⁸⁶Rb⁺ as a tracer for K⁺. In brief, cells were prelabeled overnight to isotopic equilibrium with 19-37 KBq/ml ⁸⁶Rb⁺ at 37°C. After prelabeling, the cells were washed three times with 2 ml of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 3.6 mM NaHCO₃, 1 mM MgCl₂, 30 mM HEPES, pH 7.4, and 1 mg/ml D-glucose, ~340 mOsM). Cells were then allowed to incubate in 2 ml of buffer A (370-200 mOsM; routinely rendered either hypertonic or hypotonic by an increase or decrease in NaCl concentration, respectively) in the absence or presence of agonists. In some experiments, osmolarities of the buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. Osmolarities of buffers were monitored by means of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA). At the times indicated, aliquots of the extracellular medium (1 ml) were removed and radioactivity determined after the addition of 6 ml of Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 ml of 0.1 M NaOH. The rate of efflux of ⁸⁶Rb⁺ was calculated as a fractional release/min, i.e., the

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radioactivity released/min into the extracellular medium as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay. For all measurements, radioactivity released at the zero time point was subtracted from the observed value. Throughout the study “basal” release of $^{86}\text{Rb}^+$ is defined as that which occurs at a specified osmolarity in the absence of agonists.

Measurement of K^+ influx. K^+ influx was determined using $^{86}\text{Rb}^+$ as a tracer for K^+ . SH-SY5Y neuroblastoma cells were washed twice with 2 ml of isotonic buffer A (~340 mOsM) and then incubated in buffer A (370-200 mOsM routinely rendered either hypertonic or hypotonic, unless otherwise stated, by an increase or decrease in NaCl concentration, respectively) containing $^{86}\text{Rb}^+$ (28-56 KBq/ml) with or without agonist at 37°C. In some experiments, osmolarities of the buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. At the times indicated, the extracellular medium was aspirated, cells washed three times with 2 ml of isotonic buffer A and then the cells were lysed with 2 ml of 0.1 M NaOH. Aliquots of lysate (1 ml) were removed and radioactivity determined after the addition of 6 ml Universol scintillation fluid. In all measurements, radioactivity accumulated at the zero time point was subtracted from the observed value. Protein contents of cell lysates were determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). From the measurement of $^{86}\text{Rb}^+$ uptake, K^+ influx was calculated

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as nmol/mg protein/min with the assumption that $^{86}\text{Rb}^+$ transport into the cells reflects that of K^+ .

Intracellular water space. The intracellular water space was measured essentially as previously described (Novak et al., 1999). SH-SY5Y neuroblastoma cells were washed with 5 x 2 ml of buffer A without D-glucose and then incubated in buffer A with increasing extracellular concentrations of 3-O- ^3H -methyl-D-glucose at 37°C until equilibrium had been achieved (50 min). Cells were then washed with 5 x 2 ml of ice-cold buffer A without glucose containing 0.1 mM phloretin and lysed with 2 ml of 0.1 M NaOH. Aliquots (1 ml) of lysate were removed and radioactivity determined after the addition of 6 ml Universol scintillation fluid. Intracellular concentrations of 3-O- ^3H -methyl-D-glucose were monitored at equilibrium and a plot of this parameter vs. the concentration of extracellular 3-O- ^3H -methyl-D-glucose yields a line whose slope is the volume of intracellular water with respect to protein. Determination of water space by this method requires that 3-O-methyl-D-glucose not be metabolized or actively transported, and these assumptions were validated by the linearity of the plot and its extrapolation through the origin (Kletzien et al., 1975).

K^+ mass measurements. SH-SY5Y cells were washed with 2 x 2 ml of isotonic buffer A. Cells were then incubated for 10 min in buffer A (340-230 mOsm rendered hypotonic by a reduction in NaCl concentration) at 37°C. The

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extracellular medium was then aspirated, cells were then washed with 2 ml of K⁺ free buffer A (142 mM NaCl, 2.2 mM CaCl₂, 3.6 mM NaHCO₃, 1 mM MgCl₂, 30 mM HEPES, pH 7.4, and 1 mg/ml D-glucose, ~335 mOsm) and lysed in 2 ml of 0.1 M NaOH. Protein contents of cell lysates were determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Lysates from three separate 35 mm wells were combined (total volume 6 ml) and centrifuged at 3,000 x g for 30 min at 5° C. Supernatants were then adjusted to a final pH of between 5 and 11 with 4 N HCl. K⁺ values were obtained using a glass combination K⁺ electrode (Cole Parmer) and an Acorn Series Ion 6 meter (Oakton Instruments, Vernon Hills, IL).

Data Analysis. All experiments shown were performed in duplicate or triplicate and repeated at least three times. Values quoted are given as means \pm S.E.M. for the number (n) of independent experiments indicated. A two-tailed Student's t test (paired or unpaired) was used to evaluate differences between two experimental groups (level of significance, $p < 0.05$). Ordinary or repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups. EC₅₀ values were obtained using Prism 4.0a (GraphPad Software Inc., San Diego, CA).

Results

Agonist activation of mAChRs on SH-SY5Y neuroblastoma cells enhances both $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux. When SH-SY5Y cells were exposed to hypotonic buffer A (230 mOsM; ~30% reduction in osmolarity), conditions previously determined to be optimal for the release of organic osmolytes, there was a time-dependent increase in both $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux under basal conditions ('basal' is defined as influx or efflux monitored at a specified osmolarity in the absence of agonist). Inclusion of Oxo-M (100 μM) elicited a marked enhancement of $^{86}\text{Rb}^+$ influx over basal at 3 min and thereafter and resulted in a doubling of the rate of $^{86}\text{Rb}^+$ uptake (Fig. 1A). Inclusion of Oxo-M also significantly enhanced the efflux of $^{86}\text{Rb}^+$ in an approximately linear manner up to 10 min of incubation (rate constants for $^{86}\text{Rb}^+$ efflux under basal- and Oxo-M-stimulated conditions were 0.85 and 2.61% per min, respectively; Fig. 1B). In subsequent experiments, both basal- and agonist-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux were routinely monitored after either 5 or 10 min incubations. The addition of Oxo-M resulted in a stimulation of $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux with EC_{50} values of 0.45 μM and 1.37 μM , respectively, and with Hill coefficients close to unity (Fig. 2 A,B). The inclusion of 10 μM atropine completely blocked Oxo-M-stimulation of both $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux (data not shown).

Osmolarity dependence of basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux. Because the degree of facilitation of osmolyte release observed following mAChR activation has previously been demonstrated to be dependent

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on the extent of hypoosmotic stress (Loveday et al., 2003; Heacock et al., 2004), the ability of mAChR activation to regulate $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux was monitored in SH-SY5Y cells under conditions of isotonicity (340 mOsM; defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown), mild-to-severe hypotonicity (310-200 mOsM), or mild hypertonicity (370 mOsM). Two experimental paradigms were employed to evaluate the dependence of $^{86}\text{Rb}^+$ fluxes on osmolarity. In the first, buffers were rendered either hypertonic or hypotonic by increases or decreases, respectively, in the NaCl concentration (since Na^+ and Cl^- are the primary osmolytes found in plasma and reductions in plasma osmolarity observed under pathological conditions, such as hyponatremia, principally reflect changes in the concentrations of these ions). Under these conditions, the magnitude of basal $^{86}\text{Rb}^+$ influx was constant at all osmolarities tested. The addition of Oxo-M resulted in an increase in $^{86}\text{Rb}^+$ influx of ~ 75%, compared to basal, at all osmolarities (370-200 mOsM; Fig. 3A). In contrast, the basal efflux of $^{86}\text{Rb}^+$ was enhanced over that observed under isotonic conditions (340 mOsM) when osmolarity was reduced to 200 mOsM. Moreover, although the addition of Oxo-M resulted in a relatively small increase in $^{86}\text{Rb}^+$ efflux at both 340 and 370 mOsM, the extent of Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux was significantly increased over isotonic at an osmolarity of 280 mOsM (a reduction in osmolarity of 18%) with a maximal enhancement observed at 230 mOsM (386% of basal; Fig. 3B). In the second experimental paradigm, osmolarities of buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. Under these conditions,

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the basal influx of $^{86}\text{Rb}^+$ was significantly enhanced over that observed under isotonic conditions when the osmolarity was reduced to 230 or 200 mOsM (134% and 159% of that at 340 mOsM, respectively). The extent of Oxo-M-stimulated $^{86}\text{Rb}^+$ influx was also dependent upon osmolarity and, although an increased influx was monitored under isotonic conditions, significantly greater increases were observed at 230 and 200 mOsM than at 340 mOsM (Fig. 4A). The magnitudes of both basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux were also found to be dependent upon the osmolarity of the buffer under conditions of a fixed concentration of NaCl and the values obtained for $^{86}\text{Rb}^+$ efflux were quantitatively similar for the two experimental paradigms (Fig. 4B).

$^{86}\text{Rb}^+$ influx is mediated primarily via Na^+/K^+ -ATPase and the NKCC transporter under both basal and Oxo-M-stimulated conditions. K^+ transport mechanisms, including Na^+/K^+ ATPase and the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ (NKCC) and K^+-Cl^- (KCC) co-transporters, have previously been implicated in cell volume regulation. To determine the role, if any, played by these transporters in $^{86}\text{Rb}^+$ influx in SH-SY5Y neuroblastoma cells, both basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx were monitored in the absence or presence of pharmacological inhibitors at concentrations similar to those previously employed (Yabaluri et al., 1997; Ernest et al., 2005). Inclusion of 800 μM concentrations of either bumetanide or furosemide, inhibitors of the NKCC, attenuated both basal- and Oxo-M stimulated $^{86}\text{Rb}^+$ influx by ~50%. Since furosemide inhibits both NKCC and KCC, we also evaluated the ability of DIOA, a KCC inhibitor, to attenuate $^{86}\text{Rb}^+$ influx. Inclusion

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of a 40 μM concentration of DIOA had no effect on basal $^{86}\text{Rb}^+$ influx but resulted in a 20% inhibition of the Oxo-M-mediated component. Inclusion of 30 μM ouabain, a selective inhibitor of the Na^+/K^+ -ATPase, resulted in a significant inhibition (~40%) of both basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx. When both ouabain and furosemide were present, basal- and Oxo-M stimulated $^{86}\text{Rb}^+$ influx were essentially abolished (94% and 97% reductions, respectively; Fig. 5A). In contrast, neither the inclusion of bumetanide nor furosemide had any significant effect on basal $^{86}\text{Rb}^+$ efflux (Fig. 5B). Furthermore, the inclusion of the NKCC inhibitors resulted in either no effect (bumetanide) or a modest inhibition (19%, furosemide) when Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux was monitored. Inclusion of DIOA also resulted in a small inhibition of Oxo-M-mediated $^{86}\text{Rb}^+$ efflux. Addition of ouabain had no effect on the magnitude of either basal- or Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux, but when co-administered with furosemide, an inhibition of Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux (~20%) was again observed (Fig. 5B). Taken collectively, these results suggest that $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux are mediated by distinct mechanisms.

$^{86}\text{Rb}^+$ efflux is partially mediated by K^+ channels. Evidence that $^{86}\text{Rb}^+$ efflux is mediated, in part at least, by K^+ channels was obtained from experiments in which either the addition of TEA or Ba^{2+} , general inhibitors of K^+ channels, were found to attenuate both basal- and Oxo-M-stimulated efflux. Inclusion of TEA (10 mM) or Ba^{2+} (4 mM) significantly inhibited basal $^{86}\text{Rb}^+$ release by 37 ± 7 and $38\pm 6\%$, respectively ($p < 0.05$; $n = 6-9$) and Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux by 30 ± 6

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and $41 \pm 4\%$ respectively ($p < 0.05$; $n = 6-9$). The addition of specific K^+ channel blockers such as apamin ($1 \mu\text{M}$) or iberiotoxin (100 nM), inhibitors of Ca^{2+} -activated channels, had little or no effect ($< 10\%$) on either basal or Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux. Inclusion of glibenclamide ($100 \mu\text{M}$), an inhibitor of ATP-dependent K^+ channels, had no effect on basal $^{86}\text{Rb}^+$ release but did result in a small, yet significant reduction in Oxo-M-stimulated $^{86}\text{Rb}^+$ efflux ($< 15\%$).

Basal- and Oxo-M stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux: dependence on Ca^{2+} availability and PKC activity. Previously we have demonstrated that the mAChR-stimulated osmosensitive release of the organic osmolyte, taurine, from SH-SY5Y cells is more dependent upon Ca^{2+} availability and PKC activity than that of the inorganic osmolyte, Cl^- , suggesting that the release of these osmolytes may be differentially regulated (Heacock et al., 2006; Cheema et al., 2007). For this reason, in the present study, the roles played by Ca^{2+} availability and PKC activity in $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux were evaluated. Although removal of extracellular Ca^{2+} had no effect on either basal- or Oxo-M-stimulated $^{86}\text{Rb}^+$ influx, the additional depletion of intracellular Ca^{2+} stores with $1 \mu\text{M}$ thapsigargin resulted in an increase in basal $^{86}\text{Rb}^+$ influx, whereas the ability of Oxo-M to enhance $^{86}\text{Rb}^+$ influx over the basal value was attenuated by approximately 35% under these conditions. Inclusion of $10 \mu\text{M}$ chelerythrine, a PKC inhibitor, had no effect on basal $^{86}\text{Rb}^+$ influx but significantly inhibited (50-60%) the Oxo-M-stimulated component, both in the presence or absence of Ca^{2+} /thapsigargin (Fig. 6A). Removal of extracellular Ca^{2+} resulted in an increase in basal efflux of

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$^{86}\text{Rb}^+$ whereas the ability of Oxo-M to increase efflux over the basal value was unchanged relative to control incubations. Depletion of intracellular Ca^{2+} stores with thapsigargin resulted in a further increase in the basal efflux of $^{86}\text{Rb}^+$ but significantly attenuated (~25%) the Oxo-M-mediated increase in $^{86}\text{Rb}^+$ efflux. As observed for $^{86}\text{Rb}^+$ influx, inclusion of chelerythrine had no effect on basal $^{86}\text{Rb}^+$ efflux but significantly inhibited (~60%) Oxo-M-mediated $^{86}\text{Rb}^+$ efflux. Under conditions in which intracellular stores of Ca^{2+} were depleted and PKC activity inhibited, the ability of Oxo-M to stimulate $^{86}\text{Rb}^+$ efflux was severely attenuated (86% inhibition, Fig. 6B).

Activation of multiple GPCRs can elicit both $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux under hypotonic conditions. In addition to the mAChR, activation of several other GPCRs has been shown to increase the efflux of osmolytes from SH-SY5Y cells under hypotonic conditions (Heacock et al., 2006; Cheema et al., 2007). These include the protease-activated receptor (PAR) which can be activated by thrombin and lysophospholipid receptors that can be selectively activated by either sphingosine-1-phosphate (S1P) or lysophosphatidic acid (LPA). To investigate whether activation of these receptors could also mediate changes in $^{86}\text{Rb}^+$ fluxes, thrombin (1.25 nM), S1P (5 μM) or LPA (10 μM) were added to SH-SY5Y cells under hypotonic conditions (230 mOsm) and $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux monitored after a 10 min incubation. Addition of each of the three agonists resulted in a significant increase in both $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux, with a rank

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order of efficacy for both fluxes being, thrombin = S1P > Oxo-M > LPA (Fig. 7 A,B).

Oxo-M-mediated regulation of intracellular K⁺ concentration: roles of osmolarity and K⁺ influx. The ability of Oxo-M to regulate the concentration of K⁺ in SH-SY5Y cells under conditions of isotonicity (340 mOsM), mild hypotonicity (290 mOsM) or moderate hypotonicity (230 mOsM) was evaluated in the absence or presence of inhibition of K⁺ influx following a 10 min incubation. Under isotonic conditions the intracellular K⁺ concentration, as determined by means of a K⁺-specific electrode, was 1.22 μmol K⁺/mg protein, a value consistent with previous measurements of intracellular K⁺ in the neuroblastoma 2A cell line (~1 μequiv. K⁺/mg protein: Kimelberg, 1974). Since the intracellular water space for SH-SY5Y cells was determined to be 8.5 μl/mg protein (n=3), the intracellular K⁺ concentration of SH-SY5Y cells was calculated to be approximately 140 mM. Under either isotonic (340 mOsM) or mildly hypotonic (290 mOsM) conditions, the addition of Oxo-M had no significant effect on intracellular K⁺ content. However, at both osmolarities, concurrent inhibition of K⁺ influx by inclusion of furosemide (800 μM) and ouabain (30 μM) resulted in small, but significant reductions in K⁺ mass under basal conditions (i.e. in the absence of the agonist). These reductions in K⁺ concentration were accentuated by the presence of Oxo-M (Table 1). When cells were incubated under moderately hypotonic conditions (230 mOsM), the addition of Oxo-M resulted in a significant loss of K⁺ from the cells, even when K⁺ influx was operational. However, when K⁺ influx was prevented by inclusion of furosemide and ouabain, at this

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osmolarity the addition of Oxo-M resulted in a further loss of cell K^+ (23%) during a 10 min incubation period (Table 1). This value for loss of K^+ agrees closely with measurement of $^{86}Rb^+$ efflux under Oxo-M-stimulated conditions (rate = 2.61 % per min: Fig. 1B).

Discussion

Previous studies of osmolyte loss from neural cells following their exposure to a hypoosmotic medium have focused almost exclusively on measurement of efflux. However, the net loss of an osmolyte from a cell reflects both its release and uptake. The principal finding to emanate from the present study is that, under hypoosmotic conditions, the activation of mAChRs in SH-SY5Y neuroblastoma cells can enhance the osmosensitive efflux **and** influx of $^{86}\text{Rb}^+$. Furthermore, these two fluxes exhibit similar characteristics in terms of kinetics, agonist concentration-dependence and requirements for Ca^{2+} availability and PKC activity. Because SH-SY5Y cells exhibit a relatively homogeneous population of M3 mAChRs (>80% of total: Wall et al., 1991; Slowiejko et al., 1994) it is likely that this mAChR subtype mediates the increase in $^{86}\text{Rb}^+$ fluxes, as has been previously demonstrated for the osmosensitive release of taurine (Heacock et al., 2004). In addition to the mAChR, activation of several other GPCRs that have previously been demonstrated to regulate the osmosensitive release of taurine from SH-SY5Y cells, i.e. PAR-1, S1P and LPA receptors (Heacock et al., 2006; Cheema et al., 2007) also promoted both the efflux and influx of $^{86}\text{Rb}^+$ under hypoosmotic conditions (Fig. 7). Furthermore, activation of PAR-1 and S1P receptors also facilitated both the influx and efflux of $^{86}\text{Rb}^+$ in primary cultures of rat astrocytes (data not shown). Taken collectively, these results suggest that the ability of GPCRs to regulate both the influx and efflux of $^{86}\text{Rb}^+$ under hypoosmotic conditions may be a general property of neural cells.

Although Oxo-M-stimulated efflux and influx of $^{86}\text{Rb}^+$ share similar

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characteristics, the two fluxes are mediated via distinct mechanisms. Under hypoosmotic conditions, both basal- and Oxo-M-mediated $^{86}\text{Rb}^+$ influx are abolished following administration of ouabain and furosemide, a result that indicates that $^{86}\text{Rb}^+$ influx is mediated principally through the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (NKCC) and $\text{Na}^+\text{/K}^+\text{-ATPase}$ (Fig 5A). These findings are consistent with previous studies in which the basal influx of $^{86}\text{Rb}^+$ in astrocytes and C6 glioma cells, monitored under isoosmotic or hypoosmotic conditions, was also inhibited by ouabain and furosemide (Kimelberg and Frangakis, 1985; Mongin et al., 1994, 1996). KCC may also play a minor role in $^{86}\text{Rb}^+$ influx in SH-SY5Y cells, as previously suggested for C6 glioma (Gagnon et al., 2007). In contrast to the results obtained for $^{86}\text{Rb}^+$ influx, none of the agents tested had any effect on basal $^{86}\text{Rb}^+$ efflux and only the addition of either furosemide or DIOA resulted in a small inhibition (<20%) of the Oxo-M-mediated component, a result consistent with a limited involvement of KCC in $^{86}\text{Rb}^+$ efflux (Fig. 5B). At least part of the $^{86}\text{Rb}^+$ efflux appears to be mediated by K^+ channels since inclusion of either TEA or Ba^{2+} attenuated both basal- and Oxo-M-stimulated efflux. However, the identity of the specific K^+ channel(s) involved remains to be determined.

The magnitude of basal- and Oxo-M-stimulated efflux of taurine and $^{125}\text{I}^-$ (used as a tracer for Cl^-) from SH-SY5Y cells is dependent upon the degree of osmotic stress, when monitored under conditions in which the buffers are rendered increasingly hypotonic by a reduction in NaCl concentration (Heacock et al., 2004; Cheema et al., 2007). However, under the same conditions, basal-

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and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx appeared to be independent of osmolarity (Fig. 3A). Although this experimental paradigm mimics the changes encountered under physiological conditions, it also involves alterations in three experimental variables, i.e. osmolarity and the concentrations of Na^+ and Cl^- ions. When monitored under conditions in which NaCl concentration was held constant and osmolarity varied by means of the addition of sucrose, it was evident that the magnitude of both basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx was dependent upon osmolarity (Fig. 4A). Both basal- and Oxo-M-stimulated efflux of $^{86}\text{Rb}^+$ were found to be dependent upon the degree of osmolarity, regardless of which experimental paradigm was employed (Figs. 3B, 4B). Thus, we conclude that whereas $^{86}\text{Rb}^+$ efflux occurs via an osmolarity-sensitive, but NaCl-independent mechanism, $^{86}\text{Rb}^+$ influx is mediated by a mechanism that is dependent on both osmolarity and NaCl, consistent with the involvement of NKCC and Na^+/K^+ -ATPase. Although both the influx and efflux of $^{86}\text{Rb}^+$ in SH-SY5Y cells are osmosensitive, the efflux component is more dependent on changes in osmolarity, as is evident from the observation that whereas the Oxo-M-mediated component of $^{86}\text{Rb}^+$ influx doubles when osmolarity is reduced from 340 to 200 mOsM, the corresponding increase for $^{86}\text{Rb}^+$ efflux is 6-7-fold (Figs. 4 A, B).

The osmosensitive efflux of taurine and $^{125}\text{I}^-$ from SH-SY5Y cells following activation of mAChRs (but not that monitored under basal conditions) is differentially regulated, with the efflux of $^{125}\text{I}^-$ exhibiting less dependence on Ca^{2+} availability and PKC activity than that observed for taurine (Cheema et al., 2007). Thus, whereas removal of extracellular Ca^{2+} attenuates mAChR-stimulated

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taurine efflux by >60%, and depletion of intracellular Ca^{2+} abolishes the response, $^{125}\text{I}^-$ efflux is unaffected by removal of extracellular Ca^{2+} and only minimally reduced by depletion of intracellular Ca^{2+} (~30%). Similarly, mAChR-stimulated taurine efflux is more susceptible to inhibition of PKC than is that of $^{125}\text{I}^-$ release (Heacock et al., 2006; Cheema et al., 2007). In the current study, the Ca^{2+} requirements observed for $^{86}\text{Rb}^+$ influx and efflux resembled more closely those previously obtained for $^{125}\text{I}^-$ release than for taurine efflux. Thus removal of extracellular Ca^{2+} had no effect on the magnitude of either mAChR-stimulated $^{86}\text{Rb}^+$ influx or efflux and only under conditions in which the intracellular pool of Ca^{2+} was depleted was the Oxo-M-mediated component reduced by 25-35% (Figs. 6 A, B). In contrast, both the basal influx and efflux of $^{86}\text{Rb}^+$ were increased by removal of Ca^{2+} . An increase in $^{86}\text{Rb}^+$ efflux under Ca^{2+} -depleted conditions has also been observed for astrocytes, although the mechanism remains unclear (Quesada et al., 1999). Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and efflux were also dependent on PKC activity and could be attenuated by ~50% following pre-incubation of the cells with chelerythrine. From this series of experiments, two conclusions can be drawn. First, the differential Ca^{2+} requirements observed for basal- and mAChR-stimulated $^{86}\text{Rb}^+$ release provide additional support for the proposal that distinct mechanisms underlie the swelling-activated and receptor-mediated components of osmolyte release (Mongin and Kimelberg, 2005; Heacock et al., 2006). Second, since both Oxo-M-mediated efflux of $^{86}\text{Rb}^+$ and $^{125}\text{I}^-$ exhibit requirements for Ca^{2+} and PKC activity that are distinct from those necessary for taurine release, these results

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indicate that the receptor-mediated release of inorganic ($^{86}\text{Rb}^+$ and $^{125}\text{I}^-$) and organic osmolytes (taurine) occurs via distinct mechanisms.

Although the use of the radiotracer, $^{86}\text{Rb}^+$, provides a convenient means whereby the characteristics of K^+ influx and efflux pathways are readily evaluated, this approach does not permit a quantitative assessment of the relative contributions made by each pathway to K^+ content of cells. To address this issue, we monitored changes in the concentration of K^+ in SH-SY5Y cells under hypoosmotic conditions using a K^+ -specific electrode. The results indicated that the addition of Oxo-M had no effect on the K^+ content of SH-SY5Y cells when exposed to either isoosmolarity or a mild reduction in osmolarity (290 mOsM) unless K^+ influx was concurrently prevented by inclusion of ouabain and furosemide (Table 1). This result suggests that under normal conditions, the agonist stimulation of K^+ efflux is countered by an equivalent stimulation of K^+ influx. When monitored under more hypoosmotic conditions (230 mOsM), Oxo-M addition results in a 10% reduction of K^+ content and this loss is further accentuated when K^+ influx is prevented (23% reduction). The results obtained from measurement of K^+ content are consistent with those derived from radiolabeling studies which indicate that although both K^+ influx and efflux are osmosensitive, it is the efflux pathway that is most strongly regulated by a reduction in osmolarity (Fig. 4). Thus, although an increase in K^+ efflux is offset by a comparable increase in K^+ influx under conditions of limited reductions in osmolarity, when cells are incubated under more hypoosmotic conditions, the efflux of K^+ predominates and a net loss of K^+ occurs.

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Previous studies have indicated that, under conditions of acute or chronic hyponatremia, the brain selectively retains its inorganic osmolytes (Melton et al., 1987; Pasantes-Morales et al., 2002; Massieu et al., 2004). In the present study, we have demonstrated that activation of GPCRs not only enhances the efflux of K^+ , but also its influx. Under conditions of limited reductions in osmolarity, such as those most likely to occur under pathological conditions, the basal uptake of K^+ , which is mediated via the NKCC and Na^+/K^+ -ATPase, is markedly facilitated by receptor activation and this permits K^+ to be more effectively retained by the cells. These results raise the possibility that, under hyponatremic conditions, tonic receptor activity in the CNS may serve not only to enhance the efflux of osmolytes, but also to maintain relatively high intracellular concentrations of K^+ .

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Footnotes

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- b)** Address correspondence to: Dr. Stephen K. Fisher, Molecular and Behavioral Neuroscience Institute, University of Michigan, 5039 Biomedical Science Research Building, 109 Zina Pitcher, Ann Arbor, MI 48109-2200. E-mail: skfisher@umich.edu

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Legends for Figures

Fig. 1. Kinetics of basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux. (A) For measurement of $^{86}\text{Rb}^+$ influx, SH-SY5Y cells were washed twice with 2 ml of isotonic buffer A prior to incubation in hypotonic buffer A (230 mOsM) that contained 0.5 $\mu\text{Ci/ml}$ of $^{86}\text{Rb}^+$, in the absence (\circ) or presence (\bullet) of 100 μM Oxo-M. Reactions were terminated at the times indicated and $^{86}\text{Rb}^+$ uptake monitored. Results are expressed as K^+ ($^{86}\text{Rb}^+$) influx (nmol/mg protein/min), assuming that $^{86}\text{Rb}^+$ uptake reflects that of K^+ . Values shown are the means \pm S.E.M. for three independent experiments, each performed in triplicate. Where error bars are absent, the S.E.M. fell within the symbol. Influx of $^{86}\text{Rb}^+$ at 340 mOsM was also linear with time from 0-20 min. *, $p < 0.05$, Different from basal (by unpaired Student's t test). (B) For measurement of $^{86}\text{Rb}^+$ efflux, SH-SY5Y cells that had been labeled to isotopic equilibrium in the presence of $^{86}\text{Rb}^+$ were washed three times with 2 ml of isotonic buffer A prior to incubation in hypotonic buffer A (230 mOsM), in the absence (\circ) or presence (\bullet) of 100 μM Oxo-M. Reactions were terminated at the times indicated and $^{86}\text{Rb}^+$ efflux monitored. Results are expressed as rate of K^+ ($^{86}\text{Rb}^+$) efflux (percentage of total cell radioactivity released/min). Values shown are the means \pm S.E.M. for three independent experiments, each performed in triplicate. Where error bars are absent, the S.E.M. fell within the symbol. Efflux of $^{86}\text{Rb}^+$ was also linear for at least 10 min when monitored at 340 mOsM. *, $p < 0.05$, Different from basal (by unpaired Student's t test).

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Fig. 2. Dose-response relationships for Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux. $^{86}\text{Rb}^+$ influx (A) and $^{86}\text{Rb}^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells incubated under hypotonic conditions (230 mOsM) in the presence or absence of Oxo-M at the concentrations indicated. Reactions were terminated after 10 min. Results are expressed as percentage of maximum agonist response (obtained at 1 mM Oxo-M) and are the means \pm S.E.M. for three independent experiments, each performed in triplicate. Where error bars are absent, the S.E.M. fell within the symbol. The calculated EC_{50} value for $^{86}\text{Rb}^+$ influx was 0.45 μM with a Hill coefficient of 0.80. Addition of Oxo-M stimulated the efflux of $^{86}\text{Rb}^+$ with an EC_{50} of 1.37 μM and a Hill coefficient of 0.75.

Fig. 3. Basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux as a function of osmolarity. $^{86}\text{Rb}^+$ influx (A) and $^{86}\text{Rb}^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells in buffers rendered hypertonic or hypotonic by an increase or decrease in NaCl concentration, respectively, in the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. Reactions were terminated after 5 min. Results are expressed as either K^+ ($^{86}\text{Rb}^+$) influx (nmol/mg protein/min) or as rate of K^+ ($^{86}\text{Rb}^+$) efflux (percentage of total cell radioactivity released/min). Values shown are the means \pm S.E.M. for three independent experiments, each performed in triplicate. *, $p < 0.01$, Different from basal release monitored under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, $p < 0.01$, Different from Oxo-M treatment under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed

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by Dunnett's multiple comparison test). At all osmolarities, Oxo-M addition significantly increased both $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux when compared to uptake or release monitored under basal conditions ($p < 0.05$, by paired student's t test).

Fig. 4. Basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux are volume-dependent. $^{86}\text{Rb}^+$ influx (A) and $^{86}\text{Rb}^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells in buffers of the osmolarity indicated in the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. Osmolarities of the buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. Reactions were terminated after 5 min of incubation. Results are expressed as either K^+ ($^{86}\text{Rb}^+$) influx (nmol/mg protein/min) or as rate of K^+ ($^{86}\text{Rb}^+$) efflux (percentage of total cell radioactivity released/min). Results shown are the means \pm S.E.M. for three or four independent experiments, each performed in triplicate. *, $p < 0.05$, Different from basal influx or efflux monitored under isotonic (340 mOsm) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, $p < 0.05$, Different from Oxo-M treatment under isotonic (340 mOsm) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). At all osmolarities, Oxo-M addition significantly increased both $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux when compared to basal conditions ($p < 0.05$, by paired student's t test).

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Fig. 5. Effect of ouabain, furosemide or bumetanide on basal- and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux. $^{86}\text{Rb}^+$ influx (A) and $^{86}\text{Rb}^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells under hypotonic conditions (230 mOsM) in the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. In some experiments, bumetanide (800 μM), furosemide (800 μM) and ouabain (30 μM) were also present. Reactions were terminated after 10 min. Results are expressed as either K^+ ($^{86}\text{Rb}^+$) influx (nmol/mg protein/min) or as rate of K^+ ($^{86}\text{Rb}^+$) efflux (percentage of total cell radioactivity released/min). Values shown are the means \pm S.E.M. for three to nine independent experiments, each performed in triplicate. *, $p < 0.05$, Different from control basal influx (by one-way ANOVA followed by Dunnett's multiple comparison test). #, $p < 0.05$, Different from control plus Oxo-M (by one-way ANOVA followed by Dunnett's multiple comparison test).

Fig. 6. The role of extra- and intracellular Ca^{2+} and PKC in basal and Oxo-M-stimulated $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux. $^{86}\text{Rb}^+$ influx (A) and efflux (B) were monitored in SH-SY5Y neuroblastoma cells under hypotonic conditions (230 mOsM). Cells were incubated in the absence (- Ca^{2+} : Ca^{2+} was omitted from buffer and 50 μM EGTA was added) or presence of extracellular Ca^{2+} . For some experiments, cells were pretreated for 15 min in isotonic buffer A in the presence of 1 μM thapsigargin (Thp) to deplete intracellular pools of Ca^{2+} . For evaluation of the involvement of PKC, cells were pretreated with 10 μM chelerythrine in isotonic buffer A for 15 min. Reactions were allowed to proceed for 5 min in the

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absence (open bars) or presence (closed bars) of 100 μM Oxo-M. Results are expressed as either K^+ ($^{86}\text{Rb}^+$) influx (nmol/mg protein/min) or as rate of K^+ ($^{86}\text{Rb}^+$) efflux (percentage of total cell radioactivity released/min). Results shown are the means \pm S.E.M. for four to six independent experiments, each performed in triplicate. *, $p < 0.05$, Different from control basal influx or efflux (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, $p < 0.05$, Different from control plus Oxo-M (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

Fig. 7. Activation of multiple GPCRs can induce both volume-dependent $^{86}\text{Rb}^+$ influx and $^{86}\text{Rb}^+$ efflux under hypotonic conditions. $^{86}\text{Rb}^+$ influx (A) and $^{86}\text{Rb}^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells under hypotonic conditions (230 mOsM) in the presence of either 100 μM Oxo-M, 1.25 nM thrombin, 10 μM lysophosphatidic acid (LPA) or 5 μM sphingosine-1-phosphate (S1P). Reactions were terminated after 10 min. Results are expressed as either K^+ ($^{86}\text{Rb}^+$) influx (nmol/mg protein/min) or as rate of K^+ ($^{86}\text{Rb}^+$) efflux (percentage of total cell radioactivity released/min). Values shown are the means \pm S.E.M. for four independent experiments, each performed in triplicate. *, $p < 0.01$, Different from basal (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

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TABLE 1

Ability of Oxo-M, ouabain, and furosemide to regulate intracellular K⁺ content at different osmolarities.

In three separate series of experiments, SH-SY5Y cells were exposed to either 340, 290 or 230 mOsM buffer A in the absence or presence of 100 μM Oxo-M, 30 μM ouabain and 800 μM furosemide. Reactions were terminated after 10 min and cells were washed with 2 ml of K⁺ free isotonic buffer A. Cells were then lysed and intracellular K⁺ content was measured using an ion specific electrode. Results are expressed as μmol K⁺/mg protein. Results shown are the means ± S.E.M. for four independent experiments, each performed in duplicate. Percentage reductions from basal values are indicated in parentheses. *, p<0.05, Different from basal content (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, p<0.05, different from both Oxo-M treatment and ouabain/furosemide treatment (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

	Basal	+ Oxo-M	+ Ouabain + Furosemide	+ Oxo-M + Ouabain + Furosemide
340 mOsM	1.22 ± .02	1.21 ± .03	1.16 ± .02 * (5%)	1.12 ± .05 * # (8%)
290 mOsM	1.17 ± .03	1.16 ± .03	1.10 ± .04 * (6%)	1.03 ± .03 * # (12%)
230 mOsM	1.12 ± .08	1.01 ± .08 * (10%)	1.00 ± .08 * (11%)	0.86 ± .06 * # (23%)

Figure 1

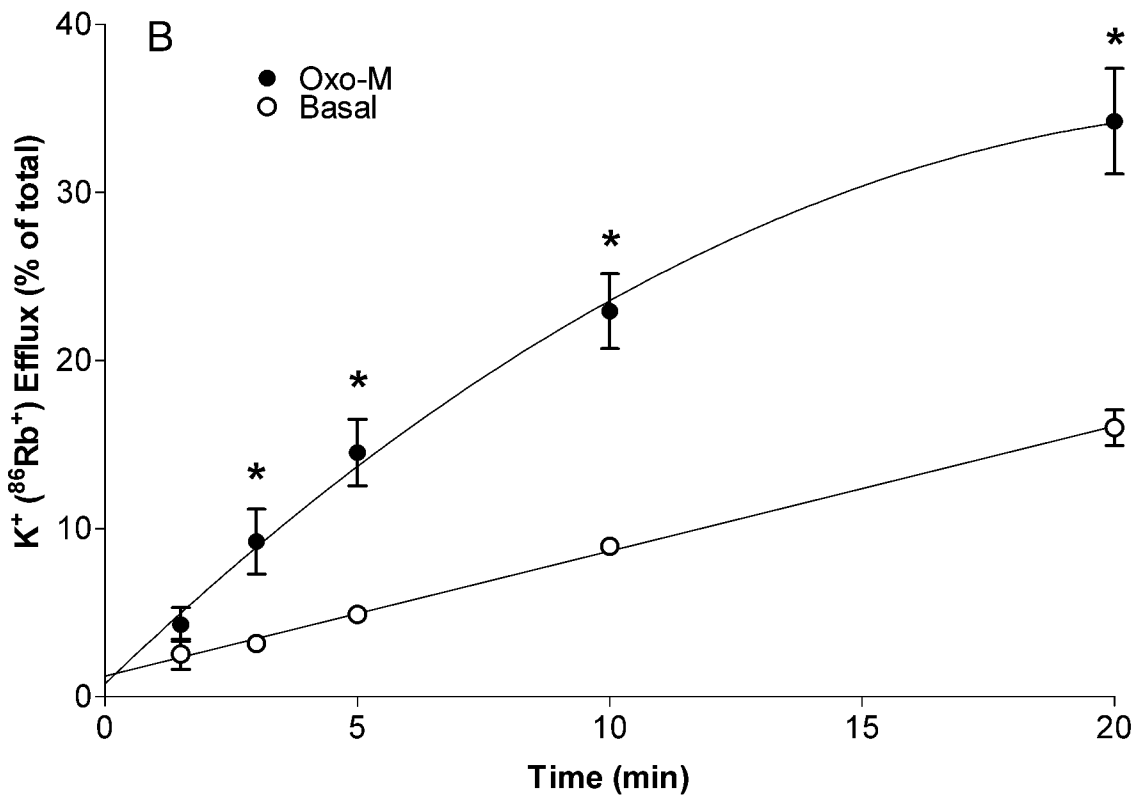
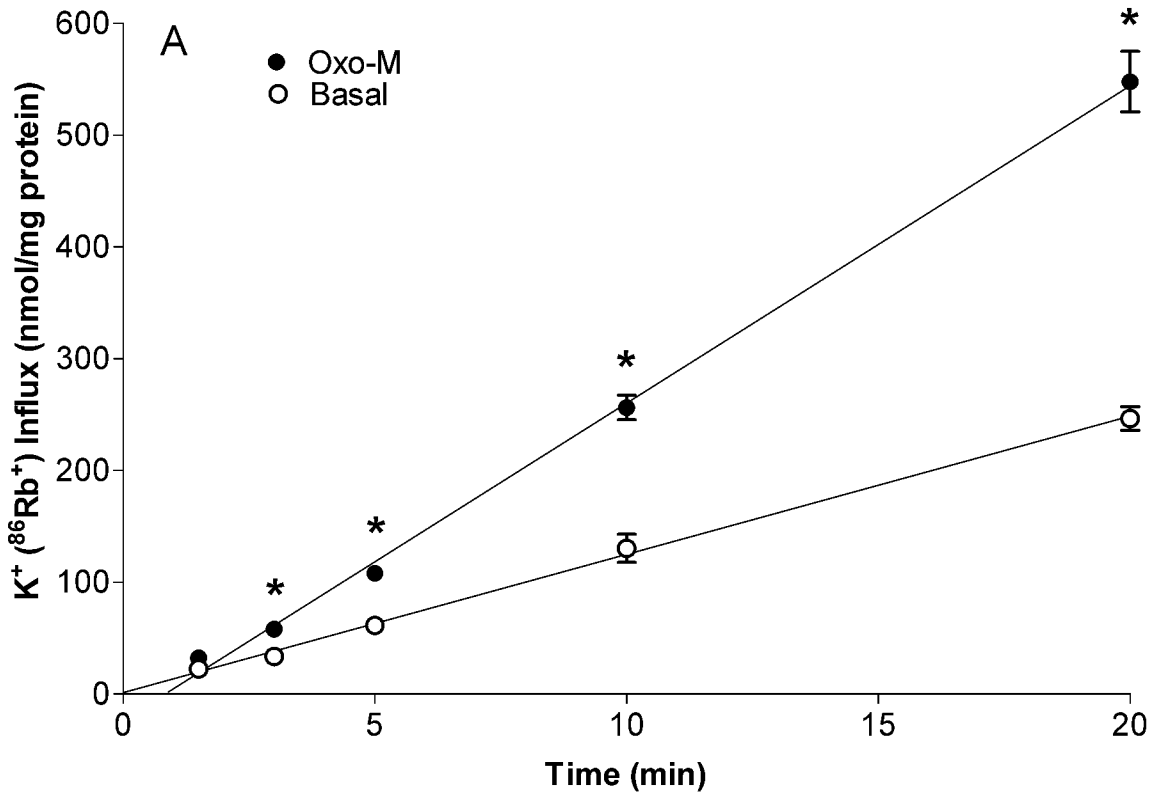


Figure 2

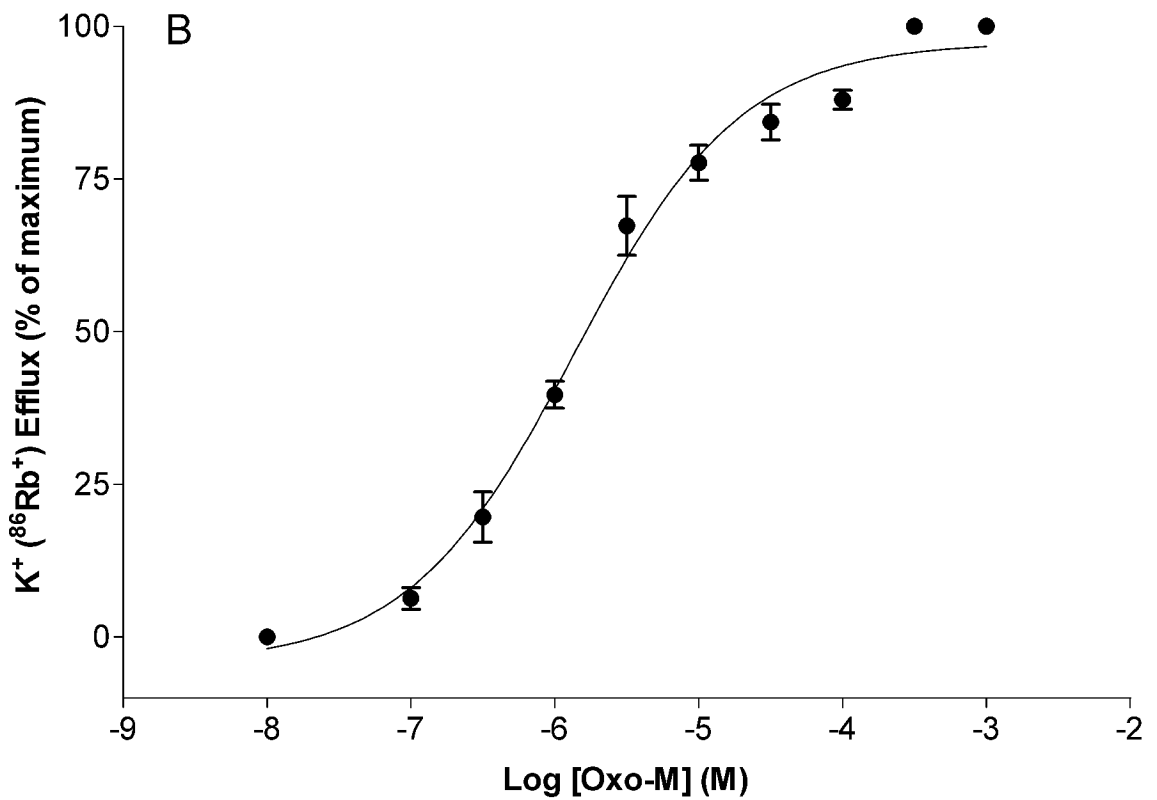
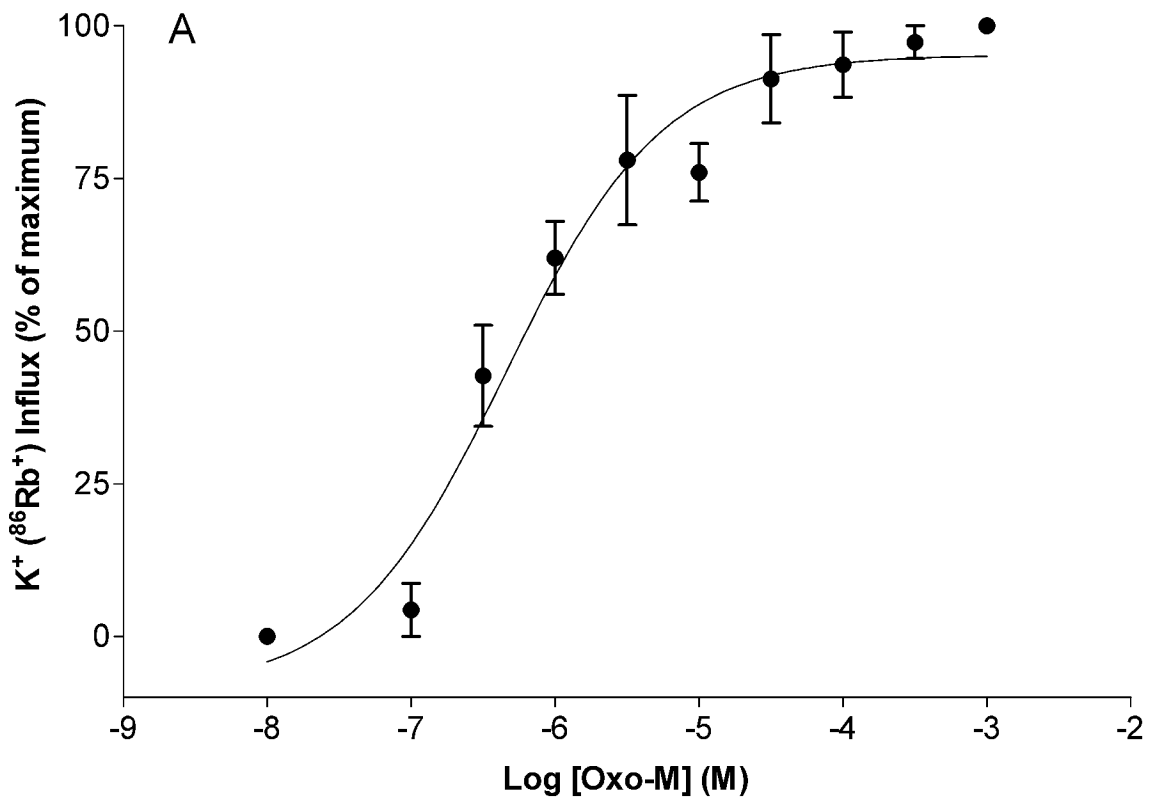


Figure 3

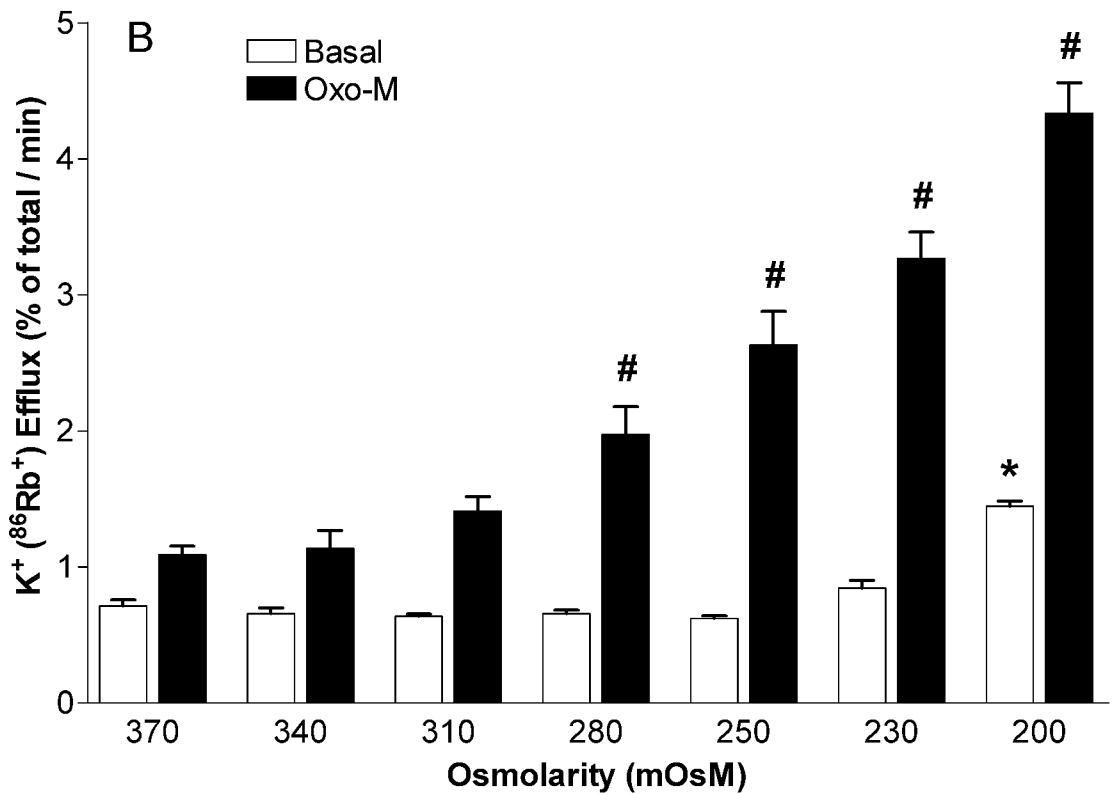
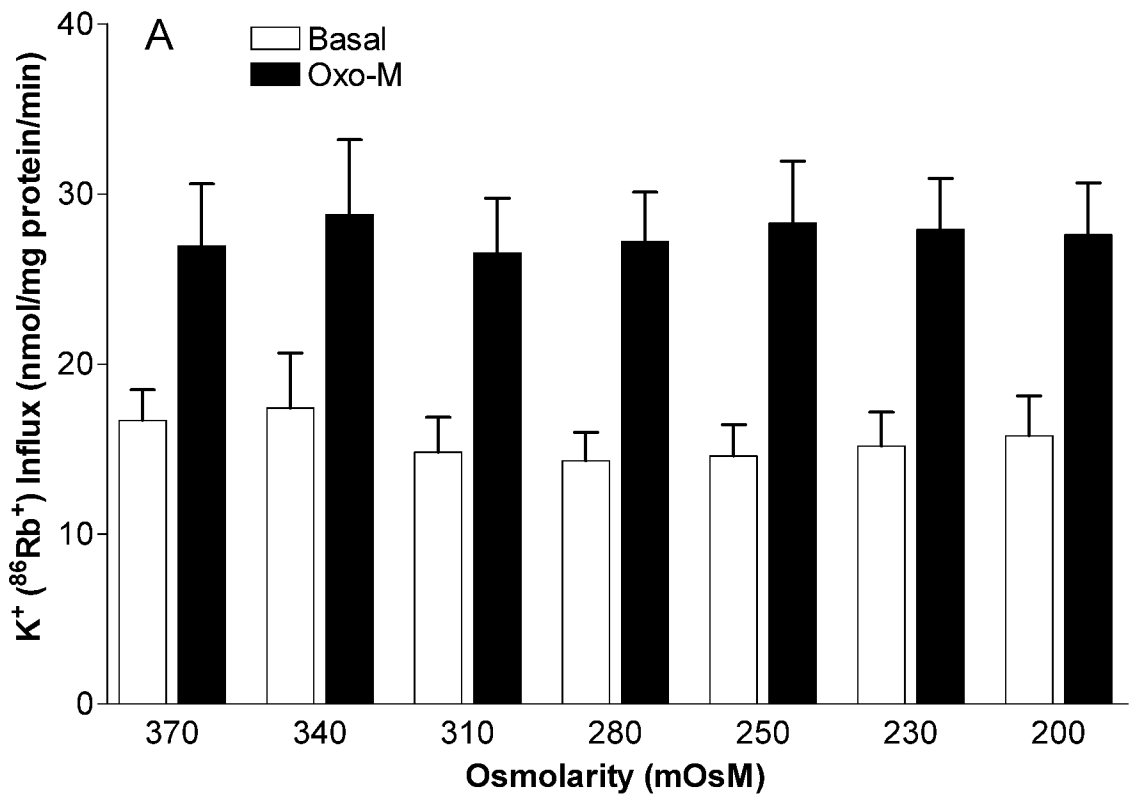


Figure 4

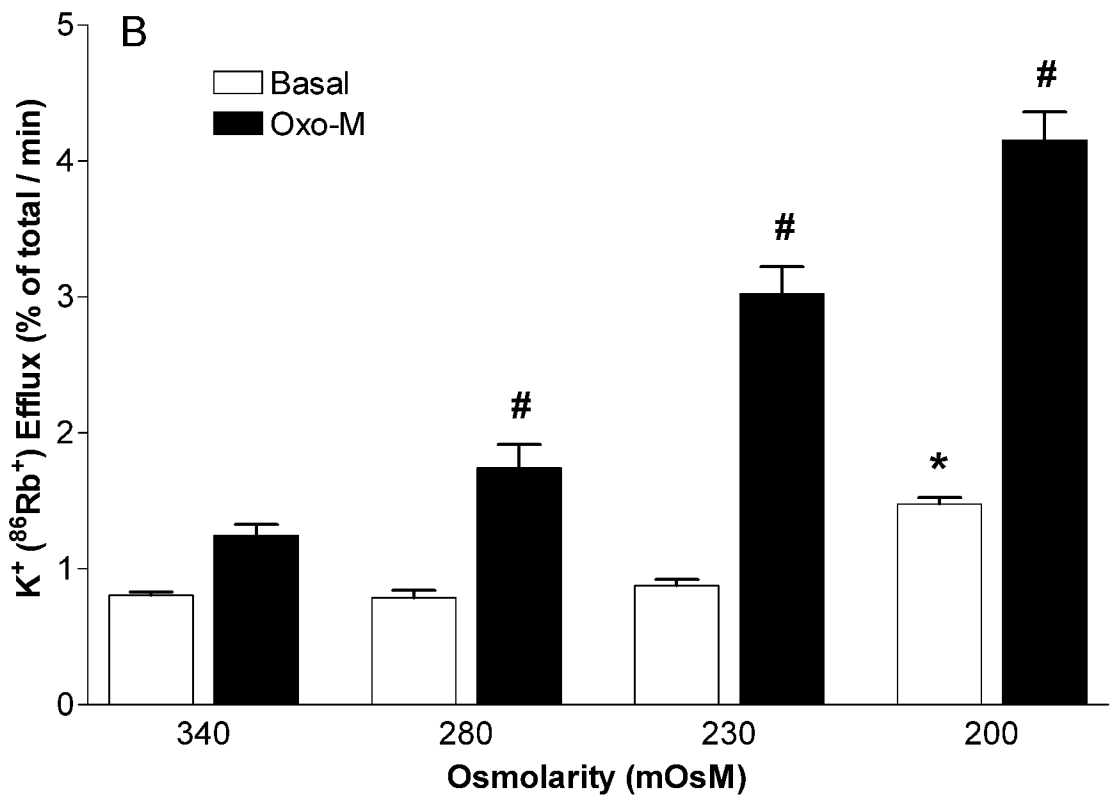
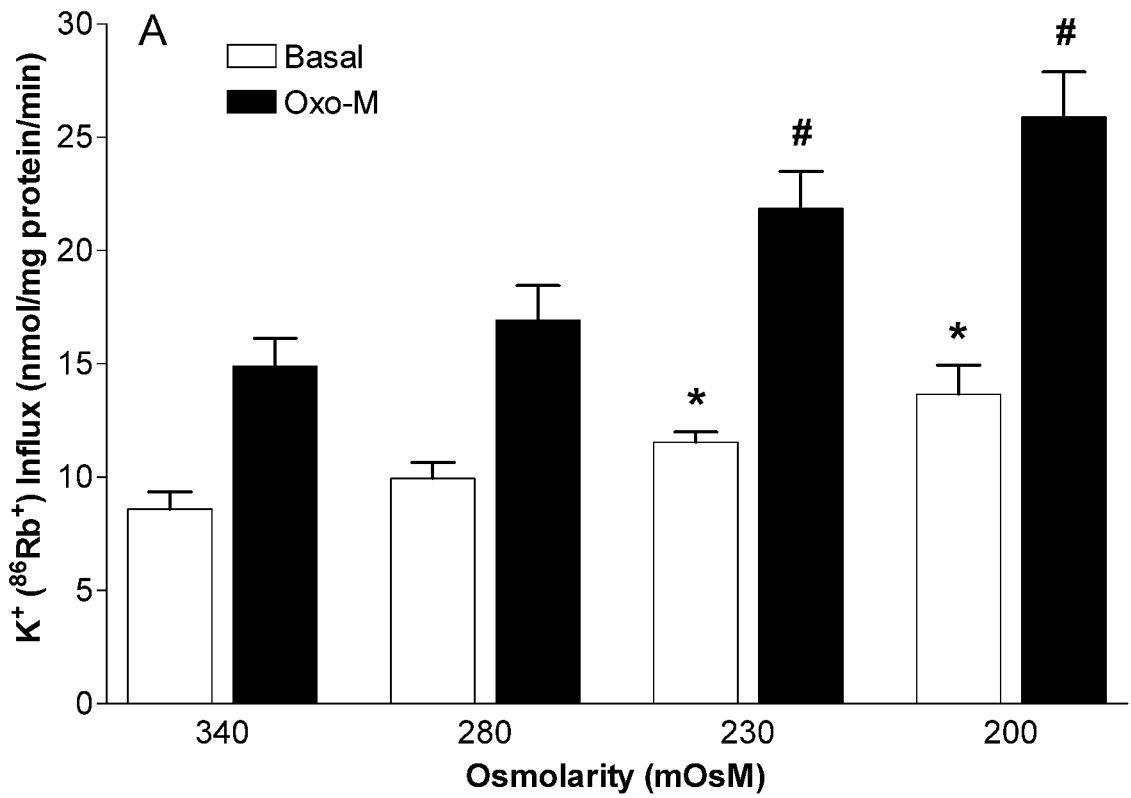


Figure 5

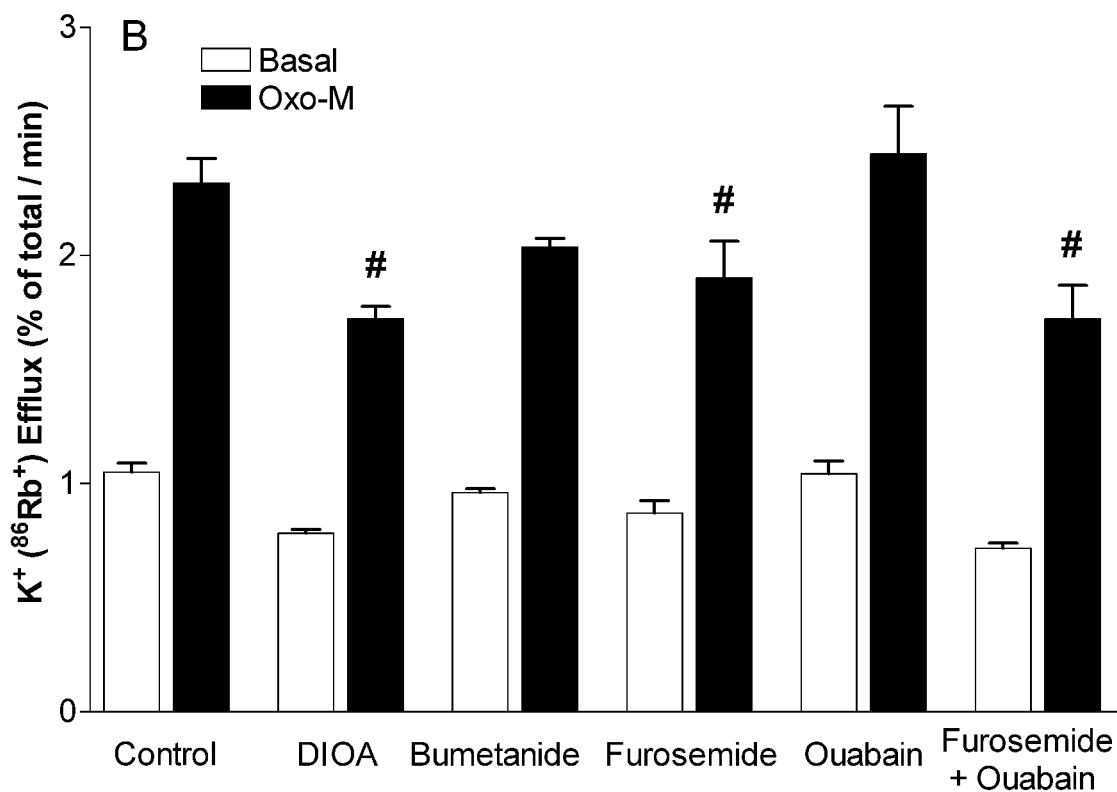
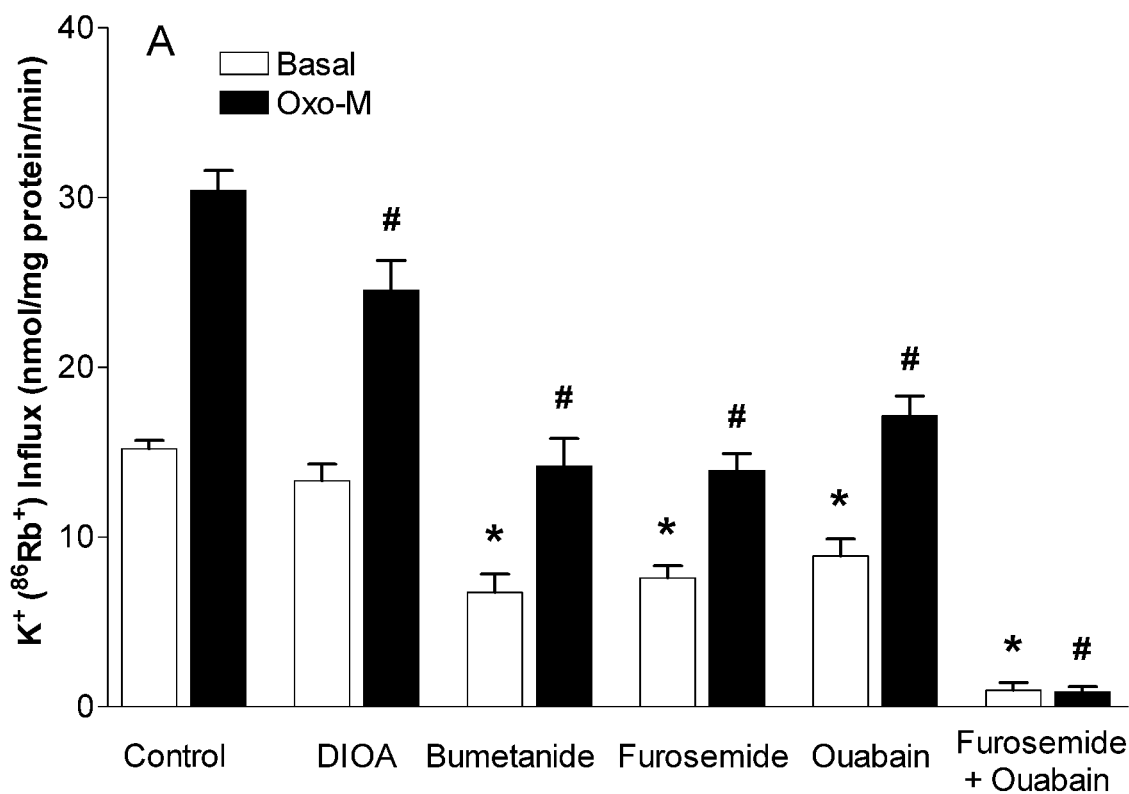


Figure 6

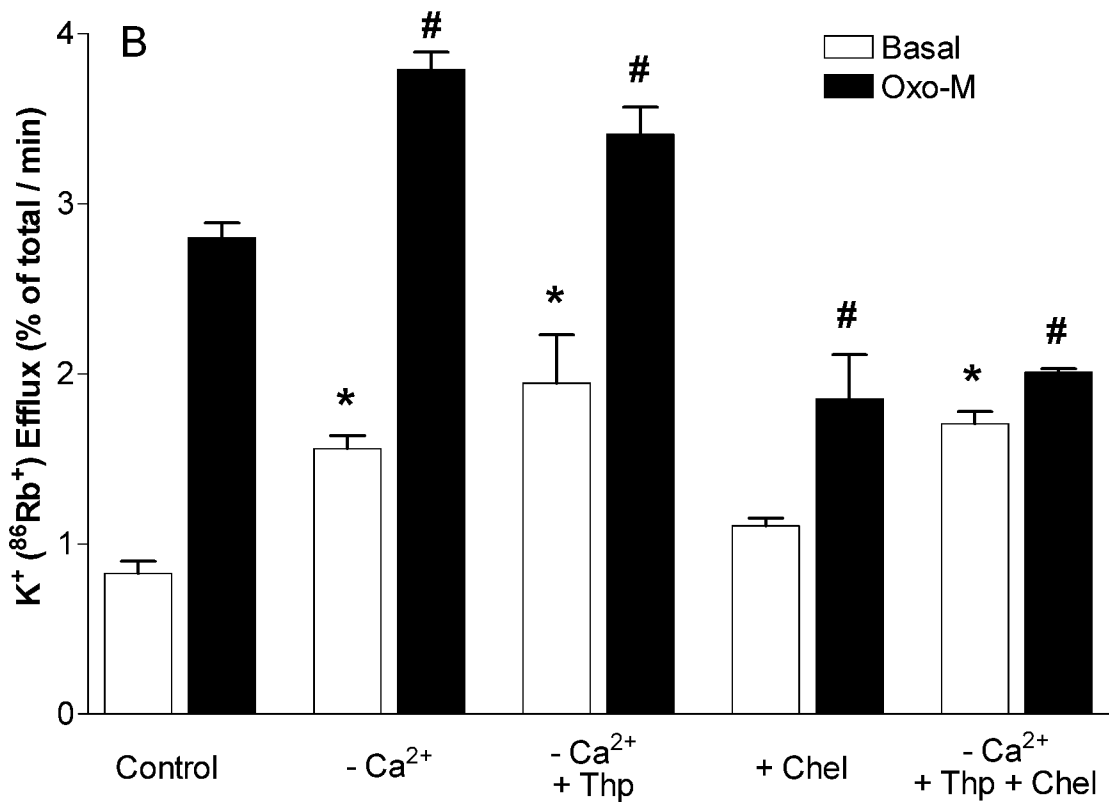
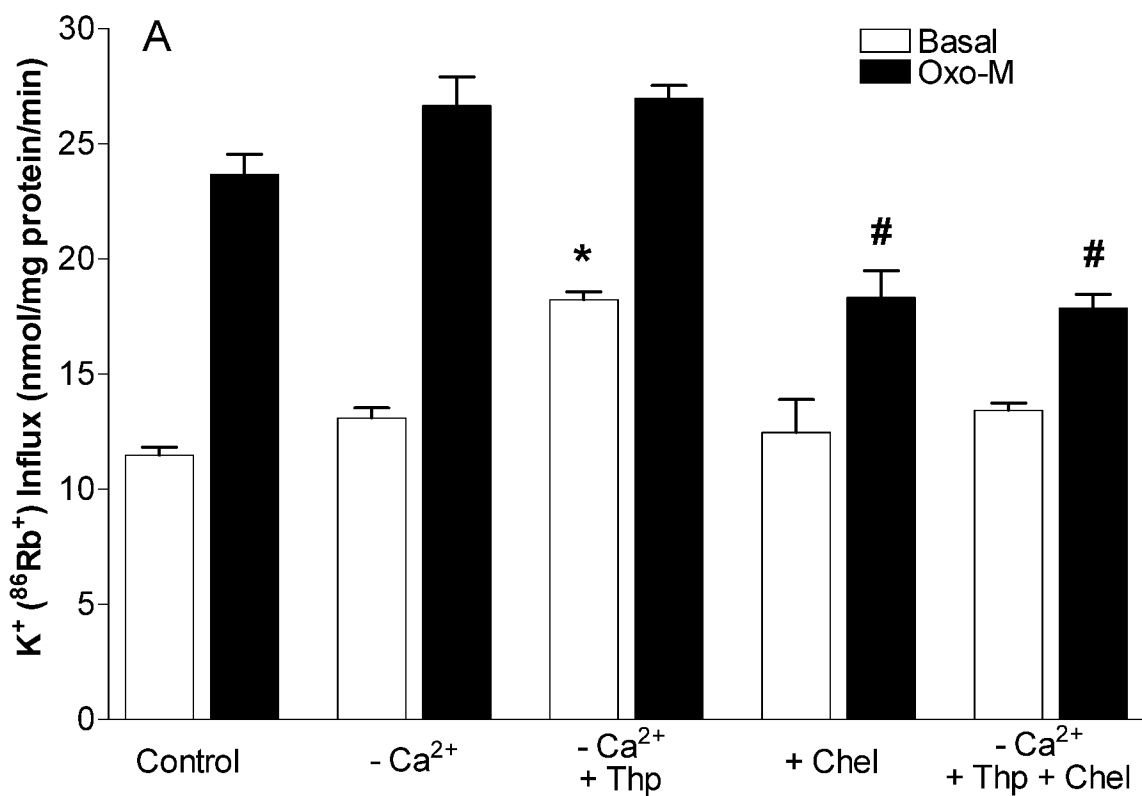


Figure 7

