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Activation of muscarinic cholinergic receptors enhances the volume-sensitive efflux of myo-inositol from SH-SY5Y neuroblastoma cells

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Abstract

A mechanism used by cells to regulate their volume under hypo-osmotic conditions is the release of organic osmolytes, one of which is myo-inositol. The possibility that activation of phospholipase-C-linked receptors can regulate this process has been examined for SH-SY5Y neuroblastoma cells. Incubation of cells with hypo-osmolar buffers (160–250 mOsm) led to a biphasic release of inositol which persisted for up to 4 h and could be inhibited by inclusion of anion channel blockers – results which indicate the involvement of a volume-sensitive organic anion channel. Inclusion of oxotremorine-M, a muscarinic cholinergic agonist, resulted in a marked increase (80–100%) in inositol efflux under hypo-osmotic, but not isotonic, conditions. This enhanced release, which was observed under all conditions of hypo-osmolarity tested, could be prevented by inclusion of atropine. Incubation of the cells with either the calcium ionophore, ionomycin, or the phorbol ester, phorbol 12-myristate 13-acetate, partially mimicked the stimulatory effect of muscarinic receptor activation when added singly, and fully when added together. The ability of oxotremorine-M to facilitate inositol release was inhibited by removal of extracellular calcium, depletion of intracellular calcium or down-regulation of protein kinase C. These results indicate that activation of muscarinic cholinergic receptors can regulate osmolyte release in this cell line.

Keywords: calcium, muscarinic cholinergic receptors, myo-inositol, osmolyte, protein kinase C, volume regulation.

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Regulation of cell volume is essential for many physiological processes and is of prime importance to the CNS, because of the restricted volume of the skull. Brain cells can swell in response to either changes in plasma osmolarity (hypo-osmotic swelling) or from changes in intracellular ion and water distribution (isotonic swelling). The latter is also referred to as cellular or cytotoxic edema (Kimelberg 2000; Pasantes-Morales *et al*. 2000). Hypo-osmotic swelling frequently occurs as a result of hyponatremia, which is associated with clinical conditions such as congestive heart failure, nephrotic syndrome and hepatic cirrhosis. Water overload may also occur in some psychiatric disorders, such as schizophrenia, or in athletes and in instances of the inappropriate secretion of anti-diuretic hormone. The majority of symptoms observed are neurological and include disorientation, mental confusion and seizures.

In response to hypo-osmotic stress, neural cells swell, and to restore osmotic balance a loss of K^+ and Cl^- ions is initially observed. However, as large changes in ion concentrations can adversely impact cell excitability, cells subsequently utilize 'compatible' or 'non-perturbing' osmolytes which are specifically designed to counter changes in osmolarity without compromising cell function. Three distinct classes of osmolytes can be identified, namely (i) amino acids, such as glutamate or taurine, (ii) methylamines, such as betaine and glycerophosphorylcholine, and (iii) polyols, such as myo-inositol or sorbitol. Of these, inositol, glutamate and taurine represent the quantitatively major

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Abbreviations used: BIM, bisindolylmaleimide; Ca^{2+}_i, cytoplasmic calcium; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid; mAChR, muscarinic cholinergic receptor; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; Oxo-M, oxotremorine-M; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C; PMA, phorbol 12-myristate 13-acetate; RVD, regulatory volume decrease; VSOAC, volume-sensitive organic anion channel.

osmolytes in the brain. Although cells may utilize a number of different transport systems for cell volume regulation (see Lang *et al*. 1998), organic osmolytes are released from neural cells via a 'volume-sensitive organic anion channel' (VSOAC). This channel, which has been extensively characterized both electrophysiologically and pharmacologically, is selectively permeable to anions (primarily Cl^-) and organic osmolytes, but not to cations (see Nilius *et al*. 1997; Lang *et al*. 1998; for reviews). The loss of these osmolytes, along with the attendant efflux of intracellular water, restores osmotic balance and effects a regulatory volume decrease (RVD) in the cell (McManus *et al*. 1995).

Evidence that inositol can function as a physiologically important osmolyte was first obtained from experiments in which animals were rendered either hyper- or hyponatremic. Hypernatremia results in a significant increase in inositol concentration (65–200%) within the brains of either experimental animals (Lien *et al*. 1990; Löhr *et al*. 1998) or humans (Lee *et al*. 1994). Conversely, a loss of inositol (40–90%) from the brains of mice, rats or humans is observed under conditions of hyponatremia (Thurston *et al*. 1989; Lien *et al*. 1991; Haussinger *et al*. 1994; Videen *et al*. 1995). Of the relatively few *in vitro* studies that have addressed the mechanism of inositol release from neural cells, nearly all have been restricted to astrocytes or glial tumor cells. In the latter, cell swelling has been shown to rapidly activate a Na^+-independent, passive, low-affinity inositol efflux mechanism (Jackson and Strange 1993), which can be blocked by the inclusion of inhibitors of anion channels such as DIDS or NPPB – a pharmacological profile consistent with the involvement of VSOAC (Strange *et al*. 1993). Although it has been assumed that inositol efflux occurs primarily from glia, recent data from this laboratory indicate that high concentrations of inositol can also be observed in some neuronal populations, e.g. NT2-N neurons (Novak *et al*. 1999; for review see Fisher *et al*. 2002). Moreover, we have recently demonstrated that hypotonicity activates the release of myo-inositol from differentiated NT2-N neurons with a pharmacological inhibition profile consistent with the presence of VSOAC on these cells (Novak *et al*. 2000). Taken collectively, these results suggest that both neurons and glia may utilize inositol efflux to regulate their cell volume.

Although a number of cell signaling pathways have been implicated in cell volume regulation (see Nilius *et al*. 1997; Lang *et al*. 1998; Pasantes-Morales *et al*. 2000; for reviews), the most consistent observation to date is that a tyrosine kinase appears to be required for activation of VSOAC in response to cell swelling (Crepel *et al*. 1998; Mongin *et al*. 1999; Deleuze *et al*. 2000; Morales-Mulia *et al*. 2001). Little is known, however, about whether osmolyte release, once initiated, is subject to neurohumoral regulation. In this regard, the ability of Ca^{2+} ionophores and phorbol esters to enhance osmolyte efflux in C6 glioma cells and NT2-N neurons (Strange *et al*. 1993; Novak *et al*. 2000) could indicate a regulatory role for phospholipase C (PLC)-linked receptors. Given that the CNS possesses a large number of PLC-linked receptors (Fisher *et al*. 1992) and the importance of cell volume regulation to brain function, this issue assumes major physiological significance.

In this study, we demonstrate that human SH-SY5Y neuroblastoma cells, which possess high concentrations of inositol (Novak *et al*. 1999), release the polyol upon hypoosmotic challenge. Moreover, this release is significantly enhanced following agonist occupancy of muscarinic cholinergic receptors (mAChRs), which results in the activation of PLC. This stimulation of inositol efflux appears to be mediated, at least in part, via a rise in cytoplasmic Ca^{2+} (Ca^{2+}_i) concentrations and activation of PKC. A preliminary account of part of this study has previously been reported (Fisher *et al*. 2003).

Materials and methods

Materials

myo-[2–^{3}H]Inositol (80 Ci/mmol) and [1,2–^{3}H]taurine (32 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL, USA). Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate, all *trans*-retinoic acid, 4,4′diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 3-[4-hydroxyphenyl]-1-[2,4,6-trihydroxyphenyl]-1-propanone (phloretin), 1-[p-dimethyl-aminoethoxyphenyl]-1,2-diphenyl 1-butene (tamoxifen), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 2-butyn-1-ammonium N,N,N-trimethyl-4-(2-oxo-1-pyrrolidinyl) iodide (oxotremorine-M; Oxo-M), atropine and pilocarpine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Thapsigargin, ionomycin and bisindolylmaleimide (BIM) were obtained from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM), 50 × penicillin/streptomycin (5000 U/mL of penicillin G sodium and 5000 μg/mL of streptomycin sulfate) were obtained from Gibco (Grand Island, NY, USA). Fetal calf serum was from BioWhittaker (Walkersville, MD, USA). Tissue culture supplies were obtained from Corning Glass Works (Corning, NY, USA), Sarstedt (Newton, NC, USA) and Becton-Dickinson (Franklin Lakes, NJ, USA). Universol liquid scintillation cocktail was from ICN (Costa Mesa, CA, USA). Dowex-1 resin (100–200 mesh; × 8 formate) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Isotetrandine was from Biomol (Plymouth Meeting, PA, USA).

Methods

Cell culture conditions

SH-SY5Y cells (passages 69–93) were grown in tissue culture flasks (75 cm^2/250 mL) in 20 mL of DMEM supplemented with 10% (v/v) of fetal calf serum. Cells were grown for 3–10 days at 37°C in a humidified atmosphere containing 10% CO_2. Cells were isolated after aspiration of the medium and incubation with a modified Puck's D_1 solution (Honneger and Richelson 1976). Cells were then resuspended in DMEM/10% fetal calf serum and subcultured into 35 mm, 6-well culture plates for 2–4 days. Experiments were

routinely conducted on cells that had reached 50–90% confluency. In some experiments, SH-SY5Y cells were differentiated into the neuronal phenotype following a 6-day incubation in the presence of 10 μM retinoic acid (Rosner *et al*. 1995).

Efflux of inositol or taurine

Osmolyte efflux from SH-SY5Y neuroblastoma cells was monitored essentially as previously described (Novak *et al*. 1999, 2000). Cells grown in 6-well plates were allowed to pre-label at 37°C for 24–48 h in the presence of 2–3 μCi/mL of myo-[2–^{3}H]inositol or for 3 h in the presence of 1 μCi/mL of [1,2–^{3}H]taurine. After pre-labeling, the medium was aspirated and cells washed with 2 × 2 mL of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$ and 30 mM HEPES, pH 7.4). Cells were then allowed to incubate in 2 mL of either isotonic buffer A (approx. 300 mOsm) or hypotonic buffer A (150–275 mOsm; rendered hypotonic by the reduction in NaCl concentration). Osmolarities of buffer A were monitored by means of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA, USA). At the times indicated, aliquots (50–200 μL) of the extracellular medium were removed and radioactivity determined after the addition of 5 mL of Universol scintillation fluid. In some experiments, the extracellular medium was centrifuged at 7800 *g* for 2 min (to account for any cell debris) and the release of radioactivity compared with that observed for non-centrifuged samples. Values for radioactivity released were comparable using the two procedures. To terminate the reactions, the medium was rapidly aspirated and the cells lysed with 2 × 1 mL of ice-cold 6% (wt/vol) trichloroacetic acid and water-soluble radioactivity determined, as previously described. Total water-soluble radioactivity present initially in the cells was calculated as the sum of that recovered in the extracellular medium and that remaining in the lysate at the end of the assay. Efflux of either inositol or taurine at any given time was then routinely expressed as the ratio of radioactivity present in the extracellular medium to total soluble radioactivity (as a percentage). In experiments in which a treatment resulted in statistically significant changes in the basal efflux of inositol (which is defined as the release of inositol under hypo-osmotic conditions), results for Oxo-M-stimulated efflux were expressed as an increase over basal (see Fig. 8). In experiments in which cells were pre-labeled with ^{3}H-inositol, more than 99% of the radioactivity released from cells co-eluted with myo-inositol, as determined by anion-exchange chromatography (Fisher *et al*. 1984).

Phosphoinositide turnover

To monitor phosphoinositide turnover, SH-SY5Y cells that had been pre-labeled with ^{3}H-inositol were incubated in hypotonic buffer A that contained 5 mM LiCl. The accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as previously described (Thompson and Fisher 1990).

Data analysis

Values quoted are means ± SEM for the numbers of independent experiments indicated. Student's two-tailed *t*-tests were used to evaluate the statistical differences of the means of unpaired or paired sets of data.

Results

Hypo-osmolarity elicits an increased efflux of inositol from SH-SY5Y cells

When SH-SY5Y cells that had been allowed to pre-label with ^{3}H-inositol were exposed to hypotonic buffer A, there was an enhancement of inositol efflux (171, 722 and 2420% of isotonic control at 251, 200 and 157 mOsm after a 20-min incubation, respectively; Fig. 1). In contrast, little or no efflux of inositol could be detected under iso-osmotic conditions (316 mOsm). At osmolarities of 200 and 157 mOsm, inositol efflux was clearly biphasic, with an initial rapid rate of release which lasted for approximately 5–10 min, followed by a slower, but sustained, release of inositol which persisted for up to 4 h. Unless indicated otherwise, in subsequent experiments, an osmolarity of approximately 160 mOsm was routinely employed and efflux monitored at either 10- or 20-min time points.

Inositol efflux is inhibited by anion channel blockers

The release of inositol that occurred under conditions of hypo-osmolarity could be substantially inhibited by agents known to block anion channels (such as VSOAC). Thus, at concentrations of 10–300 μM, inclusion of either DIDS, NPPB or phloretin inhibited inositol efflux by up to 60–70% (Fig. 2). Higher concentrations of these agents could not be tested as they resulted in detachment of the cells from the dish. Inclusion of tamoxifen, another agent purported to

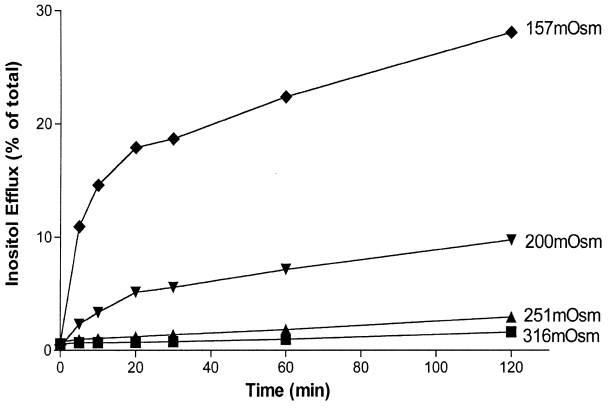

Fig. 1 Hypo-osmolarity enhances inositol efflux from SH-SY5Y neuroblastoma. Cells that had been pre-labeled overnight in the presence of ^{3}H-inositol (3 μCi/mL) were washed twice with 2 mL of isotonic buffer A prior to incubation in buffer A at the osmolarities shown. Reactions were terminated at the times indicated and inositol efflux monitored. Results are expressed as inositol efflux (per cent of total soluble radioactivity at time zero) and are the means of three replicates ± SEM. When not shown, SEM values fell within the symbol. Data shown are from one of three experiments that gave similar results.

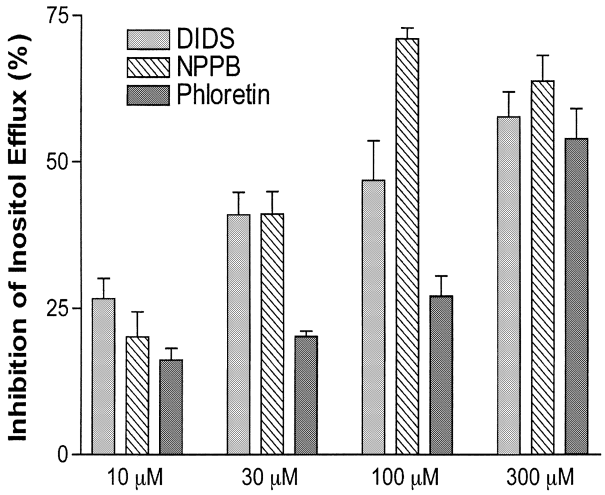

Fig. 2 Anion channel blockers inhibit inositol efflux under conditions of hypotonicity. Cells that had been pre-labeled with ^{3}H-inositol for 24 h were washed twice with isotonic buffer A and incubated in hypotonic buffer A (160 mOsm) in the presence or absence of DIDS, NPPB or phloretin at the concentrations indicated. After a 20-min incubation, reactions were terminated and inositol efflux monitored. Results are expressed as inhibition of inositol efflux relative to untreated hypotonic control. Data shown are means ± SEM for four independent experiments, each performed in triplicate.

block VSOAC in some, but not all, tissues at relatively low concentrations, had only a minimal effect on inositol release (11 ± 10% inhibition at 30 μM, $n = 3$).

mAChR activation regulates inositol efflux under hypo-osmotic conditions

When SH-SY5Y cells were incubated in hypotonic buffer A (160 mOsm), the addition of 1 mM Oxo-M resulted in a marked stimulation of inositol efflux at all times examined (Fig. 3a). In contrast, Oxo-M addition did not result in an enhanced efflux of inositol under isotonic conditions. Inclusion of atropine (10 μM) could fully block the stimulatory effect of Oxo-M, while having no effect on efflux on its own (Fig. 3b). Inclusion of 200 μM DIDS resulted in a 44 ± 8% inhibition of Oxo-M-stimulated inositol efflux ($n = 3$), indicating the involvement of a VSOAC. The presence of 1 mM Oxo-M was observed to consistently enhance inositol efflux under hypo-osmotic conditions (195 ± 8% of control at a 10-min time point, $n = 35$). Addition of the partial agonist, pilocarpine (1 mM), elicited a correspondingly smaller increase in inositol efflux (122 ± 10% of control vs. 184 ± 20% for Oxo-M in the same experiments, $n = 3$). When SH-SY5Y cells were pre-incubated with 1 mM Oxo-M for 24 h (conditions under which mAChRs undergo internalization and down-regulation, see Sorensen *et al.* 1999), these cells were found to be much less responsive to the subsequent addition of Oxo-M under hypo-osmotic conditions (112 ± 9% of control vs.

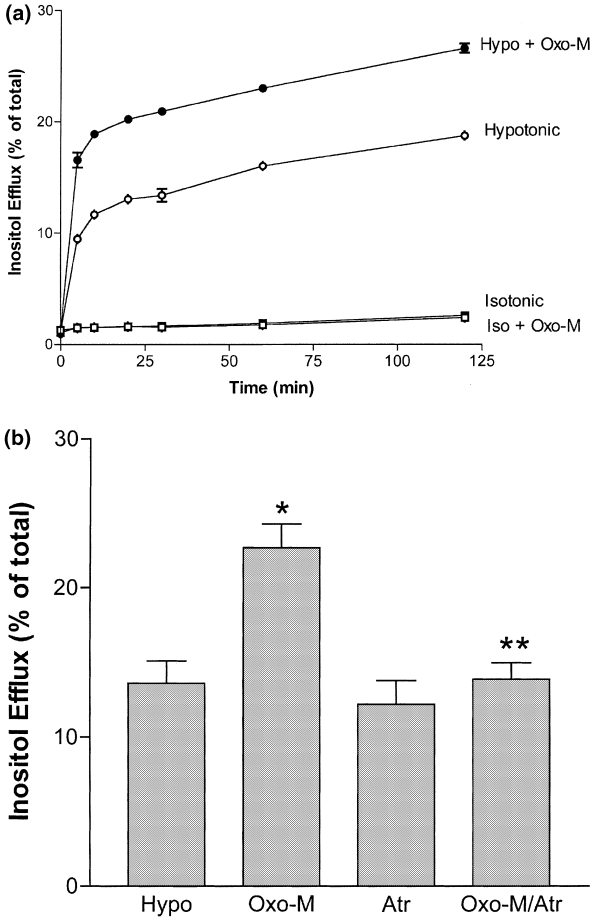

Fig. 3 Agonist occupancy of mAChRs facilitates the release of inositol from SH-SY5Y neuroblastoma. (a) Cells pre-labeled with ^{3}H-inositol were washed with isotonic buffer A and incubated in either hypotonic (Hypo: 160 mOsm) or isotonic (Iso: 310 mOsm) buffer A for the times indicated in the presence or absence of 1 mM Oxo-M and inositol efflux monitored. Results shown are expressed as inositol efflux (per cent of total radioactivity) and are the means ± SEM for triplicate replicates. Data shown are from one of three experiments that gave similar results. (b) Cells were incubated in hypotonic buffer A (160 mOsm) in the absence or presence of 1 mM Oxo-M, 10 μM atropine (Atr) or both reagents. Reactions were terminated after 20 min and inositol efflux monitored. Results shown are means ± SEM for five to six independent experiments, each performed in triplicate. *Different from hypotonic control, $p < 0.002$; **different from Oxo-M alone, $p < 0.001$.

189 ± 5% of control for untreated cells, $n = 3$). Although undifferentiated (rapidly dividing) cells were routinely used for the present studies, we also wanted to determine whether the agonist stimulation of inositol efflux could be observed in post-mitotic cells. The latter cell type would more closely resemble that present in the CNS. When SH-SY5Y cells were pre-treated with 10 μM retinoic acid for 6 days to effect their differentiation into the neuronal phenotype (Rosner *et al.* 1995), the addition of Oxo-M enhanced the efflux of inositol to 180 ± 14% of control, whereas the corresponding

value for undifferentiated cells was 202 ± 29% ($n = 3$). Values for the release of inositol under basal conditions (minus Oxo-M) were also similar (9.0 and 7.8% of total soluble radioactivity for undifferentiated and differentiated cells, respectively, after a 20-min incubation).

The ability of Oxo-M to stimulate inositol efflux could be observed at time points as early as 1 min, and in the presence of the agonist, inositol release was clearly biphasic, with the rate of inositol efflux declining after the first 5 min of incubation, as observed for control cells (Fig. 4a). If SH-SY5Y cells were first exposed to hypotonic buffer A for 20 min and then Oxo-M added (i.e. after the initial rapid phase of inositol efflux), a significant stimulation of inositol release was still observed (Fig. 4b). The EC_{50} value for Oxo-M-mediated stimulation of inositol efflux was 1.5 μM, a value similar to that observed for mAChR-mediated stimulation of inositol phosphate formation ($EC_{50} = 4.0$ μM; Fig. 5).

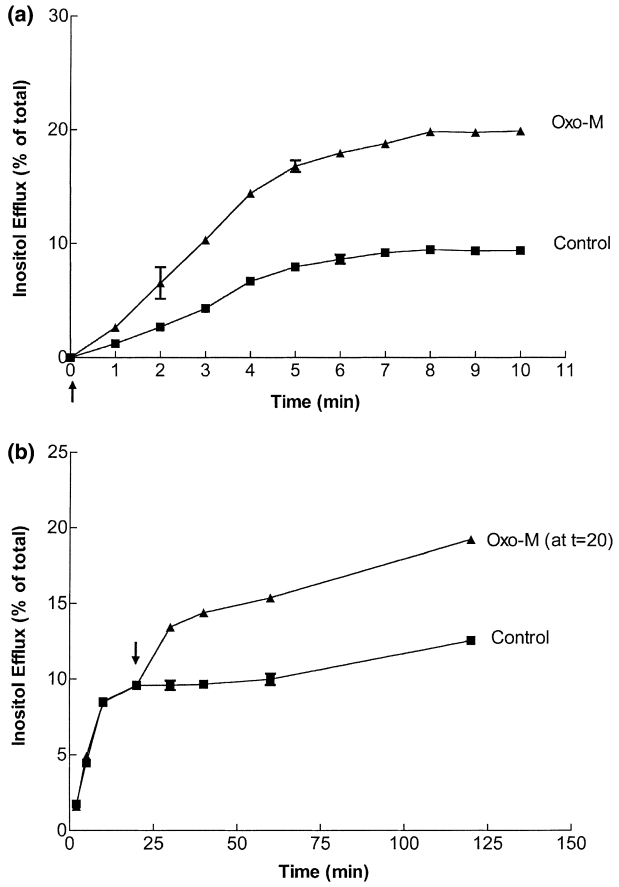

Fig. 4 Kinetics of Oxo-M-stimulated inositol efflux. (a) SH-SY5Y cells that had been pre-labeled with ^{3}H-inositol for 24 h were first washed in isotonic buffer A and then incubated in hypotonic buffer A (159 mOsm) in the presence or absence of 1 mM Oxo-M (added at time zero, as indicated by the arrow). Reactions were terminated at the times indicated and inositol efflux monitored. Values shown are the means ± SEM for triplicate replicates. Results shown are from one of two experiments that gave similar results. (b) Cells were treated as described in (a), with the exception that cells were allowed to incubate for 20 min in hypotonic buffer A prior to the addition of Oxo-M (indicated by arrow). Values shown are means ± SEM for triplicate replicates.

mAChR activation stimulates the release of either inositol or taurine under conditions of mild hypo-osmolarity

The ability of Oxo-M to enhance the release of inositol under conditions of pronounced hypo-osmolarity (160 mOsm) could also be observed when the cells were exposed to smaller reductions in osmolarity. When the osmolarity of buffer A was reduced to 250 or 227 mOsm, inositol release was increased (131 and 177% of control vs. isotonic, respectively). Inositol release was further enhanced by inclusion of Oxo-M (152 and 191% of control at 250 and 227 mOsm, respectively). In contrast, no effect of the agonist on inositol release could be observed when cells were incubated in isotonic buffer A (Fig. 6a). We also examined the ability of mAChR activation to facilitate the release of taurine, an amino acid that is well documented to serve as an

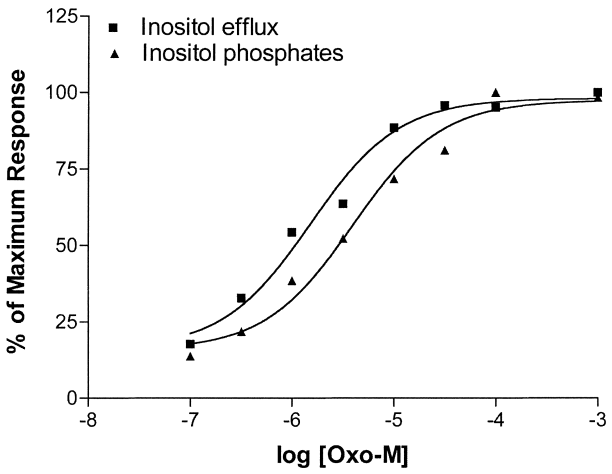

Fig. 5 Dose–response relationship for Oxo-M-stimulated inositol efflux or inositol phosphate accumulation. Cells that had been pre-labeled with ^{3}H-inositol were washed in isotonic buffer A and incubated in the presence of hypotonic buffer A (160 mOsm) that contained 5 mM LiCl. Reactions were terminated after 20 min and inositol efflux monitored. The accumulation of radiolabeled inositol phosphates in the trichloroacetic acid cell lysates was monitored as an index of stimulated phosphoinositide turnover. Results are expressed as a per cent of the maximum response (obtained at 1 mM Oxo-M) and are based upon measurement of triplicate replicates at each agonist concentration. The calculated EC_{50} values were 1.5 μM for inositol efflux and 4.0 μM for inositol phosphate accumulation. In a second experiment, values of 0.4 and 1.4 μM were obtained for the two responses, respectively.

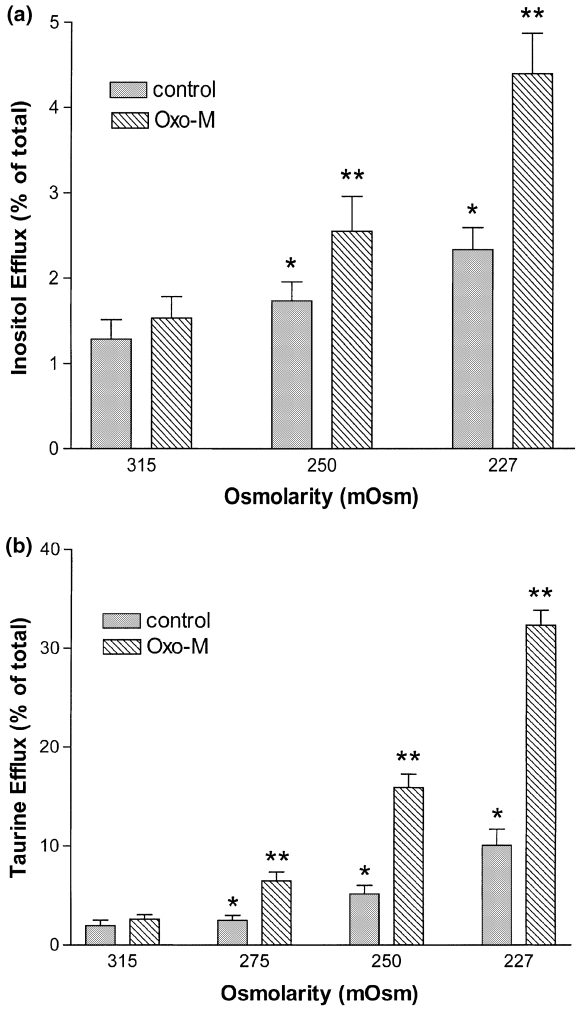

Fig. 6 Oxo-M can stimulate the efflux of either inositol or taurine under conditions of mild hypo-osmolarity. Cells that had been pre-labeled in the presence of either ^{3}H-inositol (a) or ^{3}H-taurine (b) were washed with isotonic buffer A and incubated in buffer A at the osmolarities indicated in the absence or presence of 1 mM Oxo-M. Experiments were terminated after 20 min and inositol or taurine efflux monitored. Results are expressed as inositol or taurine efflux (per cent of total radioactivity) and are means ± SEM for five (inositol release) or six (taurine release) independent experiments, each performed in triplicate. (a) *Different from isotonic control, $p < 0.02$; **different from hypotonic control (minus Oxo-M), $p < 0.03$. (b) *Different from isotonic control, $p < 0.02$; **different from hypotonic control (minus Oxo-M), $p < 0.003$.

osmolyte in neural cells (Pasantes-Morales *et al.* 2000). Under conditions of mild hypo-osmolarity (275–227 mOsm), taurine was readily released from SH-SY5Y neuroblastoma cells (273–532% of control vs. isotonic). mAChR activation resulted in a marked enhancement of taurine release under all conditions of hypo-osmolarity, but not when the cells were incubated in isotonic buffer A (Fig. 6b).

Inositol efflux can be enhanced following activation of protein kinase C (PKC) or a rise in intracellular Ca^{2+}

When SH-SY5Y cells were incubated in hypotonic buffer A (160 mOsm), the addition of either 100 nM PMA, a PKC agonist, or 1 μM ionomycin (an agent that elevates Ca^{2+}_i concentrations; see Fisher *et al.* 1989) significantly increased inositol efflux (139 ± 5 or 144 ± 4% of control, respectively, $n = 6$). In contrast, the inactive phorbol ester, 4α-phorbol was without effect (103 ± 4% of control; Fig. 7). Neither PMA nor ionomycin could facilitate inositol release when the cells were incubated under isotonic conditions. When both PMA and ionomycin were added to hypotonically treated cells, their effect on inositol release was additive (192 ± 13% of control), and efflux was comparable with that observed in the presence of Oxo-M (200 ± 19% of control).

mAChR-mediated stimulation of inositol efflux is mediated by both protein kinase C and Ca^{2+}

Although both the activation of protein kinase C (by PMA) and a rise in $[Ca^{2+}_i]$ (elicited by ionomycin) can increase inositol release, these observations do not, by themselves, demonstrate that the effects of mAChR activation are mediated by these mechanisms. To investigate the involvement of PKC in the ability of mAChRs to stimulate inositol efflux, two series of experiments were performed. In the first, SH-SY5Y cells were allowed to pre-incubate overnight in the presence of PMA – a condition that results in the down-regulation of PKC activity (Cioffi and Fisher 1990). The

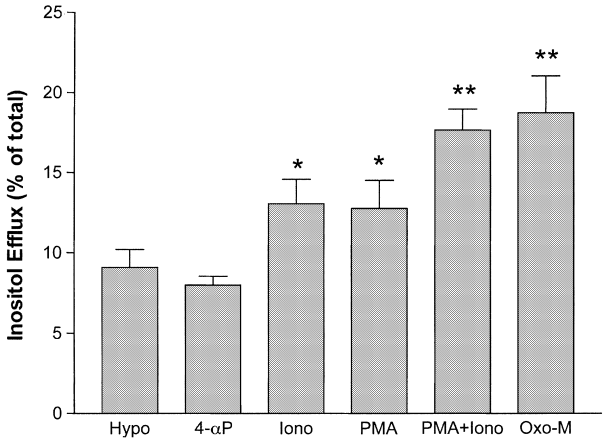

Fig. 7 Activation of either PKC, or a rise in intracellular Ca^{2+} concentration, facilitates inositol efflux from SH-SY5Y neuroblastoma. Cells that had been pre-labeled with ^{3}H-inositol were washed with isotonic buffer A and then incubated in hypotonic buffer A (160 mOsm) in the presence or absence of 4-alpha phorbol (4-αP,100 nM), PMA (100 nM), ionomycin (1 μM) or Oxo-M (1 mM). Inositol efflux was monitored after a 20-min incubation. Results shown are means ± SEM for four to five independent experiments, each performed in triplicate. *Different from hypotonic control, $p < 0.005$; **different from either PMA or ionomycin alone, $p < 0.006$.

basal release of inositol under hypotonic conditions was unimpaired in PKC down-regulated cells. In fact, a small increase in inositol efflux was consistently observed after PMA pre-treatment (127 ± 8% of control, $n = 10$, $p < 0.002$). However, following PKC down-regulation, the ability of PMA to stimulate inositol efflux was abolished while the ability of the agonist to enhance the release of the polyol was reduced by 41–46% (Figs 8a and c). In a second series of experiments, SH-SY5Y cells were pre-treated for 2 h with 3 μM BIM, a competitive antagonist of PKC (Wilkinson *et al*. 1993), and then challenged with either 100 nM PMA or 1 mM Oxo-M. Pre-treatment of cells with BIM resulted in a small increase in inositol efflux (110 ± 3% of control, $n = 5$, $p < 0.03$). Following pre-treatment with BIM, the ability of PMA to stimulate inositol release was substantially (although not completely) reduced, whereas enhanced inositol efflux in response to Oxo-M addition was only modestly impaired (16% inhibition; Fig. 8b). These results, taken together with the PMA-pre-treatment data, suggest that PKC activation plays a limited role in enhanced inositol efflux following mAChR activation.

Omission of extracellular Ca^{2+} (nominally Ca^{2+}-free buffer A) resulted in a small and variable reduction in the basal release of inositol under hypotonic conditions (−15 ± 7%, $n = 9$, $p < 0.05$). However, the ability of Oxo-M to enhance inositol efflux was substantially reduced (50 ± 6%, $n = 9$; Fig. 8c). Under the same conditions, Oxo-M stimulation of inositol phosphate formation was reduced by 49 ± 3% ($n = 4$, $p < 0.02$). An additional indication that influx of extracellular Ca^{2+} is required for agonist-stimulated osmolyte release was obtained from experiments in which the inclusion of 250 μM $LaCl_3$, an inhibitor of Ca^{2+} entry into cells (Pandol *et al*. 1987; Kwan *et al*. 1990), resulted in an inhibition of Oxo-M-stimulated

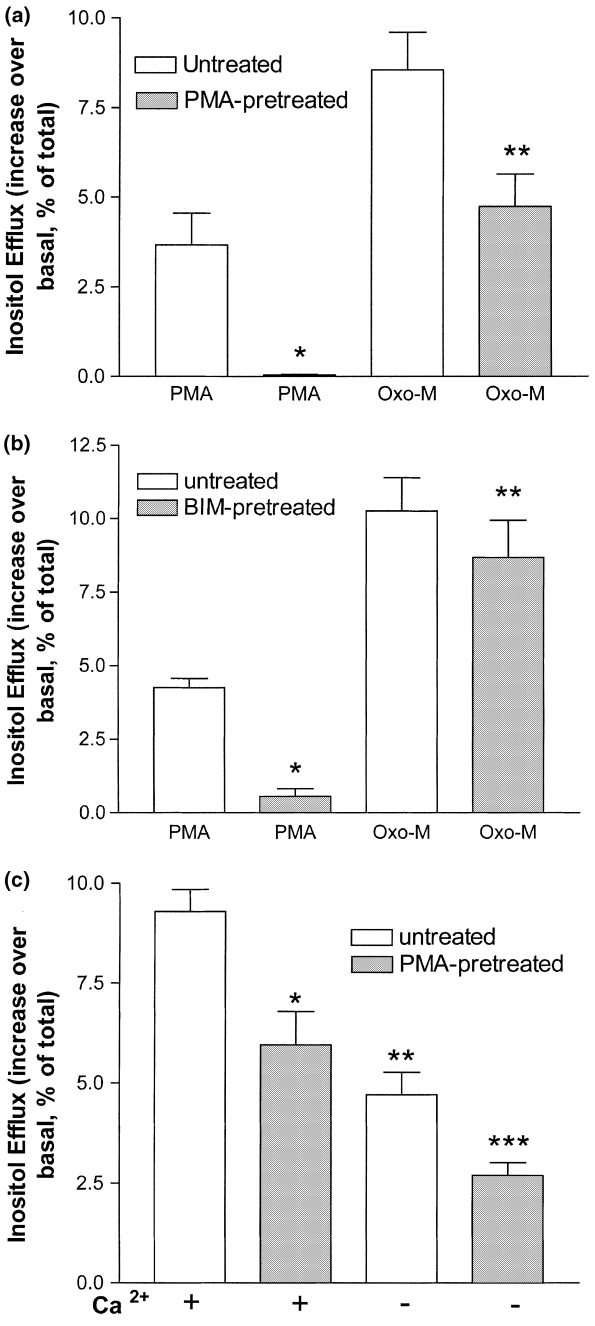

Fig. 8 PMA- or Oxo-M stimulation of inositol efflux following PKC down-regulation or inhibition. (a) SH-SY5Y cells were allowed to pre-label with ^{3}H-inositol for 48 h and then in the absence or presence of 100 nM PMA for a further 24 h. Cells (control or PMA-pre-treated) were then washed with isotonic buffer A and incubated in hypotonic buffer A (160 mOsm; basal conditions) in the presence or absence of 100 nM PMA or 1 mM Oxo-M. Reactions were terminated after 10 min and inositol efflux monitored. Results are expressed as increase in inositol efflux over corresponding control (no agent added) for either PMA or Oxo-M for the number of independent experiments performed. (b) Cells were treated as described in (a), with the exception that cells were pre-incubated with 3 μM BIM for 2 h, in place of PMA-pre-treatment. The basal release of inositol observed under hypotonic conditions was 7.5 ± 1.0% of total soluble radioactivity in (a), while the corresponding value in (b) was 11.7 ± 0.4%. *Different from control cells, $p < 0.004$; **different from control cells, $p < 0.05$ (matched-pair analysis). (c) SH-SY5Y cells were pre-labeled with ^{3}H-inositol for 24 h. Cells were then pre-treated with either PMA (100 nM) or vehicle for an additional 24 h. Cells were then washed with isotonic buffer A and incubated in hypotonic buffer A (160 mOsm) in the presence or absence of added Ca^{2+}. Inositol efflux was monitored following a 10-min incubation. Results are expressed as the percentage increase in inositol release observed in the presence of 1 mM Oxo-M, relative to the appropriate control, for the number of independent experiments indicated. The basal release of inositol observed under hypotonic conditions with Ca^{2+} present was 9.4 ± 7% of total soluble radioactivity. *Different from untreated cells, $p < 0.003$; **different from inositol release monitored in the presence of Ca^{2+}, $p < 0.002$; ***different from inositol release monitored in PMA-pre-treated cells in the presence of Ca^{2+}, $p < 0.02$.

inositol efflux (42 ± 5%, $n = 6$, $p < 0.004$) when compared with control cells (that were incubated in the presence of 2.2 mM Ca^{2+} alone). In contrast, the basal release of inositol was unaffected by La^{3+}. Inclusion of La^{3+} also inhibited the mAChR-stimulation of inositol phosphate formation to a similar extent (46 ± 2% inhibition, $n = 8$, $p < 0.0001$, when compared with control cells).

To determine whether the availability of intracellular Ca^{2+} plays a role in inositol efflux, cells were pre-incubated for 5 min in the presence of 1 μM thapsigargin, a treatment previously demonstrated to deplete intracellular stores of Ca^{2+} in these cells (Slowiejko *et al*. 1994). Depletion of intracellular Ca^{2+} had no effect on the basal release of inositol, but resulted in a reduction in Oxo-M-stimulated inositol release when assayed under nominally Ca^{2+}-free conditions (166 ± 4 vs. 144 ± 6% of basal when monitored in the absence or presence of thapsigargin pre-treatment, respectively, $n = 5$, $p < 0.01$).

To determine whether the Ca^{2+} requirement for inositol release was related to the involvement of a Ca^{2+}-activated form of PKC, the ability of the agonist to enhance inositol efflux was monitored for SH-SY5Y cells that had been pre-treated overnight with PMA and then efflux measured in the presence of nominally Ca^{2+}-free buffer. Under these conditions, a 73 ± 1% inhibition of inositol release was observed when compared with control cells (2.2 mM Ca^{2+} added, no PMA-pre-treatment; Fig. 8c). Because the inhibitory effects of Ca^{2+} depletion and PKC down-regulation on inositol efflux are less than additive, part of the Ca^{2+} requirement may reflect the involvement of a Ca^{2+}-dependent isoform of PKC. Although Ca^{2+} entry into cells can also occur via a phospholipase-A_2-mediated mechanism involving arachidonate release (Shuttleworth and Thompson 1999), in addition to the conventional PLC route, no effect of 10 μM isotetrandrine (a phospholipase A_2 inhibitor; Akiba *et al*. 1992) on the ability of Oxo-M to stimulate inositol efflux was observed (177 ± 20 and 180 ± 14% of control for control or isotetrandine-incubated cells, respectively, $n = 3$).

Discussion

When subjected to hypo-osmotic stress, SH-SY5Y neuroblastoma rapidly release inositol in a time- and osmolarity-dependent manner. The release of inositol is distinctly biphasic with a rapid release occurring during the first 5–10 min of incubation, followed by a slower, but sustained release thereafter. Similar kinetics have previously been obtained for inositol release from bovine sarcolemmal vesicles (Hale and Rubin 1995) and from cultured astrocytes (Isaacks *et al*. 1999). The pharmacological profile of inhibition of inositol release from SH-SY5Y cells by anion transport blockers is consistent with the involvement of VSOAC. Thus, inositol efflux was inhibited by both DIDS and NPPB, two inhibitors of volume-sensitive anion channels (Nilius *et al*. 1997), as well as by phloretin, an agent which inhibits these same channels, but not the Ca^{2+}-activated anion channel (Fan *et al*. 2001). A similar pharmacological profile was obtained for inositol release from C6 glioma cells (Strange *et al*. 1993) or NT2-N neurons (Novak *et al*. 2000). In contrast, tamoxifen, which appears able to discriminate between VSOACs present in different tissues (Leaney *et al*. 1997; Nilius *et al*. 1997; Brès *et al*. 2000), was unable to significantly inhibit inositol efflux from SH-SY5Y neuroblastoma. The basal release of inositol from SH-SY5Y cells was also relatively unaffected by removal of extracellular Ca^{2+}, depletion of intracellular Ca^{2+} or down-regulation of PKC – results which are consistent with those previously obtained for inositol efflux from C6 glioma cells (Strange *et al*. 1993). These results suggest that both neuronal and glial preparations release inositol upon hypo-osmotic stress as part of an adaptive mechanism to restore their volume.

The principal finding to emanate from the present study is that inositol efflux from SH-SY5Y neuroblastoma (either undifferentiated or retinoic-acid-differentiated) can be significantly enhanced following agonist occupancy of mAChRs. The ability of Oxo-M to promote inositol efflux was observed regardless of whether the agonist was added prior to, or after, osmolyte release had been initiated (Fig. 4). Inclusion of pilocarpine, a partial agonist at mAChRs (Thompson and Fisher 1990; Slowiejko *et al*. 1994), resulted in a significantly smaller stimulation of inositol efflux than that observed for Oxo-M. Confirmation of an involvement of mAChRs in the regulation of inositol efflux was obtained from experiments in which atropine was shown to fully prevent the ability of Oxo-M to promote inositol efflux. Furthermore, a prolonged incubation of SH-SY5Y cells with Oxo-M, a treatment which results in the internalization and down-regulation of mAChRs (Sorensen *et al*. 1999), resulted in a substantial attenuation of agonist-induced efflux. mAChR–mediated enhancement of inositol release observed under conditions of hypo-osmotic stress was inhibited by inclusion of DIDS. Moreover, Oxo-M was unable to facilitate inositol release under iso-osmotic conditions. Thus, a prerequisite for agonist regulation of VSOAC opening is activation of the latter by hypotonic stress. These results, taken together, indicate the involvement of a volume-sensitive channel (such as VSOAC) in inositol efflux following mAChR activation.

Only a very limited amount of information exists regarding the possibility that VSOAC, in either neural or non-neural cells, can be regulated by extracellular agonists. In this context, agonist occupancy of thrombin receptors in endothelial cells (Manolopoulos *et al*. 1997), endothelin receptors in cardiac myocytes (Du and Sorota 2000) or nucleotide receptors on epithelial cells (Tsumura *et al*. 1996), have been reported to enhance volume-activated Cl^- currents mediated via VSOAC. In addition, ATP, operating via P_{2u} and/or P_{2y}

receptors, can potently stimulate excitatory amino acid release from hypotonically stressed astrocytes (Mongin and Kimelberg 2002). However, in none of these examples have the signaling pathways yet been systematically examined. Although we and others have previously demonstrated that the activation of mAChRs in SH-SY5Y cells is linked to a number of downstream consequences, such as PLC activation and second-messenger generation, Ca^{2+} mobilization from both intra- and extracellular compartments, activation of PKC, small MW GTP-binding proteins, MAP kinase and reorganization of the actin cytoskeleton (Lambert and Nahorski 1990; Offermanns *et al*. 1993; Linseman *et al*. 1998, 2000), our present results suggest that agonist stimulation of inositol efflux is mediated via a rise in $[Ca^{2+}_i]$ and, to a lesser extent, PKC activity. The evidence for this is threefold. First, the ability of Oxo-M to enhance inositol release can be mimicked in part by the addition of either ionomycin, a calcium ionophore, or the PKC agonist, PMA. In combination these agents elicited an increase in inositol efflux that was comparable with that of the agonist. Second, both extra- and intracellular pools of Ca^{2+} appear to be required for agonist-stimulated inositol release. Thus, mAChR-stimulated release of inositol was significantly reduced (40–50%) when cells were incubated in either nominally Ca^{2+}-free buffer A, or alternatively, in regular hypotonic buffer A (containing 2.2 mM Ca^{2+}) in the presence of La^{3+}, an inhibitor of Ca^{2+} entry into cells (Pandol *et al*. 1987; Kwan *et al*. 1990). Both of these experimental paradigms resulted in a similar degree of inhibition of stimulated inositol phosphate formation. It has previously been reported that mAChR stimulation of PLC activity and inositol 1,4,5-trisphosphate formation (which is linked to Ca^{2+} mobilization) are strongly inhibited by removal of extracellular Ca^{2+} (Fisher *et al*. 1990; Lambert *et al*. 1991). Agonist occupancy of mAChRs on these cells leads to a continuous influx of extracellular Ca^{2+} and the subsequent elevation of $[Ca^{2+}_i]$ results in a sustained activation of PLC. Conversely, when Ca^{2+} entry into the cells is prevented (e.g. by omission of extracellular Ca^{2+} or the presence of calcium channels blockers such as Ni^{2+} or La^{3+}), mAChR stimulation of PLC activity declines. The present data suggest a similar requirement for the influx of extracellular Ca^{2+} in agonist-stimulated inositol efflux. Although extracellular Ca^{2+} appears to play the predominant role, the availability of intracellular Ca^{2+} also regulates stimulated inositol efflux, as indicated by the reduction in the release of the polyol following depletion of intracellular Ca^{2+} stores with thapsigargin. Third, a role for PKC can be inferred from the ability of either PMA- or BIM-pre-treatment to attenuate inositol efflux. However, it is evident that, even in the absence of PKC activity, the muscarinic agonist retains an ability to significantly enhance inositol efflux. Although the signal transduction mechanism(s) underlying the basal efflux of inositol in response to hypo-osmolarity is independent of extra- or intracellular Ca^{2+} and PKC, it is evident that the efflux mechanism can be regulated in a Ca^{2+}- and PKC-dependent manner following mAChR activation. Taken collectively, the present results suggest a role (in part, at least) for PLC activation in the regulation of inositol efflux following mAChR activation. However, a direct proof of this hypothesis remains to be established because of technical difficulties encountered with the use of two putative inhibitors of PLC. Thus, when SH-SY5Y cells were exposed to U-73122 (Bleasdale *et al*. 1990) for 10 or 20 min (standard incubation time), cell rounding and detachment from the dish were observed. Moreover, although an acute (5-min) exposure of the cells to the aminosteroid avoided cell detachment, under these conditions a significant inhibition (40–50%) of *both* basal- and stimulated efflux were observed – a result that points to a non-specific mode of inhibition of osmolyte release by this agent. As an alternative approach, we examined the ability of the aminoglycoside, neomycin, to inhibit PLC activity (Cedazo-Mínguez *et al*. 2002). However, in our hands, this agent, in the concentration range of 0.5–5 mM had little or no effect on either stimulated PLC activity or inositol efflux. Although a direct confirmation of a role for PLC in osmolyte efflux has yet to be obtained, it should be noted that our results, which implicate phosphoinositide hydrolysis in the regulation of stimulated inositol release, and by inference, cell volume regulation, are consistent with a previous study in astrocytes in which receptor-stimulated inositol lipid turnover was linked to RVD (Bender *et al*. 1993).

Although routinely monitored under conditions of pronounced hypo-osmotic stress (160 mOsm), increases in inositol efflux from SH-SY5Y cells were also detected following 15–20% reductions in osmolarity, i.e. 227–250 mOsm, values similar to plasma osmolarities encountered during hyponatremia (Andrew 1991; Haussinger *et al*. 1994). Oxo-M could significantly increase inositol efflux under conditions of hypo-osmolarity in which only a limited increase in the basal release of inositol could be observed (Fig. 6a). A similar result has recently been observed for ATP stimulation of excitatory amino acid release from astrocytes (Mongin and Kimelberg 2002). These results indicate that receptor activation facilitates the ability of cells to release osmolytes under conditions of very limited reductions in osmolarity. In this regard, it should be noted that the ability of organic osmolytes to permeate VSOAC is dependent upon their molecular dimensions. The minimum diameter of the pore channel of VSOAC lies between 5.4 and 8 Å. The molecular size of the cyclical form of inositol (7.2×5.9 Å) is at the upper limit, thereby resulting in a relatively slow rate of efflux (McManus *et al*. 1995; Nilius *et al*. 1997). In contrast, osmolytes such as glutamate, aspartate or taurine would be expected to exit the cell more readily. Consistent with this prediction, SH-SY5Y cells release radiolabeled taurine more readily than inositol in

response to mild hypo-osmotic stress, and, moreover, this release is facilitated by mAChR activation (Fig. 6b). The ability of mAChRs to enhance the release of organic osmolytes from SH-SY5Y cells may constitute part of a protective mechanism whereby cell integrity is maintained. In this context, it is of interest to note that activation of the same receptors on SH-SY5Y cells also protects against metabolic insults that lead to apoptosis (De Sarno *et al*. 2003).

Despite the fact that the occurrence of brain swelling is of major clinical importance, little is known regarding either the molecular identity of VSOAC (see Nilius *et al*. 1997 for review) or the identity of the cell signaling pathway(s) involved in the activation and regulation of VSOAC. In the present study, we demonstrate that activation of mAChRs can modulate osmolyte release, and thus the capacity of a neural cell to restore its volume in response to hypotonic stress is subject to regulation by extracellular agonists. These observations may be of interest, not only in terms of our understanding of the basic mechanisms underlying osmolyte release, but also may be of practical importance in terms of the rational design of therapeutically beneficial agents.

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