The characteristics, capacity and receptor regulation of inositol uptake in 1321N1 astrocytoma cells

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The uptake of inositol into 1321N1 astrocytoma cells was studied by measurement of the accumulation of free [^3H]inositol within the intracellular pool. Uptake occurs via a saturable transporter with apparent K_m for inositol $\sim 40\ \mu\text{M}$ and $V_{\max}$ $\sim 180\ \text{pmol/min per mg of protein}$, which permits intracellular inositol concentrations to exceed those of the medium by a factor of ~ 500 . At extracellular concentrations up to $500\ \mu\text{M}$, inositol uptake is highly dependent ($\geq 85\%$) on the presence of Na^+ in the medium, and, at physiological extracellular inositol concentrations, allows inositol to achieve an intracellular concentration of $\sim 20\ \text{mM}$, indicating an active process driven by the Na^+ gradient. Despite this, uptake was only minimally impaired or was unaffected by ouabain ($1\ \text{mM}$) or dinitrophenol ($1\ \text{mM}$). Consistent with a carrier-mediated mechanism, uptake was competitively blocked by phlorrhizin ($K_i \sim 125\ \mu\text{M}$). Uptake

was also inhibited by carbachol and histamine, which act respectively via muscarinic and H_1 receptors in these cells to stimulate phospholipase C. Inhibition by carbachol was dose-dependent ($\text{EC}_{50} \sim 3\text{--}30\ \mu\text{M}$) and blocked by atropine. Inhibition by carbachol ($1\ \text{mM}$) was non-competitive, resulting from $\sim 50\%$ decrease in the $V_{\max}$ for uptake without affecting the K_m and was persistent over 30–90 min. Inhibition by carbachol and histamine was independent of extracellular Ca^{2+} and was reproduced by phorbol ester, but not by Ca^{2+} ionophore or stimulation of adenylate cyclase. These results imply that receptors which couple to phospholipase C may mediate inhibition of inositol uptake via protein kinase C. The data are discussed in relation to inositol homeostasis in resting and stimulated cells.

INTRODUCTION

The inositol phospholipids play a central role in transmembrane signalling. For example, it is now well established that a wide variety of ligands which exert their effects through cell-surface receptors activate a phosphoinositide-specific phospholipase C which hydrolyses $\text{PtdIns}(4,5)\text{P}_2$, resulting in the simultaneous production of two second messengers: $\text{Ins}(1,4,5)\text{P}_3$, which mediates release of Ca^{2+} from intracellular stores, and *sn*-1,2-diacylglycerol, which activates protein kinase C [1]. The complex metabolism of $\text{Ins}(1,4,5)\text{P}_3$ may generate further signal molecules [2]. Emerging evidence implies an additional role for inositol lipids, as the activation of several plasma-membrane receptors possessing intrinsic tyrosine kinase activity is closely associated with the stimulation of a phosphoinositide-specific 3-kinase [3]. Recent studies [4,5] suggest that $\text{PtdIns}(4,5)\text{P}_2$ may also be the primary substrate for this enzyme, and there is considerable speculation that the product of this reaction, $\text{PtdIns}(3,4,5)\text{P}_3$, may have second-messenger functions [3,6]. Thus $\text{PtdIns}(4,5)\text{P}_2$ is the precursor of at least two, and perhaps more, intracellular signals.

The synthesis of $\text{PtdIns}(4,5)\text{P}_2$ occurs from PtdIns via $\text{PtdIns}4\text{P}$ and involves the sequential action of 4- and 5-kinases. In resting cells, concentrations of $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}4\text{P}$ are regulated by the corresponding activities of 5- and 4-phosphomonoesterases respectively. The changes in the concentration of $\text{PtdIns}(4,5)\text{P}_2$ following acute stimulation of phospholipase C are buffered by compensatory re-synthesis from PtdIns [7]. During prolonged periods of stimulation, PtdIns in turn is re-synthesized

from CMP-phosphatidate and free inositol. Thus the efficient production of inositol-containing second messengers is ultimately dependent on the availability of inositol.

Concentrations of inositol vary widely between different cell types and, although some cells have the capacity to oxidize inositol to glucuronic acid or to epimerize inositol to generate the far less abundant *scyllo*- and *neo*-inositol epimers, cellular concentrations of *myo*-inositol are generally held to be maintained through the balance of three primary routes (see [8] for review). These are: (i) synthesis *de novo*, which occurs via $\text{Ins}3\text{P}$ from glucose 6-phosphate, (ii) the re-cycling of the inositol moiety of $\text{Ins}(1,4,5)\text{P}_3$ by successive dephosphorylation, and (iii) by accumulation from the extracellular medium. The final step in both former processes is catalysed by inositol monophosphatase. This enzyme is potently inhibited by Li^+ [9], and it has been suggested that this inhibitory action may underlie the therapeutic effects of Li^+ in the treatment of manic-depression, since, by limiting inositol supply, Li^+ may down-regulate signalling through over-active receptor systems [10]. Cells which are able to obtain inositol from the medium may be relatively immune to such effects, depending on the properties of their inositol uptake system. Inositol transport has been studied in a number of tissues and cultured cell lines and appears to involve either a relatively high-affinity, saturable, Na^+ -dependent mechanism [11–13] or a low-affinity or non-saturable process [14,15], although a combination of both mechanisms has been reported in several systems [16–20].

Despite intensive study of receptor-mediated phosphoinositide turnover in recent years, the influence of inositol homeostasis

Abbreviations used: PMA, 4 β -phorbol 12 β -myristate 13 α -acetate. Inositol refers to the *myo* isomer throughout, unless otherwise specified.

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on this and the individual contributions to cellular inositol concentrations made by separate routes of supply in both resting and stimulated cells remain uncertain. As part of an ongoing series of studies in this context, we have examined the nature and capacity of the process by which 1321N1 astrocytoma cells gain access to inositol in the extracellular environment.

MATERIALS AND METHODS

Materials

All materials for tissue culture were obtained from Gibco. *myo*-[2-³H]inositol at ~18–20 Ci/mmol was from Amersham. All drugs were from Sigma. Anion-exchange resin was from Bio-Rad. All other reagents were of analytical grade and were purchased from BDH, Aldrich or Sigma.

Cell culture

The methods for culture of 1321N1 astrocytoma cells were based on those described previously [21,22]. Briefly, cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) foetal-calf serum, fungizone (2.5 µg/ml) and either penicillin+streptomycin (100 units/ml+100 µg/ml respectively) or gentamycin (10 µg/ml). Cells were maintained at 37 °C in a humidified atmosphere with CO₂ at 5% and were sub-cultured weekly and re-seeded in 80 cm² flasks at ~10000 cells/cm² for maintenance of stock cultures [23] or at approx. 5 times this density in 24-well dishes [22] for assay of inositol uptake. Medium was replaced at intervals of 3–4 days.

Assay of inositol uptake

Uptake assays were performed with cells typically at 4–6 days post-confluence. Growth medium was aspirated and cell monolayers were washed with 4 × 2 ml of Hepes-buffered modified Krebs–Henseleit buffer (118 mM NaCl or choline chloride; 4.69 mM KCl; 1.18 mM MgSO₄; 1.29 mM CaCl₂; 1.18 mM KH₂PO₄; 11.67 mM glucose; 25 mM Hepes, adjusted to pH 7.4 by addition of NaOH to ~14 mM) at 37 °C. Washed cells were allowed to equilibrate for 30–60 min at 37 °C in sufficient of the same buffer (~380–480 µl) such that a final volume of 500 µl was achieved after subsequent additions. Assays were started by the rapid successive addition of portions (10–50 µl) of [³H]inositol (1 µCi) and of vehicle or drugs as appropriate. [³H]inositol was diluted with unlabelled carrier to give a final assay concentration of 5 µM, except where otherwise indicated. Where atropine was included in assays, this was added ~15 min before agonist or buffer control.

After addition of [³H]inositol and drugs, incubations were continued at 37 °C for a further 30 min or for different intervals as indicated before reactions were quenched. This was achieved by aspiration of the incubation medium and subsequent washing of cell monolayers five times in rapid succession (total > 30 s) with 2 ml of ice-cold buffer supplemented with 1 mM unlabelled inositol. This procedure routinely decreased residual extracellular [³H]inositol concentrations by > 99.9%, as verified by the inclusion in each assay of sample blanks in which [³H]inositol was added to cells and immediately removed in this manner. Washed cells were then lysed by addition of 600 µl of ice-cold 0.5 M trichloroacetic acid. Cell debris was scraped off, and the contents of individual wells were transferred to microfuge tubes together with a wash with 250 µl of 0.5 M trichloroacetic acid to

collect residual material. Samples were briefly centrifuged, and the resulting supernatants and pellets were analysed for water and lipid-soluble [³H]inositol metabolites respectively as described below.

Measurement of intracellular [³H]inositol and metabolites

Samples (800 µl) of supernatant were neutralized by mixing with 200 µl of 10 mM EDTA (pH 7.5 with NaOH) and 800 µl of 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (1:1, v/v) as described previously [24], and samples were briefly centrifuged to facilitate phase separation. Total intracellular water-soluble ³H was then routinely measured by direct liquid-scintillation counting of samples (900 µl) of upper phase after mixing with 4 ml of Flo-scint IV (Packard). Alternatively, in some experiments water-soluble ³H was separated into its constituent free [³H]inositol and [³H]inositol phosphate fractions by Dowex anion-exchange chromatography as described previously [25].

Lipid-soluble ³H metabolites were extracted from the pelleted cell debris. Pellets were washed once with 1 ml of 5% (w/v) trichloroacetic acid/1 mM EDTA and 1 ml of water, and [³H]lipids were then extracted for 10–15 min into 750 µl of methanol/chloroform/12 M HCl (80:40:1, by vol.). Aqueous and organic phases were partitioned by addition of 250 µl of chloroform and 450 µl of 0.1 M HCl followed by brief centrifugation. Samples (200 µl) of lower phase were counted for ³H radioactivity as a measure of total [³H]inositol lipid.

In some experiments, cell pellets were dissolved in 0.5 ml of 1.0 M NaOH, and samples (50 µl) were assayed for protein as described in [26] with BSA as standard. Intracellular water was determined and related to protein content as described in [27]. One well of cells typically contained ~0.25–0.35 mg of protein, which was equivalent to 17.5 ± 0.5% of the cell wet weight and a cell volume of 1.5–2.0 µl/well.

Expression of results

[³H]inositol uptake, after correction for volume losses and sample blanks, was calculated from: (sum of water+lipid-soluble [³H]inositol/total added [³H]inositol) × (amount of extracellular inositol), although in most cases lipid-soluble ³H could be ignored (see below). Data are expressed as pmol of inositol accumulated intracellularly/unit time per well of cells or as a relative percentage of control. Assays of inositol uptake were performed in duplicate or triplicate. Where statistical significance is indicated, data were analysed by comparison of the paired means of duplicate or triplicate incubations over the number of experiments indicated, by a paired Student's *t* test. Values were taken as significant at *P* ≤ 0.05.

RESULTS

Basic characteristics of inositol uptake

Assay conditions

Preliminary experiments showed that at extracellular concentrations from 5 µM to 1 mM the uptake of [³H]inositol into 1321N1 cells was linear for at least 90 min. Within a typical 30 min incubation at 5 µM [³H]inositol the proportion of total ³H which accumulated intracellularly varied between, though not within, experiments from ~2 to 10%, of which > 99.0% was present as water-soluble material. Over extended time courses in which cells were incubated in tissue-culture medium supplemented with 40 µM or 1 mM [³H]inositol, equilibration between the intra- and extra-cellular compartments occurred only over ~24 h,

Figure 1 Dependence of inositol uptake on extracellular inositol concentration

Cells were incubated for 30 min in the presence of [3 H]inositol (1 μ Ci) in buffer supplemented with increasing concentrations of unlabelled inositol, and intracellularly accumulated water-soluble 3 H was then measured as described in the Materials and methods section. Data represent the means of triplicate determinations made in a single experiment in which S.E.M. values were typically $\leq 5\%$ of the mean. Similar data were obtained in several further experiments. The inset shows an Eadie-Hofstee transformation of the same data.

implying that inositol efflux from these cells is slow. Indeed, negligible loss of intracellular [3 H]inositol was observed over 1–2 h incubations when pre-loaded cells were subsequently placed in inositol-free buffer. In contrast, at 40 μ M [3 H]inositol in the medium, steady-state labelling of cellular phospholipid pools required ≥ 48 h, and at this time [3 H]inositol phospholipid and [3 H]inositol phosphate respectively comprised only $\sim 5\%$ and $< 1\%$ of intracellular label, despite the accumulation of ~ 50 – 60% of total [3 H]inositol within the intracellular compartment at equilibrium. These results suggest that there is limited metabolism of intracellularly accumulated [3 H]inositol by established routes in these cells, and that measurement of intracellular water-soluble radiolabel accumulated over brief incubations accurately and specifically reflects inositol uptake. Furthermore, as the ratio of medium (1.0 ml) to cell (~ 2.0 μ l) volume in equilibrium labelling experiments was $\sim 500:1$, these data demonstrate the very marked capacity of 1321N1 cells to concentrate inositol, with intracellular concentrations of ~ 20 mM being achieved when inositol concentration in the medium is 40 μ M. The properties of this system have thus been examined in detail.

Kinetics and Na^+ -dependence

The influence of extracellular inositol concentration on the rate of uptake is examined in Figure 1. This clearly demonstrates that inositol uptake into 1321N1 cells is a saturable process, with close to maximal rates being achieved at 0.5–1.0 mM. The inset shows an Eadie-Hofstee transformation of the same data, from which the affinity and capacity of the system were determined. Over several experiments, K_m and V_{max} values varied from ~ 20

to 60 μ M and 30 to 60 pmol/min per well of cells, with mean values of 39 ± 5 and 45 ± 5 respectively. Similar values have previously been reported in several other tissues [11,13,28] and cells [16–20]. However, in most of these systems [16–20] inositol transport comprises both relatively high-affinity, Na^+ -dependent, and low-affinity or non-saturable, Na^+ -independent, components. Figure 1 reveals no evidence for such duality, and it might therefore be expected that inositol transport in astrocytoma cells would be highly Na^+ -dependent. Table 1 thus illustrates the results of several experiments in which uptake was determined at different inositol concentrations both in normal buffer and buffer with decreased Na^+ concentration. These data show that decreasing extracellular Na^+ markedly attenuated inositol uptake at each concentration. It is also evident that the proportional effect of Na^+ removal was similar across a 100-fold range of extracellular inositol concentrations.

Given the high Na^+ -dependence and concentrative capacity of inositol transport in 1321N1 cells, the effects of ouabain, an inhibitor of the Na^+/K^+ -ATPase, and of dinitrophenol, an uncoupler of oxidative phosphorylation, were also examined. However, neither agent markedly influenced [3 H]inositol uptake, with rates in the presence of 1 mM ouabain and 1 mM dinitrophenol respectively 92.6 ± 2.6 and $109.1 \pm 4.0\%$ that in control cells. The failure of dinitrophenol to affect uptake suggests either a lack of, or indirect dependence on, ATP concentration and is consistent with the varying sensitivity of active inositol transport to metabolic inhibitors observed in other tissue and cell types [cf. 11,17,20]. Similarly, although the decrease in uptake by ouabain was statistically significant, the extent of this action was surprisingly limited, given the data in Table 1 and those previously reported [12,13,20] for Na^+ -dependent inositol uptake, although variable sensitivity to ouabain in different cells has been observed [17]. The present result presumably reflects either a slow dissipation of the Na^+ gradient and/or limited sensitivity of the Na^+/K^+ -ATPase to ouabain in 1321N1 cells.

Inositol uptake is carrier-mediated

Figure 2 examines the potential role of a carrier protein in inositol transport, comparing the effects of increasing concentrations of phlorrhizin, an inhibitor of facilitated sugar transport, on the rate of inositol uptake across a series of extracellular inositol concentrations. These data show that phlorrhizin competitively inhibits inositol transport, lowering the affinity for inositol without decreasing the maximal rate of uptake. Calculations of the inhibitor constant (K_i) from the data in Figure 2 or by correction of the IC_{50} [29] determined in separate experiments in which inhibition by increasing concentrations of phlorrhizin was measured against a fixed low (0.2 or 5 μ M) inositol concentration each gave values of ~ 125 μ M. This agrees closely with the inhibition by phlorrhizin of saturable Na^+ -dependent inositol uptake in hamster small intestine, where the calculated K_i (80 μ M) was suggested to reflect a > 10 -fold lower affinity of phlorrhizin for inositol compared with sugar transport [11]. Inhibition of both active [13,16,19,20] and non-saturable [14] inositol uptake by phlorrhizin and the related compound phloretin respectively have also been reported in several other systems and, although affinities were not determined, similar values might be inferred. Further, both phlorrhizin and phloretin at 0.3 mM cause $> 50\%$ inhibition of the Na^+ -dependent inositol transporter recently cloned from Madin-Darby canine kidney (MDCK) cells when expressed in *Xenopus* oocytes [30]. The present results thus clearly imply that inositol uptake in 1321N1 cells is carrier-mediated. We have not yet further examined the relationship between this and other similar proteins. However, as

Table 1 Dependence of inositol uptake on the presence of extracellular Na^+

1321N1 cells were incubated for 30 min in the presence of [^3H]inositol (1 μCi) at 5, 250 or 500 μM in normal buffer (Na^+ present) or buffer in which NaCl was replaced with equimolar choline chloride (Na^+ absent), and the accumulation of total intracellular water-soluble ^3H was then determined as described in the Materials and methods section. Data represent means $\pm$ S.E.M. derived from 6 experiments each performed in triplicate. The % Na^+ -dependence was calculated from the fraction of [^3H]inositol uptake apparent in low-sodium medium in each experiment.

Condition	Rate of inositol uptake (pmol/min per well)		
	[Inositol] ... 5 μM	250 μM	500 μM
Na^+ present	4.29 ± 0.40	35.58 ± 2.55	42.52 ± 4.54
Na^+ absent	0.16 ± 0.05	3.93 ± 0.87	6.06 ± 0.58
% Na^+ -dependence	$96.3 \pm 1.2\%$	$89.3 \pm 1.7\%$	$85.2 \pm 1.5\%$

Figure 2 Competitive inhibition by phloretin of inositol uptake

Cells were incubated for 30 min as described in the legend to Figure 1 in the presence of various concentrations of [^3H]inositol (5–500 μM) and in either the absence ($\circ$) or the presence of 0.1 ($\bullet$) or 0.3 mM ($\triangle$) phloretin before assay of intracellularly accumulated [^3H]inositol as described in the Materials and methods section. Data are presented as a double-reciprocal plot of the rate of uptake against inositol concentration and represent the mean of duplicate determinations made in a single experiment. Similar results were obtained on one further occasion (see the text).

the inositol carrier from MDCK cells shows considerable sequence identity with certain glucose transporters [30], it is important to emphasize that in the present study inositol uptake was routinely measured with glucose present in > 2000 -fold excess over inositol. Additional, preliminary data indicate that, although increasing glucose from 11.6 mM to 50.0 mM inhibits uptake at 5 μM inositol, a 10-fold decrease in glucose has little effect, implying only a limited affinity of glucose for the inositol transporter in 1321N1 cells.

Receptor regulation of inositol uptake

Phospholipase C-coupled receptors inhibit uptake

In several cell types Li^+ down-regulates signalling through phospholipase C-coupled receptors as it inhibits inositol mono-phosphatase and thereby decreases cellular inositol concentrations [10,31]. Cells with access to inositol in the medium may by-pass this effect of Li^+ , and the variation in properties of different inositol transport systems is likely to be a dominant factor in directing the cellular selectivity of Li^+ , and thus may be a major determinant of the therapeutic utility of Li^+ [32]. However, few studies have considered the potential regulatory influence of receptor stimulation on inositol transport [13,20,33]. Consequently, we have examined the effects on inositol uptake of stimulating receptors previously shown in 1321N1 cells to allow either persistent (muscarinic [34,35]) or transient (histamine H_1 [36,37]) activation of phospholipase C or which couple to activation of adenylate cyclase (β -adrenergic, [21]). Table 2 contrasts the effects of carbachol, a muscarinic cholinergic agonist, histamine, and isoprenaline, a β -adrenergic selective stimulus, each at maximally effective concentrations. These data clearly show that both carbachol and histamine decreased the rate of inositol uptake, although the effect of carbachol was generally more marked. In contrast, isoprenaline did not influence uptake. Table 2 also illustrates that, although carbachol and histamine together decreased uptake to a greater extent than did carbachol alone, their combined effect was less than additive, indicating that the underlying mechanism for both stimuli involves common components. These results are distinct from those obtained in pancreatic islets [13], where carbachol did not affect active inositol transport, but similar to those reported for phospholipase C-coupled receptors in rat parotid acinar cells [20,33], indicating that in some cell types receptors which stimulate $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis may selectively modify inositol uptake. In this respect it is important to emphasize that in 1321N1 cells agonist actions represent a true inhibition of [^3H]inositol uptake and are not the result either of re-distribution of uptaken [^3H]inositol owing to enhanced incorporation into phospholipid or of increased [^3H]inositol efflux, both of which might be expected as secondary consequences of stimulated phosphoinositide turnover. The former effect can be discounted, because in uptake assays run both with and without 1 mM carbachol, [^3H]phosphoinositide accounted for $< 2\%$ of intracellular label. Similarly, agonist effects on [^3H]inositol efflux can be eliminated for two reasons. Firstly, 1 mM carbachol had no effect on cellular inositol concentrations in 1321N1 cells labelled to isotopic equilibrium with [^3H]inositol (I. H. Batty and C. P. Downes, unpublished work). Secondly, the inclusion of 10 mM LiCl , to block any agonist-evoked increase in cellular inositol resulting from stimulated phosphoinositide breakdown, did not decrease the inhibitory impact of 1 mM carbachol on inositol uptake (as % of control, rates of uptake were: LiCl , 81.1 ± 3.4 ; carbachol, 47.0 ± 2.7 ; carbachol + LiCl , 42.2 ± 2.0 ; means $\pm$ S.E.M. of 6 experiments). The inhibitory actions shown in Table 2 thus specifically reflect regulation of inositol import.

Muscarinic-receptor-mediated inhibition of uptake

Measurement of uptake across a range of extracellular inositol concentrations both with and without 1 mM carbachol showed that inhibition by this agonist is non-competitive, resulting from a decreased maximal rate of uptake rather than a decreased affinity for inositol ($K_m = 56 \pm 4 \mu\text{M}$ with or without carbachol; $V_{\text{max}} = 35 \pm 6$ and 18 ± 4 pmol/min per well of cells respectively in control and stimulated cells; means $\pm$ S.E.M. from 3 experi-

Table 2 Effects of different receptor agonists on inositol uptake

Cells were incubated for 30 min with [3 H]inositol (5 μ M) either under control conditions or in the presence of the different stimuli indicated, and intracellular [3 H]inositol accumulation was then measured as described in the legend to Table 1. Data represent means $\pm$ S.E.M. of 7 experiments each performed in triplicate: *significantly different from control ($P \leq 0.05$); **significantly different from carbachol alone ($P \leq 0.05$).

Stimulus	Rate of inositol uptake (pmol/min per well)
Control	5.44 $\pm$ 0.49
1.0 mM Carbachol	3.61 $\pm$ 0.15*
1.0 mM Histamine	4.39 $\pm$ 0.23*
0.1 mM Isoprenaline	5.56 $\pm$ 0.34
1.0 mM Carbachol + 1.0 mM histamine	3.16 $\pm$ 0.12**

Figure 3 Concentration- and time-dependence of the inhibition of inositol uptake by carbachol

Cells were incubated in buffer containing [3 H]inositol (5 μ M) in the absence (○) or presence (●) of increasing concentrations of carbachol for 30 min before assay of total intracellular water-soluble 3 H as described in the Materials and methods section. Data are from a single experiment and represent means $\pm$ S.E.M. of 3–6 incubations. Similar data were obtained in several similar experiments (but see the text). Inset: cells were incubated for the times indicated in buffer containing [3 H]inositol (5 μ M) in the absence (○) or presence (●) of 1 mM carbachol before measurement of uptake as above. Data are mean values from triplicate incubations in a single experiment. Similar results were obtained on at least one further occasion.

ments). The inhibition of inositol uptake by carbachol was also dependent on agonist concentration, as shown in Figure 3. The maximal effect of carbachol was apparent at 0.1–1.0 mM, and over a large number of experiments ranged between 20 and 60% attenuation of uptake. The limited effect observed in some

Table 3 Influence of extracellular Ca^{2+} on agonist inhibition of inositol uptake

Cells were incubated for 30 min as described in the Materials and methods section in normal buffer (+ Ca^{2+}), buffer lacking added CaCl_2 (– Ca^{2+}) or buffer without CaCl_2 and containing 0.5 mM EGTA (+ EGTA). Incubations were then continued for a further 30 min in the presence of [3 H]inositol (5 μ M) either without (Control) or with 1 mM carbachol or 1 mM histamine before determination of [3 H]inositol uptake as described previously. Data show the rate of uptake in the presence of agonist as a relative percentage of the control rate measured in buffer of the same composition. The effect of Ca^{2+} removal on the control rate of uptake as a percentage of that in normal buffer is shown in parentheses. Data represent means $\pm$ S.E.M. of 3 experiments each performed in triplicate.

Condition	Inositol uptake (% of control)		
	Control	Carbachol	Histamine
+ Ca^{2+}	100	59.2 $\pm$ 6.7	70.0 $\pm$ 3.3
– Ca^{2+}	100 (86.0 $\pm$ 15.4)	56.5 $\pm$ 3.6	67.6 $\pm$ 6.8
+ EGTA	100 (66.0 $\pm$ 8.0)	60.1 $\pm$ 5.4	68.3 $\pm$ 3.5

experiments precluded precise definition of the EC_{50} for agonist, although half-maximal inhibition by carbachol occurred at ~ 3 –30 μ M. The concentration-dependence of this effect is very similar to the range which carbachol also exhibits for displacement of [3 H]*N*-methylscopolamine binding [38], stimulation of phospholipase C [34] and activation of cyclic nucleotide phosphodiesterase [22] in these cells. These results therefore strongly suggest that inhibition of inositol uptake by carbachol is similarly mediated via muscarinic receptors. In support of this, the muscarinic-receptor antagonist atropine did not itself affect uptake, but fully reversed the action of carbachol (as % of control: 10 μ M atropine, 106.2 $\pm$ 5.7; 1 mM carbachol, 65.6 $\pm$ 6.1; 10 μ M atropine + 1 mM carbachol, 99.4 $\pm$ 3.1; means $\pm$ S.E.M. of 3 experiments). The inset to Figure 3 further reveals that carbachol evokes a persistent inhibition of uptake, with a proportionately similar decrease apparent at 30, 60 and 90 min after agonist addition. This implies that the mechanism underlying this effect desensitizes only slowly, a property which is a notable feature of muscarinic-receptor-mediated phosphoinositide turnover in these cells [34,35], suggesting that one or more $\text{PtdIns}(4,5)\text{P}_2$ -derived signals may be involved. The potential regulation of inositol uptake by Ca^{2+} and protein kinase C was therefore examined as shown in Tables 3 and 4.

Table 3 compares the effects of decreased extracellular Ca^{2+} concentration on inositol uptake into resting cells or those exposed separately to carbachol and histamine. Removal of Ca^{2+} from the medium by omission from the incubation buffer alone had a variable effect on uptake into control cells, whereas addition of EGTA to chelate residual extracellular Ca^{2+} consistently decreased uptake. However, the inhibitory effects of neither carbachol nor histamine were influenced by removal of Ca^{2+} by either approach. This suggests that, although Ca^{2+} may be necessary for optimal uptake, the regulatory influence of receptor stimulation is independent of extracellular Ca^{2+} . These results contrast sharply with those reported in parotid acinar cells [20], where agonist inhibition of inositol uptake was abolished in the absence of Ca^{2+} from the medium.

In Table 4 the effects of raising intracellular Ca^{2+} via the Ca^{2+} ionophore ionomycin, or of activating protein kinase C via the phorbol ester 4 β -phorbol 12 β -myristate 13 α -acetate (PMA), are compared directly with those of carbachol. The data show that ionomycin caused a significant but modest increase in uptake,

Table 4 Comparative effects of carbachol, phorbol ester and Ca^{2+} ionophore on inositol uptake

Cells were incubated for 30 min in buffer containing [^3H]inositol (5 μM) and 1 mM carbachol, 1 μM PMA, 1 μM ionomycin, 1 μM each PMA + ionomycin or appropriate vehicle, buffer alone (control), 0.1% or 0.2% (v/v) dimethyl sulphoxide (DMSO). Reactions were then stopped and total intracellular water-soluble ^3H was measured as described in the Materials and methods section. Data represent means $\pm$ S.E.M. of 6 experiments performed in triplicate: *significantly different from control ($P \leq 0.05$); **significantly different from 0.2% DMSO ($P \leq 0.05$).

Condition	Rate of inositol uptake (pmol/min per well)
Control	5.14 $\pm$ 0.23
1.0 mM Carbachol	3.44 $\pm$ 0.09*
0.1% DMSO control	4.95 $\pm$ 0.21
1.0 μM PMA	3.61 $\pm$ 0.16*
1.0 μM Ionomycin	5.91 $\pm$ 0.23*
0.2% DMSO control	5.66 $\pm$ 0.32
1.0 μM PMA + 1.0 μM Ionomycin	5.14 $\pm$ 0.23**

whereas PMA evoked a very similar inhibition to that observed in response to carbachol. These opposing actions of ionophore and phorbol ester were confirmed by the ability of ionomycin to reverse partly the inhibition by PMA. The effects of both agents are thus compatible with the notion that the mechanism by which receptors modify inositol uptake is largely independent of intra- or extra-cellular Ca^{2+} , but may be mediated through protein kinase C.

DISCUSSION

The present study has established a simple assay in which the unidirectional movement of [^3H]inositol into the cellular free inositol pool allows direct measurement of inositol uptake without complications arising from the incorporation of intracellularly accumulated radiolabel into inositol lipid or phosphate and in which metabolic trapping through other potential routes of inositol utilization is unlikely (see the Introduction and [8]). By using this procedure together with a steady-state [^3H]inositol-labelling approach, 1321N1 astrocytoma cells have been shown to accumulate inositol against a concentration gradient by a mechanism which involves a saturable transporter with a relatively high affinity ($\sim 40 \mu\text{M}$) and capacity ($\sim 180 \text{ pmol/min per mg}$ of protein) for inositol. The concentrative nature of this process, its susceptibility to inhibition by phloretin and dependence on extracellular Na^+ concentration respectively imply further that inositol uptake into these cells occurs by an active carrier-mediated mechanism driven by energy derived from the electrochemical Na^+ gradient. These properties are broadly comparable with those of several other tissues and cells previously shown to accumulate inositol actively [11–13, 16–20]. Two distinguishing features of inositol uptake in 1321N1 cells, however, are its very high concentrative capacity and the absence of a second, low-affinity, component which is apparent and often characterized by its Na^+ -independence in many otherwise similar systems [16–20]. As physiological extracellular inositol concentrations are typically $< 100 \mu\text{M}$ (see [8] and references therein), however, it seems probable that such low-affinity inositol transport may play a proportionately greater role in mediating inositol efflux than import under steady-state conditions in cells which co-express a high-affinity uptake system. Thus it is perhaps significant that 1321N1 cells display little Na^+ -independent uptake and show only slow rates of inositol efflux. Whether or

not these factors are related, their combination is clearly compatible with the capacity of 1321N1 cells to concentrate inositol. Further studies in which 1321N1 cells were cultured in inositol-free medium strongly suggest that uptake rather than synthesis contributes the vast majority of cellular inositol in unstimulated astrocytoma cells (I. H. Batty and C. P. Downes, unpublished work). Thus we can estimate from the current data that, at physiological extracellular concentrations ($\sim 40 \mu\text{M}$), 1321N1 cells achieve intracellular inositol levels of $\sim 20 \text{ mM}$ by importing $> 85\%$ of inositol by the Na^+ -dependent saturable transporter operating at $> 50\%$ capacity.

The heavy dependence of 1321N1 cells on a single quantifiable route of inositol supply suggests that they are a convenient model system in which to explore the relationship between inositol homeostatic mechanisms and the demands placed on these to maintain phosphoinositide synthesis during receptor-stimulated $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis (see below). In this context, the ability of carbachol and histamine acting respectively via muscarinic and, presumably, H_1 -receptors to inhibit inositol uptake is a particularly striking feature of this process in 1321N1 cells. Earlier studies with different cell types have reported modified inositol transport in response to growth factors [39] or agents which induce differentiation [19, 40], but these may reflect adaptive changes, and the regulatory influence of acute receptor stimulation has rarely been examined. However, similar inhibition of inositol uptake exerted by substance P via NK1 receptors and by carbachol via muscarinic receptors was recently reported in rat parotid acinar cells [20, 33]. In both these and astrocytoma cells the maximal inhibitory effect on uptake is of similar magnitude, persists over prolonged exposure to agonist and results from a decrease in the capacity rather than affinity of the Na^+ -dependent transporter. Comparison of the present and these previous results thus suggests strong similarities not only between the inositol transporters in 1321N1 and parotid cells but also in their capacity for regulation. Equally, the similar characteristics of inositol uptake in 1321N1 cells to those of several other systems (see above) suggest that regulation of cellular inositol concentrations in this manner may be a common phenomenon, though it is clearly not universal, since carbachol does not modify active inositol uptake in pancreatic islets [13]. The correlation between the ability of receptors to inhibit inositol uptake and receptors which couple to phospholipase C shown here is necessarily limited, but does appear to extend to parotid acinar cells [20, 33], and implies that effects on uptake may be indirectly mediated by $\text{Ins}(1,4,5)\text{P}_3$ or diacylglycerol. The ability of PMA to decrease uptake by an extent similar to carbachol in 1321N1 cells clearly supports this and suggests that the inhibitory actions of agonists in these cells may be mediated via protein kinase C, whereas the effects of changes in Ca^{2+} concentration suggest that receptor modulation of uptake in these cells is largely Ca^{2+} -independent. This is in direct contrast with the situation in parotid acinar cells, where agonist inhibition of uptake is highly dependent on extracellular Ca^{2+} [20]. This may indicate that distinct mechanisms underlie this response in 1321N1 and parotid cells, but equally may simply reflect the involvement of different forms of protein kinase C with differing Ca^{2+} requirements. In this context it is notable that the inositol transporter cloned from MDCK cells expresses potential phosphorylation sites for both protein kinases A and C within its putative cytosolic domains [30]. This supports the current conclusions, but also emphasizes the need to define better the receptor specificity of the inhibitory effects on inositol uptake. It is also notable that, although muscarinic-receptor stimulation in 1321N1 cells evokes a sustained hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ [34, 35], H_1 -receptor activation of phospholipase C rapidly

(< 5 min) desensitizes [36,37]. This may indicate that, if stimulation of phosphoinositide turnover and inhibition of inositol uptake are causally related, the two events may be separated by some receptor reserve. However, it must be emphasized that, although the present observations strongly imply that agonist inhibition of inositol uptake may be mediated by protein kinase C, a possible direct action of carbachol or histamine on the plasma-membrane Na⁺ gradient cannot be eliminated, although an indirect action at the level of the Na⁺/K⁺-ATPase seems unlikely, given the limited inhibitory influence of ouabain.

Since the present results demonstrate a considerable capacity of 1321N1 cells to import inositol in a receptor-regulated manner, the question of the functional relationship between these processes and stimulated phosphoinositide turnover is raised. Other studies presently in progress in this laboratory suggest that, during maximal muscarinic-receptor stimulation, the rate of PtdIns(4,5)P₂ hydrolysis, measured under conditions equating to those reported here, is ~ 8–20 nmol/h per mg of protein. On the basis of data reported above, it can be estimated that the existing intracellular inositol pool, accumulated predominantly via uptake from the medium, is sufficient to sustain such a rate of PtdIns(4,5)P₂ hydrolysis for a period of several hours even under circumstances where inositol re-cycling or synthesis is impaired, for example by the presence of Li⁺. Furthermore, given access to physiological extracellular inositol during stimulation, continued uptake should supply ~ 10–40% of that inositol required to maintain phospholipase C activity, thereby further off-setting cellular inositol depletion. This illustrates the powerful homeostatic influence of inositol uptake in these cells, but also questions the physiological significance of the inhibition of uptake as a potential regulator of phosphoinositide signalling. It should be pointed out, however, that the high resting cellular inositol concentrations currently estimated in 1321N1 astrocytoma cells are a feature not only of the capacity of these cells to import inositol but also of a relatively slow rate of inositol efflux. In other systems, where the steady state between uptake and efflux results in lower cellular inositol concentrations and/or where the rate of stimulated phosphoinositide turnover is high relative to the net rate of inositol uptake, inhibition of transport may assume considerable significance. Evaluation of these factors will await further studies.

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