Subtype-specific regulation of equilibrative nucleoside transporters by protein kinase CK2

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Two subtypes of equilibrative transporters, *es* (equilibrative inhibitor-sensitive) and *ei* (equilibrative inhibitor-insensitive), are responsible for the majority of nucleoside flux across mammalian cell membranes. Sequence analyses of the representative genes, *ENT1* {equilibrative nucleoside transporter 1; also known as *SLC29A1* [solute carrier family 29 (nucleoside transporters), member 1]} and *ENT2* (*SLC29A2*), suggest that protein kinase CK2-mediated phosphorylation may be involved in the regulation of *es*- and *ei*-mediated nucleoside transport. We used human osteosarcoma cells transfected with catalytically active or inactive α' and α subunits of CK2 to assess the effects of CK2 manipulation on nucleoside transport activity. Expression of inactive CK2 α' (decreased CK2 α' activity) increased the number of binding sites (~ 1.5 -fold) for the *es*-specific probe [³H]NBMPR ([³H]nitrobenzylthioinosine), and increased (~ 1.8 -fold) the $V_{\max}$ for 2-chloro[³H]adenosine of the NBMPR-sensitive (*es*) nucleoside transporter. There was a concomitant decrease in the $V_{\max}$

of the NBMPR-resistant (*ei*-mediated) uptake of 2-chloro[³H]adenosine. This inhibition of CK2 α' activity had no effect, however, on either the K_D of [³H]NBMPR binding or the K_m of 2-chloro[³H]adenosine uptake. Quantitative PCR showed a transient decrease in the expression of both hENT1 (human ENT1) and hENT2 mRNAs within 4–12 h of induction of the inactive CK2 α' subunit, but both transcripts had returned to control levels by 24 h. These data suggest that inhibition of CK2 α' reduced *ei* activity by attenuation of hENT2 transcription, while the increase in *es*/hENT1 activity was mediated by post-translational action of CK2. The observed modification in *es* activity was probably due to a CK2 α' -mediated change in the phosphorylation state of the ENT1 protein, or an interacting protein, effecting an increase in the plasma membrane lifetime of the transport proteins.

Key words: adenosine, 2-chloroadenosine, nitrobenzylthioinosine, phosphorylation, regulation, transporter.

INTRODUCTION

Specific transport proteins are required for the transfer of hydrophilic nucleosides across cell membranes. These transporters are critical for the scavenging of nucleosides by cells lacking *de novo* purine biosynthesis, and for regulation of adenosine concentrations in the vicinity of extracellular purinergic receptors [1,2]. The cellular uptake of several chemotherapeutic drugs, such as cytosine arabinoside and gemcitabine, is also dependent on nucleoside transporters [3,4].

Both Na⁺-independent equilibrative (SLC29) and Na⁺-dependent concentrative (SLC28) nucleoside transport systems exist [5,6]. The equilibrative transporters are expressed ubiquitously, and two subtypes can be distinguished by their differential sensitivities to inhibition by NBMPR (nitrobenzylthioinosine; nitrobenzylmercaptapurine riboside). The *es* (equilibrative inhibitor-sensitive) type of transport activity is inhibited by NBMPR in the nanomolar range, while inhibition of the *ei* (equilibrative inhibitor-insensitive) subtype requires micromolar concentrations of NBMPR [1]. Genes encoding proteins with *es* and *ei* characteristics have been designated *ENT1* {equilibrative nucleoside transporter 1; also known as *SLC29A1* [solute carrier family 29 (nucleoside transporters), member 1]} and *ENT2* (*SLC29A2*) respectively. Both transporters have 11 transmembrane domains, with a large intracellular loop joining transmembrane domains 6 and 7 which contains sites for phosphorylation by protein kinase

CK2 ('casein kinase II') [7,8]. We [9], and others [10], have identified mouse ENT1 splice variants that differ in having either one or two consensus sites for phosphorylation by CK2. Preliminary data suggest that the variant with only one CK2 site has reduced transport functionality relative to the variant with both CK2 consensus sites (M. Stolk and J. R. Hammond, unpublished work). Only a single ENT1 isoform, containing both of the CK2 sites, has been identified, to date, in human cells [8].

Protein kinase CK2 is a serine/threonine protein kinase that is found in nearly all compartments of mammalian cells [11], and exists as a heterotetramer composed of two catalytic (α) subunits and two regulatory (β) subunits [12]. It appears to play an important role in the control of cell growth and proliferation [12,13], with increased CK2 activity being associated with rapidly growing cells. Nucleoside transporter expression by mammalian cells has also been directly correlated with growth rate [3,11,14–16], and CK2-mediated phosphorylation may be a factor in this regulation.

Little is known about how nucleoside transporters are regulated. It has been shown that activators and inhibitors of protein kinase C and/or protein kinase A modulate the activity of equilibrative nucleoside transporters in a variety of cell types [17–20]. Changes also occur in nucleoside uptake rates and transporter subtype expression upon cell differentiation [19,21,22]. However, the mechanisms underlying these changes have not been elucidated, and no work has been conducted on the role of CK2 in this regard.

Abbreviations used: BGS, bovine growth serum; *ei*, equilibrative inhibitor-insensitive; ENT, equilibrative nucleoside transporter; hENT, human ENT; *es*, equilibrative inhibitor-sensitive; HA, haemagglutinin A; MAP kinase, mitogen-activated protein kinase; NBMPR, nitrobenzylmercaptapurine riboside (nitrobenzylthioinosine); NBTGR, nitrobenzylthioguanosine; RT-PCR, reverse transcription-PCR; STAT, signal transduction and activators of transcription; TET, tetracycline; T_m , melting temperature.

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From a clinical perspective, understanding how cells regulate nucleoside transport activity/expression would aid in the development of protocols for reducing the toxicity to the host of nucleoside analogues used in cancer and antiviral therapies [23]. Moreover, coronary vasodilators such as dipyridamole and drafazine act by blocking the re-entry of adenosine into the cell via nucleoside transporters, thereby enhancing the cardioprotective actions of adenosine released under ischaemic conditions [2,24]. Understanding how cells regulate the number of functioning nucleoside transporters would aid in the more efficacious application of these therapies.

The present study was undertaken to determine whether manipulation of CK2 catalytic activity in human osteosarcoma cells would affect the capacity of these cells to accumulate nucleosides via equilibrative nucleoside transporters. In the absence of well defined pharmacological modifiers of CK2 activity [12,25], we employed a model consisting of human osteosarcoma cells stably transfected with active or inactive α and α' CK2 catalytic subunits under the control of a TET (tetracycline)-regulated promoter [26]. The number of *es* transporters was assessed using the *es*-specific probe [^3H]NBMPR, and transporter function was determined using both [^3H]formycin B and 2-chloro[^3H]adenosine as substrates.

Preliminary reports of these findings were presented at the XIVth World Congress of Pharmacology [27] and the 4th International Congress on Protein Kinase CK2 (London, Ontario, Canada; 29 July–1 August 2004).

EXPERIMENTAL

Materials

[G- ^3H]NBMPR (5.5–20.1 Ci/mmol), [G- ^3H]formycin B (14 Ci/mmol) and 2-chloro[8- ^3H]adenosine (9.3 Ci/mmol) were purchased from Moravex Biochemicals (Brea, CA, U.S.A.). $^3\text{H}_2\text{O}$ (1 mCi/g) was purchased from DuPont Canada Inc. (Markham, Ontario, Canada). Dulbecco's modified Eagle's medium and culture grade PBS were from GIBCO/BRL (Burlington, Ontario, Canada). BGS (bovine growth serum) was from VWR International (Mississauga, Ontario, Canada). Penicillin G and streptomycin sulphate were from ICN (Montreal, Quebec, Canada). Non-radiolabelled formycin B, NBMPR, 2-chloroadenosine, NBTGR (nitrobenzylthioguanosine), trypsin/EDTA, TET and dipyridamole {2,6-bis-(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]-pyrimidine} were supplied by Sigma (St. Louis, MO, U.S.A.). Dilazep {*N,N'*-bis-[3-(3,4,5-trimethoxybenzoyloxy)propyl]-homopiperazine} was a gift from Asta Werke (Frankfurt, Germany). Drafazine {2-(aminocarbonyl)-*N*-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis(4-fluorophenyl)-pentyl]-1-piperazineacetamide 2HCl} and solufazine {3-(aminocarbonyl)-4-[4,4-(4-fluorophenyl-3-pyridinyl)butyl]-*N*-(2,6-dichlorophenyl)-1-piperazineacetamide 2HCl} were obtained from Janssen Research Foundation (Beerse, Belgium). Polyclonal antibodies to CK2 α or CK2 α' have been described previously [26]. Goat anti-rabbit secondary antibody conjugated with horseradish peroxidase was purchased from Bio-Rad (Hercules, CA, U.S.A.), and silicone oil (550 Fluid) was from Dow-Corning Inc. (Baltimore, MD, U.S.A.).

Cell lines

U2-OS/UTA6 cells stably transfected with an inducible bi-directional plasmid expressing the CK2 β regulatory subunit coordinately with a HA (haemagglutinin A)-tagged version of the inactive α' (GV7.21), active α' (RS2.31) or inactive α (GV13.35)

catalytic subunit of CK2 were generated as described previously [26]. Cells were cultured in T175 flasks in Dulbecco's modified Eagle's medium containing 10% (v/v) BGS plus penicillin G (100 units/ml) and streptomycin sulphate (100 $\mu\text{g}/\text{ml}$) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were split into parallel cultures and grown in the presence (TET+) or absence (TET-) of 1.5 $\mu\text{g}/\text{ml}$ TET for the times specified in the Results section prior to harvesting for the assays described below. Induced expression of the HA-tagged CK2 catalytic subunits was confirmed in the TET- cells by immunoblotting with the appropriate CK2 α subunit-specific antibody [26], and by RT-PCR (reverse transcription-PCR) using primers specific for the HA-tagged CK2 transcript (see Figure 1). For radioligand binding or substrate flux assays, cells at passages 4–30 were removed from the flasks by trypsinization (0.05%, v/v; 5 min; 37°C), then diluted with medium containing 10% (v/v) BGS and pelleted by centrifugation. Cell pellets were washed once by resuspension/centrifugation in either Dulbecco's PBS (137 mM NaCl, 6.3 mM Na_2HPO_4 , 2.7 mM KCl, 1.5 mM KH_2PO_4 , 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.9 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, pH 7.4) or a modified Na^+ -free PBS (NMG^+Cl^- and K_2HPO_4 replacing NaCl and Na_2HPO_4 respectively, where NMG is *N*-methyl-D-glucamine), and then suspended in the same buffer for use in the assays. Cell concentrations were determined using a haemocytometer.

[^3H]NBMPR binding

Cells ($\sim 10^5$ cells/ml) suspended in Dulbecco's PBS were incubated with [^3H]NBMPR (in the presence or absence of inhibitors) for 45–60 min at room temperature ($\sim 22^\circ\text{C}$) to attain steady-state binding. Cells were then collected on Whatman GF/B filters using a 24-port Brandel cell harvester, washed twice with Tris buffer (10 mM Tris, pH 7.4, 4°C), and analysed for ^3H content using standard liquid-scintillation counting techniques. Non-specific binding of [^3H]NBMPR, defined as that which remained cell-associated in the presence of 10 μM NBTGR, was subtracted from total binding to obtain a measure of the site-specific binding of [^3H]NBMPR to the *es* transporter. K_D and B_{max} values were determined from non-linear (hyperbolic) curves fitted to plots of the specific binding of [^3H]NBMPR against the free [^3H]NBMPR concentration at steady state. IC_{50} values for dipyridamole, dilazep, drafazine and solufazine were calculated from variable slope sigmoid curves fitted to the specific binding of [^3H]NBMPR (0.5 nM) relative to control, against the log of the inhibitor concentration.

[^3H]Nucleoside uptake

Assays were conducted at room temperature ($\sim 22^\circ\text{C}$) in Na^+ -free buffer to ensure that uptake of [^3H]substrate was due solely to the operation of the Na^+ -independent equilibrative transporters (*es* or *ei*). In some cases, cells were depleted of ATP to reduce intracellular metabolism ('trapping') of the [^3H]nucleoside substrate as its nucleotide derivatives, by sequential incubation with rotenone (20 ng/ml; 15 min at 37°C) and 2-deoxyglucose (2 mM; 10 min at 37°C) [28]. Uptake was initiated by addition of cell suspension ($\sim 1 \times 10^6$ cells) to [^3H]substrate (0.5 ml final volume) layered over a 200 μl cushion of silicone/mineral oil (21:4, v/v) in 1.5 ml microcentrifuge tubes. Assays were terminated after defined incubation times by centrifugation (12 000 *g*) of cells through the oil. The estimated time required to pellet the cells through the oil layer (2 s) was included in all reported incubation times. The supernatant and oil were removed, and the cell pellets were digested in 250 μl of 1 M NaOH overnight (~ 16 h) at room temperature. The digest was analysed for ^3H content by standard liquid-scintillation counting techniques. Uptake

Table 1 PCR primers used in the present study

Gene		Primer sequence (5'–3')	Expected size (bp)
ENT1	Forward	TCA GCC CAC CAA TGA AA	215
	Reverse	GGC CCA ACC AGT CAA AGA TA	
ENT2	Forward	GCT CTT TGC CGT TTC TAA TGG	172
	Reverse	TCA GAG CAG CGC CTT GA	
β -Actin	Forward	CCT CTA TGC CAA CAC AGT GC	235
	Reverse	CAT CGT ACT CCT GCT TGC TG	
HA-CK2 α'	Forward	CCA TAC GAT GTT CCT GAC TAT GC	282
	Reverse	TGG TGA TAT TAA TGG CCT CAA A	

assays were conducted in the presence or absence of either 50 nM NBMPR (to inhibit *es*-mediated uptake) or 14 μ M NBMPR/dipyridamole (to inhibit both *es*- and *ei*-mediated uptake). Data are presented as intracellular [3 H]substrate concentrations (pmol/ μ l of intracellular volume; μ M) after correction for the amount of 3 H label present in the extracellular space of the cell pellet.

Total water volumes of the cell pellets were determined in each experiment by incubating cells with 3 H $_2$ O for 3 min and then processing the samples as described above. An estimate of the extracellular water space was obtained by extrapolation of the linear time course of non-mediated uptake of [3 H]substrate (in presence of 14 μ M NBMPR/dipyridamole) back to zero time. In both cases, it was assumed that the concentration of 3 H in the total (3 H $_2$ O) or extracellular ([3 H]substrate + dipyridamole) water spaces of the cell pellet was equivalent to that in the supernatant remaining above the oil layer (based on d.p.m./ μ l of standards). The intracellular water volume was calculated as the difference between the total and extracellular water space estimates.

To assess the relative contributions of *es* and *ei* to the [3 H]nucleoside uptake observed, cells were incubated with a range (0.01 nM–1 μ M) of NBMPR concentrations for at least 15 min, and then analysed for uptake of 10 μ M [3 H]nucleoside as described above. IC₅₀ values for NBMPR were calculated from two-component sigmoid curves fitted to the uptake of [3 H]nucleoside (pmol/10⁶ cells) against the log of the NBMPR concentration.

Real-time PCR

To determine whether induction of the inactive α' subunit of CK2 in GV7.21 cells led to a change in *ENT1* or *ENT2* gene expression, quantitative real-time PCR amplification of hENT1 (human ENT1), hENT2 and human β -actin was performed. Total RNA was isolated from GV7.21 cells (in the presence or absence of TET) using the GenEluteTM Total RNA mini-prep kit (Sigma) and then treated with DNase. A first-strand DNA template was generated using the SuperscriptTM First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, U.S.A.). The removal of genomic DNA from the preparations was confirmed by conducting traditional RT-PCR for hENT1 (see Table 1 for primers used) in the absence of reverse transcriptase. PCR primers were designed to achieve product sizes of 215 bp for hENT1, 172 bp for hENT2 and 235 bp for β -actin (see Table 1). A Roche LightCycler was used to perform real-time PCR in 25 μ l microcapillary tubes (Roche Diagnostics Canada, Laval, PQ, Canada) using SYBR GreenTaq ReadyMixTM (Sigma) with 2 μ l of cDNA template per reaction. This system allows amplification and detection of products in a single reaction tube. Reaction conditions included an initial activation step of 30 s at 95 °C, followed by 40 cycles of ramping at 20 °C/s to hold for 0 s at 95 °C, ramping at 20 °C/s to hold for 5 s at 54 °C (for ENT1/ENT2) or 58 °C (for β -actin), ramping at 5 °C/s to hold for 13 s at 72 °C, and ramping at 20 °C/s

to hold for 1 s at 80 °C (ENT1) or 83 °C (ENT2/ β -actin), with a single acquisition of data before the start of each cycle. Data were analysed with LightCycler Software version 3 (Idaho Tech, Salt Lake City, UT, U.S.A.). To optimize the amplification of ENT1 and ENT2, melting curve analysis was used to determine the T_m (melting temperature) of specific products and primer dimers. The following conditions were used: ramping at 20 °C/s to hold for 0 s at 95 °C, ramping at 20 °C/s to hold for 10 s at 65 °C, and continuous acquisition of data while ramping at 0.1 °C/s until reaching 95 °C. An additional signal acquisition step, 2–3 °C below the T_m , was added after the elongation phase of PCR to ensure signal acquisition from specific target products.

Standard curves for amplification of hENT1, hENT2 and human β -actin were constructed using different amounts of cDNA template derived from GV7.21 cells. The levels of ENT1 and ENT2 transcripts were then assessed using duplicate sets of cDNAs derived from three distinct lots of RNA isolated from GV7.21 cells grown in the presence or absence of TET for the times indicated in the Results section. The cycle number at which the fluorescence signal was significantly higher than baseline (the crossing point; Cp) was used to calculate the relative concentrations of ENT1, ENT2 and β -actin from their standard curves. These data were then normalized to β -actin to account for differences in reverse transcription efficiencies and the amount of template in each reaction mixture. The products from the reactions described above were also run on a 1% (w/v) agarose gel, along with a DNA ladder (GeneRulerTM DNA ladder mix; Fermentas, Burlington, ON, Canada), to confirm that all products were of the correct length for the primers used (Table 1).

Data analysis

Experiments were conducted at least four times in duplicate, and results are expressed as means $\pm$ S.E.M. Non-linear regression analyses were performed using GraphPad Prism 4. Statistical differences between data sets were defined using Student's *t* test for paired or unpaired data, as appropriate, with *P* < 0.05 considered significant (Graphpad Instat 3.05).

RESULTS

It has been shown previously that removal of TET from GV7.21 cell cultures (induction of inactive CK2 α') resulted in significant changes in cell proliferation, presumably through inhibition of endogenous CK2-mediated phosphorylation activity [26]. Thus the degree of cell confluence was controlled in the present study, such that cells cultured in either the presence or the absence of TET were harvested from flasks at 60–80% confluence. The expression of the recombinant HA-tagged catalytic CK2 subunit RNA was confirmed by RT-PCR (Figure 1A), and Western blots confirmed the induction of the catalytically active or inactive CK2 α' protein when cells were grown in the absence of TET (Figure 1B). HA-tagged CK2 α' transcript was observed by RT-PCR within 2 h of removal of TET from the cell medium. A very small amount of HA-tagged transcript was consistently observed in GV7.21 cells grown in the presence of TET (Figure 1A), suggesting minor 'leakage' of the TET-OFF transcriptional control system. However, there was no detectable HA-tagged CK2 α' protein expressed under TET+ conditions (Figure 1B).

[3 H]NBMPR binding

Binding of the high-affinity *es* transporter probe [3 H]NBMPR [29,30] was saturable, with non-specific binding comprising less than 15% of total binding at the highest concentration used (Figure 2A). GV7.21 and RS2.31 cells grown in the presence of TET

Figure 1 Induced expression of HA-tagged CK2 α' subunits in GV7.21 and RS2.31 cells

(A) Polyadenylated RNA was prepared, in parallel, from similar numbers of U2-OS/UTAG6 (negative control) and GV7.21 cells grown in the presence (+) or absence (–) of TET for 48 h. RT-PCR was performed using a sense primer specific for the HA tag and an antisense primer to the CK2 α' sequence (see Table 1). The results shown are representative of those derived from three independent GV7.21 $\pm$ TET pairs. The level of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression, determined for each template preparation, is shown for comparison. Samples were resolved on 1.2% (w/v) agarose gels with DNA size markers shown in the right lane. The HA-CK2 PCR product was of the expected size (282 bp). (B) Whole-cell extracts containing equal amounts of protein were prepared from RS2.31 and GV7.21 cells ($\pm$ TET) and analysed by immunoblotting with antibodies to CK2 α' as described previously [26].

Figure 2 Equilibrium binding of [3 H]NBMPR to GV7.21 cells

(A) Cells were grown in the absence (open symbols) or presence (solid symbols) of TET for 48 h, and then exposed to a range of concentrations of [3 H]NBMPR in the absence (total binding) or presence (non-specific binding) of 10 μ M NBTGR. Specific binding was calculated as the difference between total and non-specific binding. Each point is the mean $\pm$ S.E.M. of 14 experiments performed in duplicate. (B) GV7.21 cells were grown in the absence of TET for 1–48 h, and then compared with control (TET+) cells for the number of [3 H]NBMPR binding sites. Data are plotted as the percentage change in $B_{\max}$, relative to control, upon induction of the inactive CK2 α' . Each bar represents the mean $\pm$ S.E.M. of four experiments conducted in duplicate. * $P < 0.05$ for significant difference between TET+ and TET– conditions (Student's t test).

had approx. 90 000 NBMPR binding sites per cell, with a K_D of 0.13 nM (Table 2). The GV13.35 cells had a higher number of binding sites ($121\,000 \pm 19\,000$ sites/cell), but a lower binding affinity ($K_D = 0.25$ nM), than the other two cell lines under TET+ conditions. Induction of the expression of the recombinant α or α' CK2 subunits had no effect on the K_D of [3 H]NBMPR (Figure 3, Table 2). However, decreasing CK2-mediated phosphorylation by the induction of the kinase-dead α' subunit in GV7.21 cells led

to a significant increase in the maximum number of [3 H]NBMPR binding sites ($B_{\max}$) from $92\,000 \pm 9000$ to $125\,000 \pm 11\,000$ sites/cell, representing an increase of $49 \pm 17\%$ (calculated from paired experiments; $\pm$ TET for 48 h). This increase in [3 H]-NBMPR binding was observed within 8 h of removing TET from the cells, with a maximum increase seen at 16 h (Figure 2B). Interestingly, relative to the TET+ condition, there was a slight, but statistically significant, decrease ($10 \pm 6\%$) in [3 H]NBMPR $B_{\max}$ prior (2–4 h after TET washout) to the longer-term increase (Figure 2B). In contrast with that seen in the GV7.21 cells, there was no significant change in [3 H]NBMPR $B_{\max}$ upon expression of active CK2 α' (RS2.31 cells) or inactive CK2 α (GV13.35 cells) (Figure 3, Table 2). Hill coefficients for [3 H]NBMPR binding to each of the three cell lines ($\pm$ TET) were consistently between 0.9 and 1.0 (results not shown).

Since this was the first study to describe the interaction of ligands with the *es* transporter of human osteosarcoma cells, we confirmed that this system possessed the requisite pharmacological properties. Experimentally derived IC_{50} values (results not shown) were used along with the K_D of [3 H]NBMPR binding (Table 2) to calculate the K_i for each inhibitor using the Cheng–Prusoff relationship [31]. The most effective inhibitor tested was dilazep ($K_i = 0.17 \pm 0.04$ nM), followed by drafazine (0.47 ± 0.10 nM), dipyrindamole (1.6 ± 0.4 nM), and solufazine (2.2 ± 0.3 nM). With the exception of dipyrindamole ($n_H = 0.61 \pm 0.13$), all of the inhibitors had pseudo Hill coefficients not different from unity. Similar results were obtained for these inhibitors with and without induction of the inactive CK2 catalytic subunits in both the GV7.21 (inactive α') and GV13.35 (inactive α) cell lines.

2-Chloro[3 H]adenosine uptake

Time-course profiles (Figure 4) showed that dipyrindamole-sensitive uptake of 2-chloro[3 H]adenosine (10 μ M) by ATP-replete cells occurred with an initial rate (V_i) of 2.6 ± 0.4 pmol/s per μ l, to a maximum concentration of 9.5 ± 0.6 pmol/ μ l of intracellular water (μ M). This steady-state concentration was not significantly different from the initial medium concentration of 2-chloro[3 H]adenosine. Under ATP-depleted conditions, the initial rate of influx (3.1 ± 0.9 pmol/s per μ l) and the maximum achievable intracellular concentration (9.0 ± 0.5 pmol/ μ l) of 10 μ M 2-chloro[3 H]adenosine were similar to those seen in the ATP-replete cells. Hence all subsequent studies were done using ATP-replete cells. Incubation of cells with 50 nM NBMPR (Figure 4), to block uptake via the *es* transporter, resulted in a 4-fold reduction in the rate of transporter-mediated (dipyrindamole-sensitive) uptake of 2-chloro[3 H]adenosine ($V_i = 0.6 \pm 0.1$ pmol/s per μ l at 10 μ M).

The uptake of a range of concentrations of 2-chloro[3 H]adenosine was then assessed using a 5 s incubation time (minimum attainable) in GV7.21 cells ($\pm$ TET; Figure 5). Parallel assays were conducted in the absence and presence of 50 nM NBMPR or 14 μ M dipyrindamole/NBMPR to allow the calculation of total, *es*- and *ei*-mediated uptake of 2-chloro[3 H]adenosine. Induction of the inactive CK2 α' subunit resulted in no significant change in the K_m of 2-chloro[3 H]adenosine for the *es* transporter (~ 25 μ M), but did increase the $V_{\max}$ of uptake by 80%, from 6.7 pmol/s per μ l in control cells to 12.1 pmol/s per μ l in the cells expressing catalytically inactive CK2 α' (Table 3). In parallel, the maximum rate of uptake of 2-chloro[3 H]adenosine by the *ei* system was reduced by 28% upon inhibition of CK2 activity; there was also a significant increase in the affinity of 2-chloro[3 H]adenosine for the *ei* transporter upon induction of the inactive CK2 α' (Table 3, Figure 5). These changes resulted in a shift in the *es/ei* uptake ratio

Table 2 [^3H]NBMPR binding by GV7.21, RS2.31 and GV13.35 cells with and without induction of inactive and active CK2 catalytic subunits

Binding of [^3H]NBMPR was assessed as shown in Figure 2(A). K_D (nM) and $B_{\max}$ (10^3 sites/cell) were determined from hyperbolic curves fitted to the specific binding data of each experiment. Values are the means $\pm$ S.E.M. of the number of paired ($\pm$ TET) experiments shown in parentheses. Significance of differences: * $P < 0.05$ compared with [^3H]NBMPR binding to cells grown in the presence of TET (Student's t test for paired samples); † $P < 0.05$, significantly different from 1 (Student's t test).

	GV7.21 (14)		RS2.31 (11)		GV13.35 (7)	
	K_D	$B_{\max}$	K_D	$B_{\max}$	K_D	$B_{\max}$
TET+	0.13 ± 0.01	92 ± 9	0.13 ± 0.01	83 ± 6	0.25 ± 0.03	121 ± 19
TET-‡	0.15 ± 0.02	$125 \pm 11^*$	0.13 ± 0.02	74 ± 7	0.25 ± 0.02	125 ± 13
TET-/TET+	1.1 ± 0.1	$1.5 \pm 0.2^\dagger$	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.1 ± 0.1

‡ Induction of catalytically inactive CK2 α' (GV7.21), inactive CK2 (GV13.35) or active CK2 α' (RS2.31) upon removal of TET.

Figure 3 Mass law analysis of the specific binding of [^3H]NBMPR to GV7.21, RS2.31 and GV13.35 cells

Cells were grown in either the presence (■) or the absence (□) of TET to induce expression of the respective inactive and active CK2 catalytic subunits as shown in each panel. Specific binding of [^3H]NBMPR was assessed as described in the legend to Figure 2. Data are represented as Scatchard plots, where the site-specific binding of [^3H]NBMPR ('Bound'; abscissa) is plotted against the ratio of specific binding to equilibrium free concentration of [^3H]NBMPR ('Bound/Free'; ordinate). All data fit best to a linear function, and each point is the mean of at least eight experiments performed in duplicate.

from approx. 3:2 in control cells to almost 4:1 upon inhibition of CK2 activity.

[^3H]Formycin B uptake

To confirm that the data obtained using 2-chloro[^3H]adenosine were not peculiar to the substrate used, we assessed the ability of NBMPR to inhibit the uptake of $10 \mu\text{M}$ [^3H]formycin B by both GV7.21 (Figure 6A) and RS2.31 (Figure 6B) cells grown in the presence or absence of TET for 48 h. Formycin B is well established as a poorly metabolized substrate for both the *es* and

Figure 4 2-Chloro[^3H]adenosine uptake by GV7.21 cells

Cells cultured in the presence of TET to suppress expression of recombinant CK2 α' were incubated with $10 \mu\text{M}$ 2-chloro[^3H]adenosine in the absence (Total) or presence of 50 nM NBMPR (+NBMPR; selective inhibition of *es*-mediated uptake) or $14 \mu\text{M}$ dipyrindamole/NBMPR (+DY; complete inhibition of transporter-mediated uptake) for the times indicated. Uptake is represented as pmol of 2-chloro[^3H]adenosine accumulated per μl of intracellular water (μM). Each point is the mean $\pm$ S.E.M. from five experiments.

ei transporter subtypes [32]. NBMPR inhibition of [^3H]formycin B uptake in both GV7.21 and RS2.31 cells was biphasic, with approx. 50% of the uptake being sensitive to sub-nanomolar concentrations of NBMPR, and the remainder inhibited by NBMPR only at concentrations in excess of 300 nM . Induction of inactive CK2 α' in GV7.21 cells resulted in a 2-fold increase in *es*-mediated (NBMPR-sensitive) uptake, from $5.6 \pm 0.4 \text{ pmol}/10^6$ cells under baseline conditions to $11 \pm 2 \text{ pmol}/10^6$ cells upon inhibition of CK2 α' activity. In contrast, induction of the active α' subunit in RS2.31 cells had no effect on the relative amount of *es* transport activity.

Analysis of ENT1 and ENT2 expression by real-time PCR

The amplification plots (fluorescence against cycle number) derived for hENT1, hENT2 and β -actin are shown in Figure 7(A). The cycle number at which the fluorescence signal was significantly different from baseline (the crossing point; C_p) was used to compute the relative concentrations of the target gene products from their standard curves. Melting curves for all products had a single peak, indicating a high degree of product specificity. The lowest T_m was for hENT1 at 83°C , followed by hENT2 at 87°C and human β -actin at 88°C (Figure 7B). The PCR products obtained using these primers were all of the expected size (Table 1, Figure 7C). When normalized to β -actin, there was ~ 3 -fold more hENT1 than hENT2 mRNA present in these cells.

Figure 5 Concentration dependence of 2-chloro[³H]adenosine uptake by the *es* and *ei* transporters of GV7.21 cells

GV7.21 cells, grown in either the presence (Control; **A**) or the absence (↓CK2α' activity; **B**) of TET, were incubated for 5 s with the indicated concentrations of 2-chloro[³H]adenosine. Parallel assays were conducted in the absence (total influx) and presence of either 50 nM NBMPR (NBMPR-resistant influx) or 14 μM dipyrindamole/NBMPR (non-mediated uptake). The total transporter-mediated uptake of substrate was calculated as the total influx minus the non-mediated component. The *ei*-mediated uptake was calculated as NBMPR-resistant uptake minus the non-mediated component, and the *es*-mediated uptake was estimated as the difference between the total uptake and that seen in the presence of NBMPR. Results are plotted as pmol of 2-chloro[³H]adenosine accumulated/s per μl of cell water (*V_i*; ordinate) against the concentration of 2-chloro[³H]adenosine used (abscissa). Each point is the mean ± S.E.M. from five experiments. The transporter kinetic constants derived from these data are shown in Table 3.

Table 3 Effects of inhibiting CK2α' activity on the uptake of 2-chloro[³H]adenosine by GV7.21 cells

Cells were grown in the presence or absence of TET, as described in the text, and assessed for their capacity to accumulate 2-chloro[³H]adenosine in the presence and absence of 50 nM NBMPR or 14 μM dipyrindamole/NBMPR, as shown in Figure 4. The substrate affinities (*K_m*) and maximum uptake rates (*V_{max}*) were derived from hyperbolic curves fitted to data plotted as shown in Figure 5. The relative amount of *ei*-mediated uptake was also calculated for each experiment, and is given in parentheses. Each value represents the mean ± S.E.M. of five independent experiments. Significance of differences: **P* < 0.05 compared with cells grown in the presence of TET (Student's *t* test)

Uptake	<i>K_m</i> (μM)		<i>V_{max}</i> (pmol/s per μl)	
	TET+	TET–	TET+	TET–
Total	22 ± 4	24 ± 3	11.0 ± 0.5	13.9 ± 0.8*
<i>es</i> -mediated	27 ± 9	37 ± 5	6.7 ± 0.3	12.1 ± 0.9*
<i>ei</i> -mediated	29 ± 3	16 ± 5*	4.3 ± 0.3 (40 ± 3 %)	3.1 ± 0.4* (20 ± 2 %*)

There was a significant decrease (*P* < 0.05, paired *t* test, *n* = 6) in both *hENT1* and *hENT2* mRNAs within 4–12 h after induction of the catalytically dead CK2α', relative to the TET+ controls, but both transcripts had returned to control (TET+) levels by 16 h (Figure 8).

Figure 6 NBMPR inhibition of transporter-mediated [³H]formycin B uptake

GV7.21 (**A**) and RS2.31 (**B**) cells were cultured for 48 h in the presence or absence of TET to induce the expression of inactive and active CK2α' respectively. The uptake of 10 μM [³H]formycin B (17 s incubation) was assessed in the presence of the indicated concentrations of NBMPR. Non-mediated uptake, determined as described for Figure 4, was subtracted from all values measured. Each point is the mean ± S.E.M. of at least five paired (± TET) experiments conducted in duplicate. The *IC₅₀* values, derived from these data, for NBMPR inhibition of *es*- and *ei*-mediated uptake are shown as insets. **P* < 0.05 for significant difference from [³H]formycin B uptake by cells grown in the presence of TET (Student's *t* test for paired samples).

DISCUSSION

The osteosarcoma cell variants used in the present study exhibited both NBMPR-sensitive (*es*) and NBMPR-insensitive (*ei*) equilibrative nucleoside transport activity. These uptake activities were probably mediated by transporters encoded by the genes *hENT1* and *hENT2* respectively (see Figure 7). Using the binding of [³H]NBMPR as a stoichiometric indicator of *es* transporters expressed at the plasma membrane [30], it was apparent that these cells have a high density of *es* nucleoside transporters (~100 000 per cell), typical of rapidly proliferating cells [3,15,33]. Hill coefficients for [³H]NBMPR binding were near unity, and Scatchard transformations of [³H]NBMPR binding data were linear (Figure 3), indicating non-co-operative, one-site binding similar to that seen for this radioligand in many other systems. Furthermore, the affinity of the NBMPR binding site for a series of recognized nucleoside transport inhibitors (diazepam > drafazine > dipyrindamole > solufazine) was consistent with that found in previous studies on the *es* transporter in human cell lines [6,29,34].

Induction of the catalytically inactive CK2α' subunit in GV7.21 cells led to a 1.5-fold increase in the number of [³H]NBMPR binding sites. In contrast, no change in binding was observed upon induction of the inactive CK2α subunit in GV13.35 cells (Table 2). These data infer that the α and α' subunits of CK2 are differentially involved in the phosphorylation pathways that impact on the

Figure 7 Quantification by real-time PCR of hENT1 and hENT2 mRNAs in GV7.21 cells

Polyadenylated RNA was isolated from GV7.21 cells grown in the presence (solid symbols) or absence (open symbols) of TET for 48 h. (A) Representative PCR amplification plots for hENT1, hENT2 and β -actin were generated as described in the text. Crossing point (Cp) values, obtained from SYBR Green I fluorescence signals, were used to compute relative concentrations of β -actin, hENT1 and hENT2 from their respective standard curves. (B) PCR products were subjected to melting-curve analyses to determine the specificity of the products. All samples showed a single product, with T_m values of 83°C, 87°C and 88°C for hENT1, hENT2 and β -actin respectively. (C) Products obtained from the real-time PCR amplifications were resolved on a 1.2% (w/v) agarose gel, along with a DNA ladder (left lane; bp). The products obtained were of the expected size (hENT1, 215 bp; hENT2, 172 bp).

activity of *es* nucleoside transporters. Few functional differences have been found between the CK2 catalytic subunit isoforms. However, other studies involving the disruption of α'/α subunit activities suggest that CK2 α' function is most important for maintaining optimal cell proliferation [35].

Given that the number of *es* transporters (NBMPR binding sites) increased in response to a decrease in CK2 α' -mediated activity, it was expected that increasing CK2 α' activity (i.e. in RS2.31 cells) would produce a decrease in [3 H]NBMPR binding. However, this was not observed (Table 2), probably because the RS2.31 cells already had sufficient CK2 activity for cellular regulation, such that inducing the expression of more CK2 α' was inconsequential. Nor was there any difference in either *es*- or *ei*-mediated uptake of [3 H]formycin B by RS2.31 cells upon induction of active CK2 α' expression (Figure 6B). It has been reported that cancer cell lines often have high levels of CK2 activity due to the important role of this enzyme in cell proliferation [12], and hence functional nucleoside transporters may normally exist in a CK2-mediated phosphorylated state.

The increase in *es* transporter density implied by these NBMPR binding data was corroborated by measuring the uptake of 2-chloro[3 H]adenosine in the presence and absence of NBMPR or dipyrindamole. This adenosine derivative has been described previously as an adenosine deaminase-resistant [36] high-affinity substrate for the *es* transporter in human erythrocytes [37] and fibroblast-like synoviocytes [38]. In the present study, it was observed that the maximum intracellular concentration of 2-chloro[3 H]adenosine did not exceed the extracellular concentration, and that cellular ATP depletion had no effect on the initial rate or the maximum level of accumulation of 2-chloro[3 H]adenosine. These data suggest that 2-chloroadenosine was not phosphorylated by intracellular adenosine kinases, under the conditions of these

Figure 8 Time course of the effect of CK2 inhibition on hENT1 and hENT2 mRNA levels

GV7.21 cells were grown in either the presence (control) or absence (decreased CK2 α' activity) of TET for the indicated times (abscissa), and then harvested for RNA isolation. The amounts of hENT1 (A) and hENT2 (B) mRNAs, normalized to the β -actin signal, were determined as described for Figure 7. Each bar is the mean $\pm$ S.E.M. of two amplification runs from each of three independent isolations. * $P < 0.05$ for significant difference between the TET+ and TET- conditions (Student's *t* test for paired data).

assays, making it a useful substrate for the kinetic analysis of equilibrative nucleoside transporter function. The V_{max} for 2-chloro[3 H]adenosine uptake via the *es* transporter increased 1.8-fold upon induction of inactive CK2 α' , with no significant change in K_m (Table 3). The *es*-mediated uptake of [3 H]formycin B, a well established substrate for *es* and *ei* transporters, was also enhanced approx. 2-fold in GV7.21 cells upon induction of inactive CK2 α' (Figure 6A). These data correlated reasonably well with the 1.5-fold increase in [3 H]NBMPR binding seen upon induction of inactive CK2 α' in the GV7.21 cells, and suggest that the enhanced transport activity was due to an increase in the number of *es* transporters at the plasma membrane, as opposed to a change in substrate affinity. This appears to be a post-transcriptional effect of CK2 α' on the *es* transporter, possibly involving a decreased rate of transporter internalization in the absence of CK2-mediated phosphorylation, since there was not a corresponding increase in hENT1 mRNA observed over the 48 h analysis period (Figure 8A). In fact, there was a significant decrease in hENT1 mRNA levels observed within 4 h after TET wash-out that had returned to control levels by 16 h. This transient inhibition of hENT1 transcription might have contributed to the slight decrease in [3 H]NBMPR binding observed at the 4 h time point (see Figure 2B). Therefore, inhibition of CK2 α' activity

appears to have a dual effect on ENT1/*es* activity: a delayed post-translational increase in the number of *es* transporters functioning at the plasma membrane, overlaid with a transient inhibition of ENT1 transcription. The attenuated increase in [³H]NBMPR binding observed at the 8 and 12 h time points, relative to the later time points (see Figure 2B), may be a consequence of these overlapping effects of CK2 α' inhibition.

Approx. 40% of the equilibrative uptake of 2-chloro[³H]-adenosine by the osteosarcoma cells was mediated by the *ei* sub-type of nucleoside transporter. This is similar to the ratio of NBMPR-sensitive to -insensitive [³H]formycin B uptake by these cells (see Figure 6), which further supports the use of 2-chloro[³H]adenosine as a substrate for the equilibrative transporters. This is the first study to show that 2-chloro[³H]adenosine is a substrate for the *ei* system, with a K_m ($29 \pm 3 \mu\text{M}$) comparable with that seen for the *es* transporter ($27 \pm 9 \mu\text{M}$). These K_m values are similar to that reported previously for 2-chloro[³H]adenosine uptake by the *es* transporter of human erythrocytes [37]. Inhibition of CK2 activity by induction of the inactive CK2 α' subunit in GV7.21 cells for 48 h decreased significantly the V_{max} of *ei*-mediated 2-chloro[³H]adenosine uptake. There was also a significant decrease in mENT2 mRNA levels within 4 h of induction of the inactive CK2 α' (Figure 8B). However, the ENT2 mRNA level in the TET- cells had returned to control levels by 12 h, and was maintained at this level for up to 48 h (the time at which the cells were harvested for the functional uptake studies). It is, nevertheless, possible that this early transient inhibition of hENT2 transcription led to the subsequent reduction of *ei* transport activity observed. The overall effect of changing the relative contributions of *es* and *ei* transporters on the cell nucleoside balance is unknown, but *ei* is able to transport nucleobases such as hypoxanthine, in addition to nucleosides [39], and has a lower affinity for cytidine and guanosine than does the *es* system [40,41].

This is the first report describing the modification of nucleoside transport activity by CK2 manipulation. However, other protein kinases, such as protein kinase A and protein kinase C, have been shown to exert at least indirect effects on nucleoside transport activity [17–20]. While there was a transient transcriptional effect of CK2 α' inhibition on *es* function, the timing and direction of this transcriptional change did not correlate with the changes in *es* activity. Thus the predominant effect of CK2 α' on *es* appears to be post-translational. The simplest interpretation of these data is that phosphorylation of the *es* transporter by CK2 α' leads to internalization, and thereby functional deactivation, of the transporter. It is also possible that CK2 might phosphorylate other cellular proteins which could then phosphorylate or interact with the *es* transporter to modify function or membrane localization. For example, there is recent evidence that nucleoside transport activity is sensitive to changes in the MAP kinase (mitogen-activated protein kinase) pathway [42], and CK2 has been reported to interact with both protein phosphatase 2A [43] and MAP kinase phosphatase 3 [44], and thereby modify MAP kinase signalling cascades. In addition, ENT1 activity in macrophages can be repressed post-transcriptionally by interferon- γ via a STAT1 (signal transduction and activators of transcription 1)-dependent mechanism [45]. It is known that interferon- γ can also stimulate CK2 activity [46], and it is thus possible that CK2 might be involved in the aforementioned STAT1-dependent inhibition of ENT1.

NBMPR-sensitive (*es*-like) nucleoside transport proteins have been shown to exist in intracellular compartments of mammalian cells [47–50]. As seen for glucose transporters [51], there is likely to be an intracellular pool of nucleoside transporters that is in dynamic equilibrium with those in the plasma membrane. The

data obtained from the present study suggest that the lifetime of *es* transport proteins in the plasma membrane and/or their trafficking between subcellular membrane compartments is regulated, in part, by CK2-mediated phosphorylation.

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REFERENCES

- Hyde, R. J., Cass, C. E., Young, J. D. and Baldwin, S. A. (2001) The ENT family of eukaryote nucleoside and nucleobase transporters: recent advances in the investigation of structure/function relationships and the identification of novel isoforms. *Mol. Membr. Biol.* **18**, 53–63.
- Van Belle, H. (1993) Nucleoside transport inhibition: a therapeutic approach to cardioprotection via adenosine? *Cardiovasc. Res.* **27**, 68–76.
- Belt, J. A., Marina, N. M., Phelps, D. A. and Crawford, C. R. (1993) Nucleoside transport in normal and neoplastic cells. *Adv. Enzyme Regul.* **33**, 235–252.
- Damaraju, V. L., Damaraju, S., Young, J. D., Baldwin, S. A., MacKey, J., Sawyer, M. B. and Cass, C. E. (2003) Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy. *Oncogene* **22**, 7524–7536.
- Gray, J. H., Owen, R. P. and Giacomini, K. M. (2004) The concentrative nucleoside transporter family, SLC28. *Pflügers Arch.* **447**, 728–734.
- Baldwin, S. A., Beal, P. R., Yao, S. Y., King, A. E., Cass, C. E. and Young, J. D. (2004) The equilibrative nucleoside transporter family, SLC29. *Pflügers Arch.* **447**, 735–743.
- Griffiths, M., Yao, S. Y., Abidi, F., Phillips, S. E., Cass, C. E., Young, J. D. and Baldwin, S. A. (1997) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (*ei*) equilibrative nucleoside transporter from human placenta. *Biochem. J.* **328**, 739–743.
- Griffiths, M., Beaumont, N., Yao, S. Y. M., Sundaram, M., Boumah, C. E., Davies, A., Kwong, F. Y. P., Coe, I., Cass, C. E., Young, J. D. and Baldwin, S. A. (1997) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nat. Med.* **3**, 89–93.
- Kiss, A., Farah, K., Kim, J., Garriock, R. J., Drysdale, T. A. and Hammond, J. R. (2000) Molecular cloning and functional characterization of inhibitor-sensitive (mENT1) and inhibitor-resistant (mENT2) equilibrative nucleoside transporters from mouse brain. *Biochem. J.* **352**, 363–372.
- Handa, M., Choi, D., Caldeiro, R. M., Messing, R. O., Gordon, A. S. and Diamond, I. I. (2001) Cloning of a novel isoform of the mouse NBMPR-sensitive equilibrative nucleoside transporter (ENT1) lacking a putative phosphorylation site. *Gene* **262**, 301–307.
- Faust, M. and Montenarh, M. (2000) Subcellular localization of protein kinase CK2. A key to its function? *Cell Tissue Res.* **301**, 329–340.
- Litchfield, D. W. (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem. J.* **369**, 1–15.
- Guerra, B. and Issinger, O. G. (1999) Protein kinase CK2 and its role in cellular proliferation, development and pathology. *Electrophoresis* **20**, 391–408.
- Ahmed, K., Davis, A. T., Wang, H., Faust, R. A., Yu, S. and Tawfic, S. (2000) Significance of protein kinase CK2 nuclear signaling in neoplasia. *J. Cell. Biochem. Suppl.* **35**, 130–135.
- Pennycooke, M., Chaudary, N., Shuralyova, I., Zhang, Y. and Coe, I. R. (2001) Differential expression of human nucleoside transporters in normal and tumor tissue. *Biochem. Biophys. Res. Commun.* **280**, 951–959.
- Cass, C. E., Dahlig, E., Lau, E. Y., Lynch, T. P. and Paterson, A. R. (1979) Fluctuations in nucleoside uptake and binding of the inhibitor of nucleoside transport, nitrobenzylthioinosine, during the replication cycle of HeLa cells. *Cancer Res.* **39**, 1245–1252.
- Coe, I. R., Dohrman, D. P., Constantinescu, A., Diamond, I. and Gordon, A. S. (1996) Activation of cyclic AMP-dependent protein kinase reverses tolerance of a nucleoside transporter to ethanol. *J. Pharmacol. Exp. Ther.* **276**, 365–369.
- Coe, I., Zhang, Y., McKenzie, T. and Naydenova, Z. (2002) PKC regulation of the human equilibrative nucleoside transporter, hENT1. *FEBS Lett.* **517**, 201–205.
- Lee, C.-W. (1994) Decrease in equilibrative uridine transport during monocytic differentiation of HL-60 leukaemia: involvement of protein kinase C. *Biochem. J.* **300**, 407–412.

- 20 Soler, C., Felipe, A., Mata, J. F., Casado, F. J., Celada, A. and Pastor-Anglada, M. (1998) Regulation of nucleoside transport by lipopolysaccharide, phorbol esters and tumor necrosis factor- α in human B-lymphocytes. *J. Biol. Chem.* **273**, 26939–26945
- 21 Jones, K. W., Rylett, R. J. and Hammond, J. R. (1994) Effect of cellular differentiation on nucleoside transport in human neuroblastoma cells. *Brain Res.* **660**, 104–112
- 22 Meckling-Gill, K. A. and Cass, C. E. (1992) Effects of transformation by *v-fps* on nucleoside transport in Rat-2 fibroblasts. *Biochem. J.* **282**, 147–154
- 23 Baldwin, S. A., Mackey, J. R., Cass, C. E. and Young, J. D. (1999) Nucleoside transporters: molecular biology and implications for therapeutic development. *Mol. Med. Today* **5**, 216–224
- 24 Cook, M. A. and Karmazyn, M. (1996) Cardioprotective actions of adenosine and adenosine analogs. *EXS* **76**, 325–344
- 25 Sarno, S., Moro, S., Meggio, F., Zagotto, G., Dal Ben, D., Ghisellini, P., Battistutta, R., Zanotti, G. and Pinna, L. (2002) Toward the rational design of protein kinase casein kinase-2 inhibitors. *Pharmacol. Ther.* **93**, 159–168
- 26 Vilg, G., Saulnier, R. B., St Pierre, R. and Litchfield, D. W. (1999) Inducible expression of protein kinase CK2 in mammalian cells. Evidence for functional specialization of CK2 isoforms. *J. Biol. Chem.* **274**, 14406–14414
- 27 Stolk, M., Farah, K., Cooper, E., Vilg, G., Litchfield, D. W. and Hammond, J. R. (2002) Regulation of equilibrative nucleoside transporters by protein kinase CK2. *Pharmacologist* **44** (Suppl. 1), A40–A41
- 28 Hammond, J. R. and Johnstone, R. M. (1989) Solubilization and reconstitution of a nucleoside-transport system from Ehrlich ascites-tumour cells. *Biochem. J.* **262**, 109–118
- 29 Hammond, J. R. (2000) Interaction of a series of drafazine analogues with equilibrative nucleoside transporters: species differences and transporter subtype selectivity. *Naunyn Schmiedeberg's Arch. Pharmacol.* **361**, 373–382
- 30 Jarvis, S. M. and Young, J. D. (1980) Nucleoside transport in human and sheep erythrocytes: evidence that nitrobenzylthioinosine binds specifically to functional nucleoside-transport sites. *Biochem. J.* **190**, 377–383
- 31 Cheng, Y. and Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108
- 32 Plagemann, P. G. W. and Woffendin, C. (1989) Use of formycin B as a general substrate for measuring facilitated nucleoside transport in mammalian cells. *Biochim. Biophys. Acta* **1010**, 7–15
- 33 Goh, L. B., Mack, P. and Lee, C. W. (1995) Nitrobenzylthioinosine-binding protein overexpression in human breast, liver, stomach and colorectal tumour tissues. *Anticancer Res.* **15**, 2575–2579
- 34 Griffith, D. A. and Jarvis, S. M. (1996) Nucleoside and nucleobase transport systems of mammalian cells. *Biochim. Biophys. Acta Rev. Biomembr.* **1286**, 153–181
- 35 Litchfield, D. W., Bosc, D. G., Canton, D. A., Saulnier, R. B., Vilg, G. and Zhang, C. (2001) Functional specialization of CK2 isoforms and characterization of isoform-specific binding partners. *Mol. Cell. Biochem.* **227**, 21–29
- 36 Yamanaka, H., Kamatani, N., Nishida, Y., Nishioka, K. and Mikanagi, K. (1984) Relationship between phosphorylation and cytotoxicity of 2-chloroadenosine and 6-methylmercaptopurine riboside in human cells. *Biochim. Biophys. Acta* **798**, 291–294
- 37 Jarvis, S. M., Martin, B. W. and Ng, A. S. (1985) 2-Chloroadenosine, a permeant for the nucleoside transporter. *Biochem. Pharmacol.* **34**, 3237–3241
- 38 Koshiba, M., Kosaka, H., Nakazawa, T., Hayashi, N., Saura, R., Kitamura, N. and Kumagai, S. (2002) 2-Chloroadenosine but not adenosine induces apoptosis in rheumatoid fibroblasts independently of cell surface adenosine receptor signalling. *Br. J. Pharmacol.* **135**, 1477–1486
- 39 Osses, N., Pearson, J. D., Yudilevich, D. L. and Jarvis, S. M. (1996) Hypoxanthine enters human vascular endothelial cells (ECV 304) via the nitrobenzylthioinosine-insensitive equilibrative nucleoside transporter. *Biochem. J.* **317**, 843–848
- 40 Hammond, J. R. (1991) Comparative pharmacology of the nitrobenzylthioguanosine-sensitive and -resistant nucleoside transport mechanisms of Ehrlich ascites tumor cells. *J. Pharmacol. Exp. Ther.* **259**, 799–807
- 41 Ward, J. L., Sherali, A., Mo, Z. P. and Tse, C. M. (2000) Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. ENT2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. *J. Biol. Chem.* **275**, 8375–8381
- 42 Sperlagh, B., Baranyi, M., Hasko, G. and Vizi, E. S. (2004) Potent effect of interleukin-1 β to evoke ATP and adenosine release from rat hippocampal slices. *J. Neuroimmunol.* **151**, 33–39
- 43 Lebrin, F., Bianchini, L., Rabilloud, T., Chambaz, E. M. and Goldberg, Y. (1999) CK2 α -protein phosphatase 2A molecular complex: possible interaction with the MAP kinase pathway. *Mol. Cell. Biochem.* **191**, 207–212
- 44 Castelli, M., Camps, M., Leroy, D., Arkinstall, S., Rommel, C. and Nichols, A. (2004) MAP kinase phosphatase 3 (MKP3) interacts with and is phosphorylated by protein kinase CK2 α . *J. Biol. Chem.* **279**, 44731–44739
- 45 Soler, C., Felipe, A., Garcia-Manteiga, J., Serra, M., Guillen-Gomez, E., Casado, F. J., MacLeod, C., Modolell, M., Pastor-Anglada, M. and Celada, A. (2003) Interferon- γ regulates nucleoside transport systems in macrophages through signal transducer and activator of transduction factor 1 (STAT1)-dependent and -independent signalling pathways. *Biochem. J.* **375**, 777–783
- 46 Mead, J. R., Hughes, T. R., Irvine, S. A., Singh, N. N. and Ramji, D. P. (2003) Interferon- γ stimulates the expression of the inducible cAMP early repressor in macrophages through the activation of casein kinase 2. A potentially novel pathway for interferon- γ -mediated inhibition of gene transcription. *J. Biol. Chem.* **278**, 17741–17751
- 47 Jimenez, A., Pubill, D., Pallas, M., Camins, A., Llado, S., Camarasa, J. and Escubedo, E. (2000) Further characterization of an adenosine transport system in the mitochondrial fraction of rat testis. *Eur. J. Pharmacol.* **398**, 31–39
- 48 Mani, R. S., Hammond, J. R., Marjan, J. M., Graham, K. A., Young, J. D., Baldwin, S. A. and Cass, C. E. (1998) Demonstration of equilibrative nucleoside transporters (hENT1 and hENT2) in nuclear envelopes of cultured human choriocarcinoma (BeWo) cells by functional reconstitution in proteoliposomes. *J. Biol. Chem.* **273**, 30818–30825
- 49 Pisoni, R. L. and Thoene, J. G. (1989) Detection and characterization of a nucleoside transport system in human fibroblast lysosomes. *J. Biol. Chem.* **264**, 4850–4856
- 50 Lai, Y., Tse, C. M. and Unadkat, J. D. (2003) Mitochondrial expression of the human equilibrative nucleoside transporter 1 (hENT1) results in enhanced mitochondrial toxicity of antiviral drugs. *J. Biol. Chem.* **279**, 4490–4497
- 51 Baldwin, S. A., Barros, L. F. and Griffiths, M. (1995) Trafficking of glucose transporters – Signals and mechanisms. *Biosci. Rep.* **15**, 419–426