Dehydroascorbic acid uptake by coronary artery smooth muscle: effect of intracellular acidification

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Dehydroascorbic acid (DHAA) enters cells via Na^+-independent glucose transporters (GLUT) and is converted to ascorbate. However, we found that Na^+ removal inhibited [^{14}C]DHAA uptake by smooth-muscle cells cultured from pig coronary artery. The uptake was examined for 2–12 min at 10–200 μM DHAA in either the presence of 134 mM Na^+ or in its absence (*N*-methyl D-glucamine, choline or sucrose replaced Na^+). This inhibition of DHAA uptake by Na^+ removal was paradoxical because it was inhibited by 2-deoxyglucose and cytochalasin B, as expected of transport via the GLUT pathway. We tested the hypothesis that this paradox resulted from an inefficient intracellular reduction of [^{14}C]DHAA into [^{14}C]ascorbate upon intracellular acidosis caused by the Na^+ removal. Consistent with this hypothesis: (i) the Na^+/H^+-exchange inhibitors ethylisopropyl amiloride and cariporide also decreased the uptake, (ii) Na^+ removal and Na^+/H^+-exchange inhibitors lowered cytosolic pH, with the decrease being larger in 12 min than in 2 min, and (iii) less of the cellular ^{14}C was present as ascorbate (determined by HPLC) in cells in Na^+-free buffer than in those in Na^+-containing buffer. This inability to obtain ascorbate from extracellular DHAA may be detrimental to the coronary artery under hypoxia-induced acidosis during ischaemia/reperfusion.

Key words: ischaemia/reperfusion, membrane transport, oxidative stress, vitamin C.

INTRODUCTION

Vitamin C or ascorbate not only aids in protecting cells against oxidative stress but is also an important cofactor in many enzymic reactions [1,2]. Ascorbate is required in collagen hydroxylation, carnitine biosynthesis and formation of catecholamines [3]. Ascorbate is a recyclable antioxidant. It plays a key role in protecting cellular membranes and proteins against oxidative stress, with substances such as tocopherol being involved as intermediates [4]. In the process, ascorbate is converted into dehydroascorbic acid (DHAA). DHAA can in turn be reduced by various metabolites such as glutathione and NADPH [5]. In humans and some other mammals, ascorbate is an essential vitamin and daily dietary intake results in 50–100 μM of this substance in plasma [6]. However, the extracellular ascorbate may be oxidized non-enzymically to DHAA, thereby decreasing the availability of ascorbate in ischaemic areas.

Ascorbate is absorbed by many cell types via Na^+–ascorbate symporters such as SVCT1 and SCVT2 [7–12]. Ascorbate uptake by cells is inhibited by depletion of extracellular Na^+ and anion-transport inhibitors such as sulfinpyrazone and 4,4′-di-isothiocyanatostilbene-2,2′-disulphonate (DIDS) [13,14]. It is not inhibited by extracellular glucose and its analogue 2-deoxyglucose (DOG), or by cytochalasin B [6,15]. DHAA enters cells via the GLUT family of Na^+-independent glucose transporters [16–18]. Once inside the cells, DHAA is converted into ascorbate, thus obviating the need for a DHAA gradient for accumulation of ascorbate via this pathway [19]. DHAA uptake is thus supposedly independent of extracellular Na^+ and not affected by anion-transport inhibitors, but is inhibited by glucose, DOG or cytochalasin B. Here we report, using [^{14}C]DHAA, that ascorbate accumulation by smooth-muscle cells cultured from pig coronary artery is inhibited not only by DOG and cytochalasin B, as expected, but also by removal of extracellular Na^+ and by Na^+/H^+-exchange inhibitors. DHAA uptake occurs via the expected pathway but its conversion into ascorbate may be inefficient during intracellular acidosis.

MATERIALS AND METHODS

Coronary artery smooth-muscle cell cultures

Pig coronary artery smooth-muscle cells were isolated and plated in Dulbecco's modified Eagle's medium (Gibco) supplemented with 0.5 mM Hepes, pH 7.4, 2 mM glutamine, 50 mg/l gentamicin, 0.125 mg/l amphotericin B and 10% fetal bovine serum, and then allowed to grow to confluence as described previously [20,21]. An aliquot of cells in passage 3 was thawed and grown to confluence, trypsinized and replated at a density of 1.4×10^4 cells/cm^2. The medium was changed after 2 days and the cells were used 5–8 days after plating, in passage 4.

[^{14}C]DHAA uptake

Measurement of the uptake of [^{14}C]DHAA was carried out in glucose-free Na^+/Hepes buffer containing 134 mM NaCl, 5.4 mM KCl, 10 mM *N*-methyl D-glucamine$^+$ (NMG; to replace glucose), 0.8 mM $MgSO_4$, 20 mM Hepes and 1.8 mM $CaCl_2$,

Abbreviations used: DHAA, dehydroascorbic acid; DOG, 2-deoxyglucose; NMG, *N*-methyl D-glucamine$^+$; EIPA, ethylisopropyl amiloride.

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pH 7.3. The osmolarity of this solution was 290–300 mOsm. To obtain Na^+-free solutions, Na^+ was replaced with an equimolar amount of NMG. [^{14}C]DHAA was prepared by incubating 10 mM L-[^{14}C-carboxyl]ascorbate (specific activity, 5.1 mCi/mmol; Amersham Bioscience) with 2 units of ascorbate oxidase (Sigma)/μl of [^{14}C]ascorbate in glucose-free, Na^+-free Hepes buffer at 37 °C for 2 min. The [^{14}C]DHAA was diluted typically to a final concentration of 10 μM in the various solutions used. Unlabelled DHAA (Sigma) was stored as a powder under nitrogen at −80 °C and dissolved just before use.

[^{14}C]DHAA-uptake assay was carried out as described previously [7]. Tissue-culture dishes (60 mm) containing the cells were placed in a 37 °C shaking water bath (at 25 rev./min), rinsed twice with the appropriate Hepes buffer (either glucose-free, Na^+-free Hepes or glucose-free Na^+/Hepes buffer). [^{14}C]DHAA-uptake solution (2 ml, 10 μM) was added to these cells. After a 2 min incubation period, the cells were rinsed six times with ice-cold sucrose/Tris/HCl (320 mM sucrose/10 mM Tris/HCl, pH 7.3) and then harvested by scraping into 1 ml of ice-cold water. Some of the resulting suspension (0.9 ml) was used for scintillation counting in 5 ml of Ready Safe cocktail (Beckman), and the remainder was saved for protein estimation. Background due to non-specific binding was measured by performing the uptake assay on ice, for 0 min. This typical procedure was modified by changing the uptake time, DHAA concentration, and use of inhibitors and solutions of different ionic compositions, as described in the Results section. In some experiments, [^{14}C]ascorbate and [^{3}H]DOG uptake were also examined in the presence of 134 mM Na^+. Protein estimation was carried out on each sample using Bradford Reagent (Bio-Rad). A 60 mm dish of the cells typically contained $(1.4 \pm 0.1) \times 10^6$ cells [cell density, $(5.0 \pm 0.4) \times 10^4$ cells/cm^2], which equated to 0.2 ± 0.02 mg of protein.

HPLC

Ascorbate was quantified by HPLC-based electrochemical assay with a Waters M460 amperometric detector and assay sensitivity was 2 pmol of ascorbate as described previously [14]. The ascorbate concentrations of experimental samples were determined by intrapolation on an external standard curve. To assess the conversion of ascorbate into DHAA with ascorbate oxidase, the level of ascorbate in the uptake buffers was monitored in each experiment. The buffer (450 μl) was mixed with 50 μl of a chilled solution of 8.5 % metaphosphoric acid and frozen immediately at −80 °C until use. With 10 μM initial ascorbate, less than 0.1 μM routinely remained after the enzymic conversion. For determining cellular ascorbate content, the cells were scraped into 1 ml of ice-cold water. Of this, 100 μl was used for protein estimation, 500 μl for scintillation counting and the remainder was frozen immediately with metaphosphoric acid at −80 °C until analysis by HPLC. As these cells do not contain any ascorbate when cultured under standard conditions, samples without added DHAA or ascorbate were used as blanks.

Intracellular pH measurement

Intracellular pH was determined by microfluorometry as described previously [22]. Smooth-muscle cells cultured on a glass coverslip were incubated with serum-free Dulbecco's modified Eagle's medium for 2 h and then loaded for 30 min with 1 μM 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein/acetoxymethyl ester (BCECF/AM; Molecular Probes, Eugene, OR, U.S.A.). The coverslip was then mounted on to a Zeiss microscope thermostatic holder maintained at 37 °C. Fluorescence intensity in single cells was measured by adjusting the diaphragm in front of the photomultiplier tube attached through a Zeiss microscope to an M series dual-wavelength excitation system from Photon Technologies. The pair of excitation wavelengths used was 495 and 440 nm, and the emitted fluorescent light was measured at 530 nm. The fluorescence signals were processed using ImageMaster Felix software and the ratio of the F_{495}/F_{440} obtained. The fluorescence of cells was monitored first for 20 min in glucose-free Na^+-Hepes buffer and then in either glucose-free, Na^+-free Hepes buffer or in Na^+/Hepes buffer with 10 μM cariporide. Intracellular pH calibration was carried out in glucose-free buffer with 15 μM nigericin, high K^+ at different pH (containing 130 mM KCl, 19.4 mM NaCl, 0.8 mM $MgSO_4$, 20 mM Hepes and 1.8 mM $CaCl_2$) and 50 μM 2,3-butanedione monoxime (to prevent the cells from contracting during the calibration).

ATP measurements

ATP was measured as described previously [23] using a luciferase ATP assay kit (Sigma-Aldrich Canada, Oakville, Canada). Cells were first incubated and washed as for the DHAA-uptake experiments and then placed in 1 ml of 1.6 % trichloroacetic acid for 30 min at 0 °C. The trichloroacetic acid solution was removed and 80 μl of 1 M Tris base plus 120 μl of Tris/EDTA (100 mM Tris/10 mM EDTA, pH 7.7) were added to 800 μl of this solution. The solution was diluted 5-fold in the Tris/EDTA buffer and 10 μl of the diluted solution was added to 100 μl of the luciferase/luciferin as per the instructions of the manufacturer. Luminescence was monitored immediately for 20 s. A standard curve using known ATP concentrations was used to determine the ATP content of the samples. Cells were suspended in 0.1 M NaOH and used for protein estimation.

Data analysis

Null hypotheses were tested using Student's t test. P values of < 0.05 were considered to be statistically significant. Each experiment was replicated at least three times.

RESULTS

We will first describe the total accumulation of [^{14}C]DHAA, monitored as the amount of radioactivity retained by the cells, the effects of Na^+ removal and various inhibitors on this activity, the effects of Na^+ removal and Na^+/H^+ exchange inhibitors on intracellular pH, and finally compare the cellular radioactivity and ascorbate as determined using HPLC. Note that [^{14}C]DHAA accumulation refers to accumulation of ^{14}C from solution containing [^{14}C]DHAA but does not signify that the radioactive material in the cell is DHAA.

Time course and concentration dependence of [^{14}C]DHAA accumulation

[^{14}C]DHAA was taken up by smooth-muscle cells in both Na^+-containing and Na^+-free buffers (Figure 1). The accumulation continued to increase for at least 12 min. At all times examined, the accumulation of radiolabel was significantly greater in the presence of Na^+ than in its absence ($P < 0.05$). The inset in Figure 1 compares the percentage inhibition of ^{14}C accumulation due to Na^+ removal obtained on uptake over 2 min with that over 12 min from 16 experiments. The percentage inhibition increased slightly with time. As the ascorbate uptake was Na^+-dependent, we routinely checked with HPLC that our solutions did not contain any detectable amounts of ascorbate. We also routinely included a control experiment with 10 mM DOG, which is known

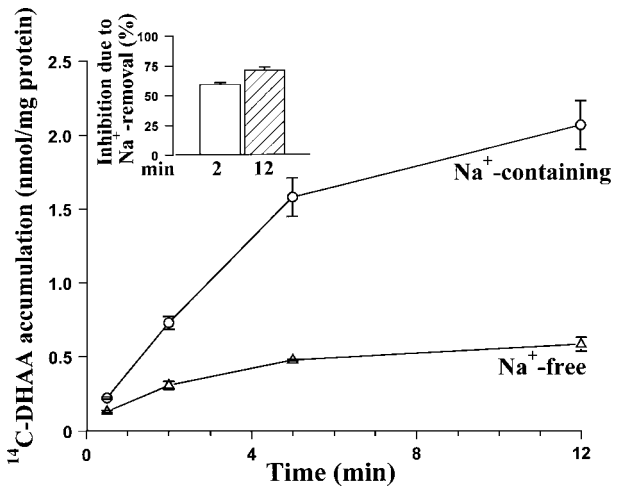

Figure 1 Time course of [^{14}C]DHAA accumulation

Time course of 10 μM [^{14}C]DHAA uptake in Na^+-containing and Na^+-free Hepes buffers. At each incubation time tested, cells showed less uptake in Na^+-free medium than in Na^+-containing medium ($P < 0.05$). The values are the means $\pm$ S.E.M. from five replicates. Repeating this experiment gave similar results. Inset: inhibition of DHAA uptake due to Na^+ depletion at 2 and 12 min from several experiments. Values are means $\pm$ S.E.M. from 15 (2 min) and four (12 min) experiments. In all paired experiments, the inhibition due to Na^+ removal at 12 min was greater than that at 2 min ($P < 0.05$).

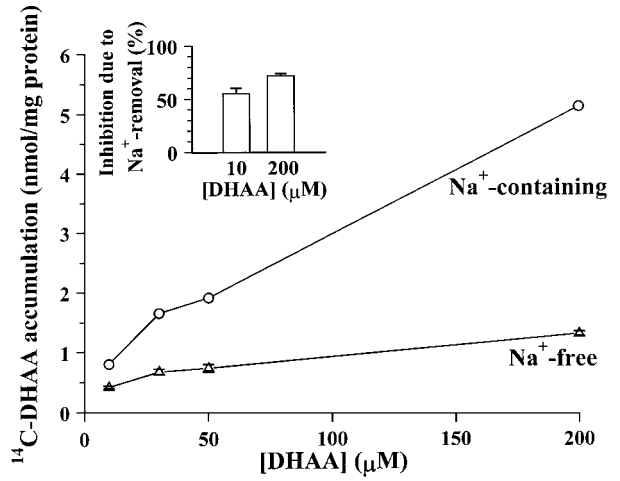

Figure 2 Concentration dependence of DHAA accumulation

The cells were incubated for 2 min in the presence or absence of 134 mM Na^+ (NMG), 10 μM [^{14}C]DHAA plus unlabelled DHAA to obtain the specified values of DHAA concentrations. The values are means $\pm$ S.E.M. from five replicates. The inset shows the percentage inhibition due to Na^+ removal (with Na^+-containing solution as a control) from three experiments. The percentage inhibition due to Na^+ removal was greater at 200 μM DHAA than at 10 μM ($P < 0.05$).

to inhibit the uptake of DHAA but not that of ascorbate. These controls rule out any contributions due to ascorbate transport or due to incomplete conversion of ascorbate into DHAA.

We then examined in a 2 min uptake experiment whether the effect of Na^+ removal depended on the DHAA concentration in the uptake medium. [^{14}C]DHAA accumulation continued to increase with increasing DHAA concentration (10–200 μM; Figure 2). This was expected, as these concentrations of DHAA are below the reported K_m value for transport of DHAA by

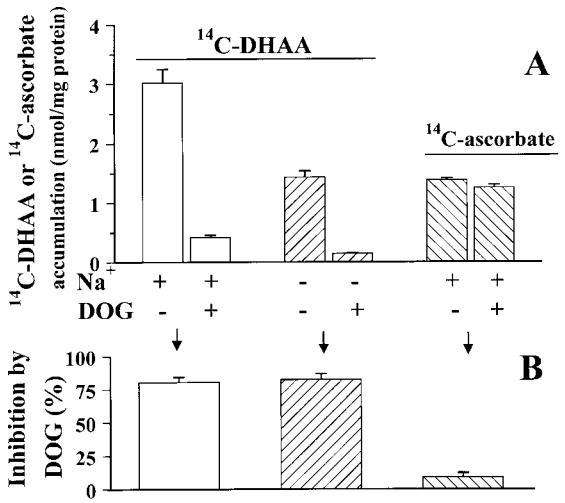

Figure 3 Effect of 2-DOG on [^{14}C]DHAA accumulation

[2-DOG] = 0 (control) or 10 mM, [[^{14}C]DHAA] or [[^{14}C]ascorbate] = 10 μM, [Na^+] = 134 or 0 mM (replacement with NMG), uptake time = 12 min. (**A**) Values of ^{14}C uptake were the means $\pm$ S.E.M. from six replicates for uptake with and without DOG. (**B**) Inhibition by DOG was determined for each group as 100 $\times$ [(uptake without DOG $-$ uptake in presence of DOG)/uptake without DOG]. The percentage inhibition produced by DOG in Na^+-containing medium was not significantly different from that obtained in Na^+-free medium ($P > 0.05$). DOG did not inhibit [^{14}C]ascorbate uptake ($P > 0.05$).

GLUT-type transporters [9]. Inhibition of ^{14}C accumulation due to Na^+ removal was observed at each DHAA concentration. The inset in Figure 2 shows that the percentage inhibition due to Na^+ removal was slightly greater at 200 μM DHAA than at 10 μM. We also determined the effect of Na^+ removal on [^{3}H]DOG accumulation over 2 min. The accumulation was not inhibited by Na^+ removal, as the average value of DOG uptake in Na^+-free solution was $8 \pm 7\%$ higher than in solution containing Na^+ (not statistically significant).

Effects of inhibitors on [^{14}C]DHAA uptake

We examined the effects of inhibitors of GLUT and anion transporters on [^{14}C]DHAA uptake. DOG is a substrate of GLUT transporters and hence it would compete for the uptake of DHAA [24]. DOG inhibited [^{14}C]DHAA accumulation but not ascorbate uptake (Figure 3A), suggesting that DHAA entered the cells via the GLUT pathway. Figure 3(B) shows that the percentage inhibition of [^{14}C]DHAA accumulation by DOG was similar whether the uptake was carried out in the presence or the absence of Na^+. We also showed this in uptake experiments for only 2 min (results not shown). GLUT transporters are inhibited by cytochalasin B but not cytochalasin D [24]. Since both cytochalasins may disrupt the cytoskeleton, we compared their effects (Figure 4). Figure 4(A) shows that the presence of cytochalasin B inhibited the [^{14}C]DHAA uptake better than cytochalasin D in the presence and absence of Na^+. Figure 4(B) shows that the percentage inhibition of [^{14}C]DHAA accumulation by cytochalasin B was similar whether the uptake was carried out in the presence or the absence of Na^+. In uptake experiments for 2 min, the results were similar (not shown). As expected, cytochalasin B inhibited [^{3}H]DOG uptake (Figure 4D) but not [^{14}C]ascorbate uptake (Figure 4C), providing further evidence that [^{14}C]DHAA entered the cells via the GLUT pathway.

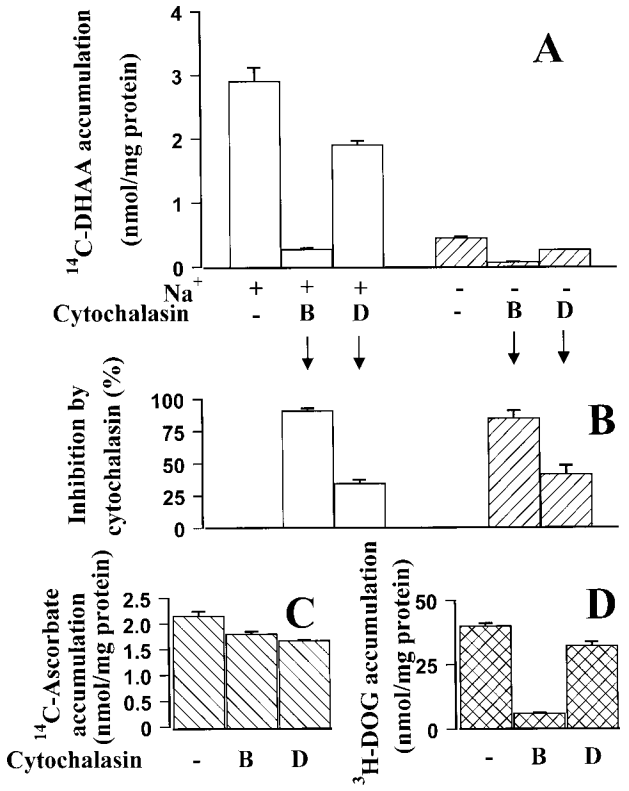

Figure 4 Effect of cytochalasin B on [^{14}C]DHAA accumulation

[Cytochalasin B or D] = 0 (control) or 3 μM, [[^{14}C]DHAA] or [[^{14}C]ascorbate] = 10 μM, [[^{3}H]DOG] = 200 μM, [Na^+] = 134 or 0 mM (replacement with NMG), uptake time = 12 min. (**A**) Uptake values for [^{14}C]DHAA shown are means $\pm$ S.E.M. from four to six replicates. (**B**) Inhibition by cytochalasin B and D was determined for each group as $100 \times$ [(uptake without cytochalasin $-$ uptake in presence of cytochalasin)/uptake without cytochalasin]. Both cytochalasin B and D produced an inhibition, but that produced with cytochalasin B was significantly greater than with D ($P < 0.05$). The percentage inhibition produced by cytochalasin B or D in Na^+-containing medium was not significantly different from that obtained in Na^+-free medium ($P > 0.05$). (**C**) Effect of cytochalasins on [^{14}C]ascorbate uptake in the presence of Na^+. Both cytochalasin B and D produced marginal inhibition, with cytochalasin D being slightly more inhibitory ($P < 0.05$). (**D**) Effect of cytochalasins on [^{3}H]DOG uptake in the absence of Na^+. Both cytochalasin B and D inhibited the uptake but the inhibition by cytochalasin B was significantly greater ($P < 0.05$).

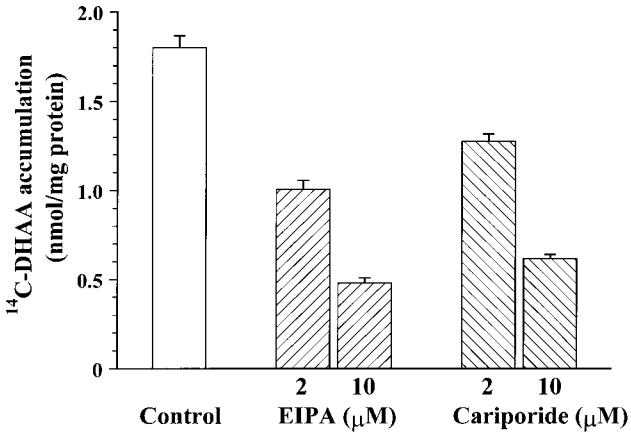

Figure 5 Effect of EIPA and cariporide on [^{14}C]DHAA uptake

[[^{14}C]DHAA] = 10 μM, [Na^+] = 134 mM, EIPA or cariporide = 0 (control), 2 or 10 μM, uptake time = 2 min. Both agents caused significant inhibition at 2 and 10 μM, with inhibition at 10 μM being greater than at 2 μM ($P < 0.05$).

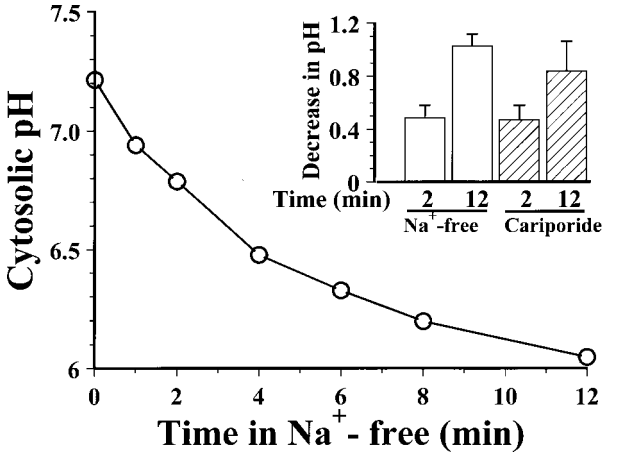

Figure 6 Cytosolic pH measurement

The main graph shows the pH in one cell at different times after changing from Na^+/Hepes buffer to Na^+-free Hepes. Basal pH in 10 cells was 7.3 ± 0.1. Inset: the decrease in pH in five cells at 2 and 12 min after changing to Na^+-free buffer and for four cells after changing to Na^+/Hepes plus 10 μM cariporide (means $\pm$ S.E.M.). See the Materials and methods section for details of measurements.

[^{14}C]DHAA accumulation and Na^+/H^+-exchange inhibition

Na^+ removal in arterial smooth-muscle cultures may decrease intracellular pH by inhibiting Na^+/H^+ exchange, especially in a nominally carbonate-free buffer (carbonate was not used since it has been reported to destabilize DHAA) [25–27]. Na^+/H^+-exchange inhibitors also lower intracellular pH [25]. Therefore, we examined the effects of the Na^+/H^+-exchange inhibitors ethylisopropyl amiloride (EIPA) and cariporide on [^{14}C]DHAA accumulation [28,29]. We examined the effects of the two inhibitors, at 2 and 10 μM, on the accumulation of [^{14}C]DHAA in Na^+-containing buffer. Both EIPA and cariporide inhibited the accummulation, with 10 μM being more effective than 2 μM (Figure 5).

Intracellular pH measurement

We next tested the hypothesis that replacing Na^+ with NMG or inhibition of Na^+/H^+ exchanger decreased intracellular pH. The pH of the cells in the Na^+/Hepes buffer was 7.3 ± 0.1 (mean $\pm$ S.E.M. from 10 cells). There was a time-dependent decrease in pH after the buffer bathing the cells was changed to Na^+-free buffer containing 10 μM DHAA (Figure 6). When several cells were examined the pH decreased by 0.5 ± 0.1 units within the first 2 min and by 1.0 ± 0.1 units in 12 min (Figure 6, inset). Similarly, when the cells were perfused with glucose-free Na^+/Hepes buffer containing 10 μM DHAA and 10 μM cariporide, the pH decreased by 0.5 ± 0.1 units in the first 2 min and by 0.8 ± 0.2 units in 12 min. EIPA also produced a similar decrease in intracellular pH (results not shown). Thus Na^+ removal or inhibition of the Na^+/H^+ exchanger decreased intracellular pH.

Comparison of uptake using HPLC and scintillation counting

To determine the nature of the radioactive material accumulated in the cell after [^{14}C]DHAA uptake, we determined the amount

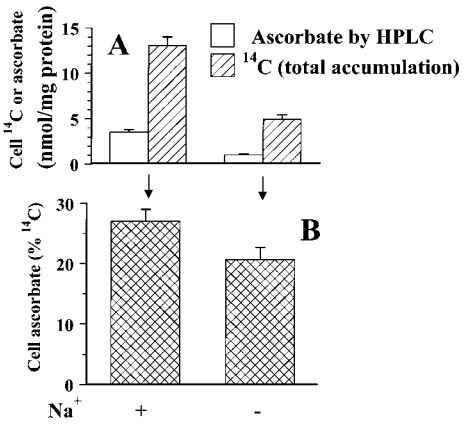

Figure 7 Comparison of uptake measurements using HPLC and ^{14}C-scintillation counting

[^{14}C]DHAA = 10 μM, unlabelled DHAA = 190 μM, [Na^+] = 134 or 0 mM (replacement with NMG), uptake time = 2 min. (**A**) Uptake values determined with HPLC and ^{14}C shown are means ± S.E.M. from three to five replicates. (**B**) For each group the cell ascorbate was determined as a percentage of ^{14}C uptake in the samples in that group. The values for percentage ascorbate were significantly greater when the uptake was carried out in Na^+-containing medium than in Na^+-free medium ($P < 0.05$).

Table 1 ATP content of cells incubated under different conditions

Cells were incubated for 2 or 12 min in the presence of 200 μM DHAA in Na^+-containing or Na^+-free Hepes buffer, as in Figure 1. Cariporide (10 μM) was included in the incubation buffer where shown. The values shown are means ± S.E.M. from five replicates. The ATP levels did not differ significantly ($P > 0.05$) in any of the groups. The experiment was replicated three times with similar results.

Buffer/treatment	ATP (nmol/mg of cell protein)
No incubation	7.2 ± 0.5
Na^+-containing, 2 min	7.7 ± 1.1
Na^+-free Hepes, 2 min	8.2 ± 0.5
Na^+-containing, + cariporide, 2 min	7.2 ± 0.8
Na^+-containing, 12 min	7.3 ± 0.4
Na^+-free Hepes, 12 min	6.7 ± 0.5
Na^+-containing, + cariporide, 12 min	7.1 ± 0.5

of ascorbate in the cells after 2 min using an HPLC-electrochemical assay (Figure 7). The ratio of ascorbate as a percentage of the total ^{14}C accumulated was used to determine whether the cells were more efficient at converting DHAA into ascorbate under certain conditions. The percentage of ascorbate was significantly greater ($P < 0.05$) for the Na^+-containing (27 ± 2 %, $n = 5$) than for the Na^+-free (21 ± 2 %, $n = 3$) solution, suggesting that Na^+ removal led to a decrease in the conversion of DHAA into ascorbate. In another experiment, we determined the uptake over a 12 min period. We expected that [^{14}C]ascorbate would accumulate in the cells but that the unconverted [^{14}C]DHAA would be constantly lost. Hence the ratio of cellular ascorbate to ^{14}C would be higher in 12 min of uptake than in 2 min. The value of this ratio increased significantly ($P < 0.05$) between 2 and 12 min, from 27 ± 2 % to 53 ± 3 % in Na^+/Hepes buffer, and from 21 ± 2 % to 46 ± 4 % in NMG/Hepes buffer.

ATP measurements

We determined whether placing the cells into a Na^+-free solution depleted them of ATP. The cells that had not been incubated in any buffer contained 7.2 ± 0.5 nmol/mg of cell protein (Table 1). Incubating them under the conditions of the DHAA-uptake experiments for 2 or 12 min in Na^+-containing or Na^+-free buffers did not significantly change their ATP content ($P > 0.05$; Table 1).

DISCUSSION

The results show that in pig coronary artery smooth-muscle cells, DOG and cytochalasin B decreased the accumulation of [^{14}C]DHAA, consistent with DHAA entering the cells via a GLUT transporter. However, uptake was inhibited partially by Na^+ removal and by Na^+/H^+-exchange inhibitors, which also lowered the intracellular pH and may have decreased the conversion of cellular DHAA into ascorbate.

The properties of [^{14}C]DHAA accumulation by smooth-muscle cells are consistent with the entry of DHAA via a GLUT transporter. It was inhibited by the competitor DOG and by the GLUT inhibitor cytochalasin B preferentially over cytochalasin D. This is in contrast with the transport of ascorbate. The only property shared with the ascorbate transport system was inhibition by removal of extracellular Na^+. This was the apparent paradox that required resolution

Accumulation of DHAA via GLUT transporters is Na^+-independent in human melanoma cells and melanocytes, fibroblasts, lymphoblasts and human placental choriocarcinoma cells [17,18,30,31]. Replacing Na^+ with NMG, sucrose or choline did not affect the retention of DHAA by these cells. However, in simian virus-40-transformed retinal pigment epithelial cells, Na^+ removal inhibited [^{14}C]DHAA, but only at extremely high DHAA concentrations [9]. In HL-60 cells, Na^+ substitution by choline, Li^+ or sucrose did not affect the initial velocity of [^{14}C]DHAA accumulation but decreased the maximum uptake over 120 min [33]. In hepatocytes, an effect of Na^+ on vitamin C recycling was suggested but no evidence was provided [34]. From these studies, it appears that the effect of Na^+ removal on DHAA uptake may be cell- or tissue-specific. Where an effect of Na^+ on DHAA accumulation was observed previously, the underlying mechanism was not reported. The inhibitory effect of Na^+ removal on the [^{14}C]DHAA uptake at first glance is paradoxical because the transport of DOG via the GLUT transporters is Na^+ independent. However, cellular entry is only the first step in the facilitated transport of [^{14}C]DHAA, and its conversion into ascorbate is essential for its intracellular retention and/or continued influx. We showed that Na^+ removal or inhibiting Na^+/H^+ exchange lead to intracellular acidosis that is associated with the impairment in the conversion of DHAA into ascorbate. As energy would be required to reduce DHAA, we determined whether the Na^+ depletion accompanies a decrease in the cellular ATP content. However, the ATP content of the cells was not affected. We cannot rule out that in Na^+-free buffer the conversion was poorer, because either the NADPH- and GSH-dependent DHAA reductase activities [35–39] are compromised or the levels of these cofactors have decreased. This issue needs resolution to understand fully the effect of acidosis on these cells. Even though Na^+ removal affects the ratio of ascorbate to ^{14}C content in Figure 7, it is not clear if the small but statistically significant difference is sufficient to produce a 2–3-fold difference in the ^{14}C retained during the DHAA uptake. We previously reported that Na^+ depletion is known to cause an increase in the [^{14}C]ascorbate efflux from pig coronary artery smooth-muscle cells [8]. Therefore, Na^+ depletion may decrease the [^{14}C]DHAA accummulation due to poorer conversion into ascorbate and also via a loss of the [^{14}C]ascorbate formed after the conversion from DHAA inside the cells. An inability to convert DHAA into ascorbate at the

lower cytosolic pH could lead to a shallower DHAA gradient across the cell membrane. This decrease in the DHAA gradient or the lower cytosolic pH itself may contribute to a decrease in the influx of [^{14}C]DHAA.

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