

## Activation of L-arginine transport by protein kinase C in rabbit, rat and mouse alveolar macrophages

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1. The role of protein kinase C in controlling L-arginine transport in alveolar macrophages was investigated.
2. L-[<sup>3</sup>H]Arginine uptake in rabbit alveolar macrophages declined by 80% after 20 h in culture. 4 $\beta$ -Phorbol 12-myristate 13-acetate (PMA), but not 4 $\alpha$ -phorbol 12-myristate 13-acetate ( $\alpha$ -PMA), present during 20 h culture, enhanced L-[<sup>3</sup>H]arginine uptake more than 10-fold. Staurosporine and chelerythrine opposed this effect.
3. L-[<sup>3</sup>H]Arginine uptake was saturable and blockable by L-lysine. After PMA treatment  $V_{\max}$  was increased more than 5-fold and  $K_m$  was reduced from 0.65 to 0.32 mM.
4. Time course experiments showed that PMA increased L-[<sup>3</sup>H]arginine uptake almost maximally within 2 h. This short-term effect was not affected by cycloheximide or actinomycin D.
5. L-[<sup>3</sup>H]Arginine uptake and its stimulation by PMA was also observed in sodium-free medium.
6. L-Leucine (0.1 mM) inhibited L-[<sup>3</sup>H]arginine uptake by 50% in sodium-containing medium, but not in sodium-free medium. At 1 mM, L-leucine caused significant inhibition in sodium-free medium also. L-Leucine showed similar effects on PMA-treated cells.
7. N-Ethylmaleimide (200  $\mu$ M, 10 min) reduced L-[<sup>3</sup>H]arginine uptake by 70% in control cells, but had no effect on PMA-treated (20 or 2 h) cells.
8. In alveolar macrophages, multiple transport systems are involved in L-arginine uptake, which is markedly stimulated by protein kinase C, probably by modulation of the activity of already expressed cationic amino acid transporters.

The amino acid L-arginine, besides being involved in protein synthesis, is the substrate of nitric oxide (NO) synthase and arginase, two enzymes which play a particular role in alveolar macrophages and other cells of the monocyte/macrophage lineage. Arginase, which catalyses the hydrolysis of L-arginine to L-ornithine, a precursor in polyamine synthesis (Tabor & Tabor, 1984), appears to be expressed constitutively in macrophages including alveolar macrophages, although the level of its expression can be modulated by different stimuli (e.g. Hey *et al.* 1995; Modolell *et al.* 1995; Wang *et al.* 1995). On the other hand, NO synthase is not expressed constitutively in macrophages and alveolar macrophages, but can be induced; and typical inductive stimuli are bacterial toxins and/or pro-inflammatory cytokines (e.g. Jorens *et al.* 1991; Hey *et al.* 1995; for review see Förstermann & Kleinert, 1995). However, marked species differences exist with regard to the mechanisms controlling the induction of NO synthase in

macrophages. Thus, in macrophages or alveolar macrophages of rats and mice NO synthase can easily be induced, whereas in other species, such as rabbits and humans for example, the induction of NO synthase appears to be much more complex (e.g. Hey *et al.* 1995; for review see Dugas *et al.* 1995).

In macrophages the intracellular availability of L-arginine may become rate limiting for both the NO synthase and arginase pathways, particularly under the conditions when high levels of NO synthase are induced (e.g. Hibbs *et al.* 1987; Hey *et al.* 1997). The transport of L-arginine into cells is mediated via specific cationic amino acid transporters and several systems have been differentiated, both functionally (e.g. system y<sup>+</sup>, y<sup>+</sup>L, b<sup>0,+</sup> or B<sup>0,+</sup>) and at the molecular level (e.g. White, 1985; van Winkle *et al.* 1985; van Winkle *et al.* 1988; Devés *et al.* 1993; for review see Closs, 1996; Malandro & Kilberg, 1996). The cellular uptake of L-arginine may be one important factor controlling the intracellular availability of this amino acid. Therefore, it appeared to be of particular

interest to elucidate the mechanisms involved in the control of L-arginine transport in macrophages. In murine macrophage J774 cells it has already been observed that induction of NO synthase is associated with an increase in L-arginine transport (Bogle *et al.* 1992). However, induction of NO synthase, but not L-arginine transport, was inhibited by dexamethasone (Baydoun *et al.* 1993), suggesting that the activity of L-arginine transport in macrophages may be controlled via specific and distinct pathways.

The protein kinase C signal transduction pathway plays a key role in the activation of cells of the monocyte/macrophage lineage (e.g. Shapira *et al.* 1994; Mayer *et al.* 1995; Tapper & Sundler, 1995). In addition, protein kinase C appears also to be one of the pathways involved in the induction of NO synthase (Paul *et al.* 1995). Therefore, the primary aim of the present study was to characterize L-arginine transport in alveolar macrophages and to elucidate the role of protein kinase C in the control of its activity.

In the present study, alveolar macrophages of rat and rabbit were used, as these two species represent examples in which marked differences with regard to the expression of inducible NO synthase have been described in alveolar macrophages (Hey *et al.* 1995). As most of the molecular knowledge about cationic amino acid transporters is currently available for the mouse species (for review see Closs, 1996; Malandro & Kilberg, 1996), a few additional comparative experiments were also carried out on mouse alveolar macrophages.

## METHODS

### Preparation and culture of alveolar macrophages

Mongrel rabbits (2–2.5 kg, Lammers, Euskirchen), Sprague–Dawley rats or NMRI mice (both bred at the University of Bonn) of either sex were killed by stunning followed by exsanguination. Lung and trachea were excised *en bloc*, washed with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (D-PBS) and lavaged three times by instilling about 60 ml (rabbit lung), 15 ml (rat lung) or 1–2 ml (mouse lung) of cold (4 °C) D-PBS (Hey *et al.* 1995). Usually for one preparation of alveolar macrophages, lavage fluids of one or two rabbit lungs, six to eight rat lungs or about forty mice lungs were pooled and centrifuged at 400 *g* for 5 min. The cells were washed three times with D-PBS and thereafter resuspended in Dulbecco's modified Eagle's medium–Ham's F-12 medium (DMEM–F-12 medium) supplemented with 5% fetal calf serum, 100 U ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin and 5 µg ml<sup>-1</sup> amphotericin B and plated in sterile 6-well dishes (NUNC; 3 × 10<sup>6</sup> cells well<sup>-1</sup>). The alveolar macrophages were allowed to adhere for 2 h (37 °C, 5% CO<sub>2</sub>). After 2 h the medium was renewed to remove non-adherent cells. The adherent cells consisted of more than 95% alveolar macrophages according to morphological criteria (May Grünwald-Giemsa staining). Thereafter, cells were either used directly for uptake studies or cultured for 20 h in the absence or presence of test substances followed by the uptake studies. In some experiments, *N*-ethylmaleimide (NEM, 200 µM) was added for the last 10 min of the culture period followed by the uptake studies. Finally, in some experiments test substances were added to the culture medium after the 20 h culture period and cells were cultured for an additional 0.25–6 h followed by the uptake studies. Cell

viability assessed by Trypan Blue exclusion was always greater than 95%.

### Measurement of L-[<sup>3</sup>H]arginine uptake in alveolar macrophages

L-[<sup>3</sup>H]Arginine uptake was measured immediately after the adherence period of the alveolar macrophages (no culture) or after different time periods in culture, mostly 20 h. Adherent alveolar macrophages were rinsed once with 2 ml of prewarmed Krebs–Hepes solution of the following composition (mM): NaH<sub>2</sub>PO<sub>4</sub>, 0.001; NaCl, 118.5; KCl, 5.57; CaCl<sub>2</sub>, 1.25; MgCl<sub>2</sub>, 1.2; Na<sub>2</sub>EDTA, 0.03; L-(+)-ascorbic acid, 0.06; Hepes, 20.0 (adjusted to pH 7.4 using NaOH); and D-(+)-glucose, 11.1.

In most experiments alveolar macrophages were incubated with Krebs–Hepes solution containing 0.1 or 100 µM L-[<sup>3</sup>H]arginine (37 kBq) for 2 or 5 min, respectively. Incubation was terminated by placing the plates on melting ice and rinsing alveolar macrophages three times with 2 ml ice-cold Krebs–Hepes solution containing 10 mM unlabelled L-arginine. Finally, alveolar macrophages were extracted in 1 ml 0.4 M HClO<sub>4</sub> and radioactivity in the cell extracts was measured by liquid scintillation spectrometry in a Beckman LS 5000TD. External standardization was used to correct for counting efficiency. In one initial series of experiments the time of exposure to L-[<sup>3</sup>H]arginine was varied from 1 to 25 min (Fig. 1). L-[<sup>3</sup>H]arginine uptake was expressed as d.p.m. per 3 × 10<sup>6</sup> cells, as picomoles (taking into account the specific activity of the labelled amino acid) or as a percentage of the uptake observed in controls of the respective cell preparation.

The sodium dependency of L-[<sup>3</sup>H]arginine uptake was studied by replacing NaCl by equimolar choline chloride. When the temperature dependency of the L-[<sup>3</sup>H]arginine uptake was studied, the culture medium was removed and the cells were incubated for 10 min in Krebs–Hepes medium at 37, 30 or 23 °C immediately followed by the uptake protocol at the respective temperature. The temperature dependency of L-[<sup>3</sup>H]arginine was calculated using Arrhenius's rate equation. The ln rate of arginine uptake  $k$  (pmol min<sup>-1</sup>) was plotted against the reciprocal of absolute temperature (10<sup>3</sup> T<sup>-1</sup>) using linear regression analysis. The activation energy ( $\mu$ ) was calculated from the regression equation,  $\ln k = \ln k_0 - \mu R^{-1} T^{-1}$ , which was transformed to  $\mu = RT(\ln k_0 - \ln k)$ ,  $R$  being the gas constant and  $T$  the absolute temperature. The temperature coefficient  $Q_{10}$  between the temperatures  $T_1$  and  $T_2$  was obtained from  $\ln Q_{10} = 10\mu R^{-1} T_1^{-1} T_2^{-1}$  (Muscholl & Ritzel, 1980).

For determination of the kinetic parameters of L-[<sup>3</sup>H]arginine uptake alveolar macrophages were incubated with different concentrations of L-[<sup>3</sup>H]arginine (10 nM to 3 mM) and accumulation was analysed using the computer program GraphPad Prism (GraphPad Software, San Diego, CA, USA) by fitting the data to Michaelis–Menten kinetics (Fig. 3).

### Extraction of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from mouse and rat alveolar macrophages using Trizol reagent. The first strand cDNA was synthesized from 2 µg of total RNA using oligo(dT)<sub>18</sub> primer and avian myoblastosis virus (AMV) reverse transcriptase under the conditions recommended by the supplier (Promega, Mannheim, Germany). The cDNA product was used for subsequent amplification by PCR. Oligonucleotide primers were constructed based on the European Molecular Biology Laboratory (EMBL) sequences for rat (r)  $\beta$ -actin (accession number V01217; J00691), mouse (m)  $\beta$ -actin (M12481; X03765), rCAT-1 (L10151; one mismatch for mCAT-1) and m/rCAT-2B (M62838; M32485; U53927; 100% homology between

the corresponding sequences mCAT-2B and rCAT-2B). Amplification of the cDNAs with the  $\beta$ -actin primers served as a positive control for the quality and quantity of the cDNAs. Primer pairs for r $\beta$ -actin were: 5'-GCACCAGGGTGTGATGGTGGGTA-3' (sense; nucleotides 117–139) and 5'-AGAGGTCCTTACGGATGTCAACG-3' (antisense; 880–858); m $\beta$ -actin: 5'-GTTACCAACTGGACGACATGGA-3' (sense; 148–170 partial sequence) and 5'-CTTCATGGTGTAGGAGCCAGAG-3' (antisense; 900–878 partial sequence); rCAT-1: 5'-GCTGCCTCAACACCTATGATCTGG-3' (sense; 89–112) and 5'-ACGATGCCACAGGAATGGC-3' (antisense; 857–838); m/rCAT-2B: 5'-ATGGTGGCTGGGTTTG-TGAAAG-3' (sense; 616–637) and 5'-CAACCCATCCTCCGCCATAGC-3' (antisense; 1116–1096). PCR amplification was performed using Taq DNA polymerase and specific primers in a programmable thermal reactor (Hybaid, Middlesex, UK) with initial heating for 3 min at 94 °C, followed by thirty-five cycles of 45 s denaturation at 94 °C, annealing at 56 °C (30 s), extension at 72 °C (1 min), and a final extension for 10 min at 72 °C. PCR products were separated by 1.2% agarose gel electrophoresis and documented by a video documentation system (MWG, Ebersberg, Germany).

### Statistics

If not stated otherwise, values are means  $\pm$  s.e.m. of  $n$  experiments; usually one to three determinations of the respective test protocol were carried out on one cell preparation, but at least three separate cell preparations were used for each protocol. Statistical significance of differences was evaluated by Student's  $t$  test. When multiple comparisons were performed the significance of differences was evaluated by ANOVA followed by the modified  $t$  test according to Bonferroni using the computer program GraphPad InStat (GraphPad Software). When groups of different variances were compared, Welch's test was applied.  $P < 0.05$  was accepted as being significant.

### Drugs and special chemicals

Actinomycin D, amphotericin B, L-arginine HCl, chelerythrine HCl, cycloheximide, cytochalasin B, Dulbecco's phosphate-buffered saline without calcium and magnesium (D-PBS), DMEM-F-12 medium,

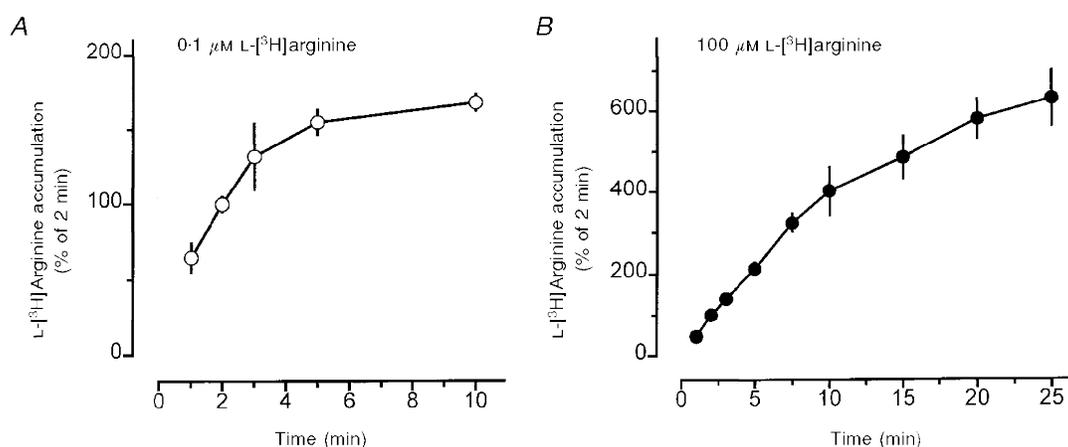
*N*-ethylmaleimide (NEM), L-leucine, L-lysine HCl, 4 $\beta$ -phorbol 12-myristate 13-acetate (PMA), and staurosporine were all from Sigma; AMV reverse transcriptase was from Promega (Mannheim, Germany); L-[2,3- $^3$ H]arginine HCl (36–40 Ci mmol $^{-1}$ ) was from Sigma or Dupont; choline chloride was from Fluka; fetal calf serum was from Vitromex (Vilshofen, Germany); 4 $\alpha$ -phorbol 12-myristate 13-acetate ( $\alpha$ -PMA) was from Biomol (Hamburg, Germany); all primers for RT-PCR were from MWG (Ebersberg, Germany); Trizol reagent and Taq DNA polymerase were from Gibco.

Stock solutions (10 mM) were prepared in dimethyl sulphoxide (DMSO) for actinomycin D, cytochalasin B, PMA,  $\alpha$ -PMA and staurosporine, and in ethanol for NEM. Lipopolysaccharide (LPS; 500  $\mu$ g ml $^{-1}$ ) was dissolved in D-PBS. None of the solvents applied alone at the concentration which was applied with the highest concentration of the respective drug affected L-[ $^3$ H]arginine uptake (each  $n = 3$ , data not shown).

## RESULTS

### Characteristics of L-[ $^3$ H]arginine uptake in rabbit alveolar macrophages

When freshly prepared rabbit alveolar macrophages were incubated immediately after a 2 h adherence period ('no culture') with L-[ $^3$ H]arginine (37 kBq ml $^{-1}$ , 0.1  $\mu$ M or 100  $\mu$ M) for different time intervals, a time-dependent, saturable accumulation of radioactivity was observed (Fig. 1). Accumulation of 0.1  $\mu$ M L-[ $^3$ H]arginine into rabbit alveolar macrophages increased almost linearly between 1 and 3 min. Thereafter, the rate of increase decreased rapidly reaching a plateau by between 5 and 10 min (Fig. 1A). When 100  $\mu$ M L-[ $^3$ H]arginine was used, the linear phase of accumulation was more prolonged, up to about 10 min (Fig. 1B). Therefore, in subsequent experiments an incubation period of 2 or 5 min was used to study initial uptake of 0.1 and 100  $\mu$ M L-[ $^3$ H]arginine, respectively. In



**Figure 1.** Time-dependent accumulation of L-[ $^3$ H]arginine in rabbit alveolar macrophages

Freshly prepared alveolar macrophages were allowed to adhere in culture dishes for 2 h ( $3 \times 10^6$  cells well $^{-1}$ ). Thereafter, alveolar macrophages were incubated for 1–10 min (A) or 1–25 min (B) in amino acid-free Krebs–Hepes solution to which either 0.1  $\mu$ M (○) or 100  $\mu$ M (●) L-[ $^3$ H]arginine (37 kBq) was added. Ordinate: radioactivity in cell extracts expressed as a percentage of the mean accumulation observed during 2 min incubation of the respective cell preparation (for absolute values see text and Table 1). Values are means  $\pm$  s.e.m. of 4–7 experiments.

**Table 1.** Effect of 20 h culture in the absence or presence of PMA on L-[<sup>3</sup>H]arginine uptake in rabbit, rat and mouse alveolar macrophages

	Rabbit	Rat	Mouse
No culture			
Uptake in pmol ( $3 \times 10^6$ cells) <sup>-1</sup>	0.56 ± 0.08 (33)	1.28 ± 0.16 (16)‡	3.13 ± 0.31 (6)
Uptake as % of mean of individual cell preparation	100 ± 3 (33)	100 ± 2 (16)	100 ± 5 (6)
20 h culture control			
Uptake in pmol ( $3 \times 10^6$ cells) <sup>-1</sup>	0.13 ± 0.01 (116)**	1.05 ± 0.11 (15)	8.84 ± 2.22 (10)*
Uptake as % of mean of individual 'no culture'	19 ± 4 (17)**	54 ± 4 (15)**	246 ± 43 (4)**
20 h culture + PMA			
Uptake in pmol ( $3 \times 10^6$ cells) <sup>-1</sup>	0.91 ± 0.07 (96)†	2.51 ± 0.19 (22)†	15.77 ± 3.93 (8)
Uptake as % of mean individual '20 h culture control'	1475 ± 157 (76)†	268 ± 20 (22)†	169 ± 12 (8)†

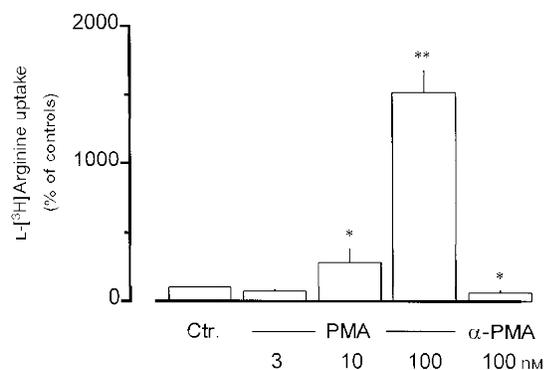
L-[<sup>3</sup>H]Arginine uptake in rabbit, rat and mouse alveolar macrophages was studied immediately after adherence (no culture) or after 20 h culture in the absence or presence of PMA (100 nM). L-[<sup>3</sup>H]Arginine uptake was measured by determining the cellular radioactivity after 2 min incubation in amino acid-free Krebs–Hepes solution to which L-[<sup>3</sup>H]arginine (37 kBq, 0.1 μM; see Fig. 1A) was added. L-[<sup>3</sup>H]Arginine uptake per 2 min is expressed as pmol ( $3 \times 10^6$  cells)<sup>-1</sup> or as a percentage of the mean uptake observed in the respective individual cell preparation 'no culture' or '20 h culture control'. Results are means ± s.e.m. with the number of experiments (*n*) in parentheses. Usually, 2–3 individual experiments of a treatment protocol were carried out on the same cell preparation, and one cell preparation was obtained from lungs of 1–2 rabbits, 6–8 rats or about 40 mice. \**P* < 0.05, \*\* *P* < 0.01 compared with the respective 'no culture' value; † *P* < 0.01 compared with the respective '20 h culture control' value.

the kinetics study, however, a 2 min incubation period was used for all concentrations of L-[<sup>3</sup>H]arginine. The absolute amounts of radioactivity accumulated in freshly prepared, non-cultured rabbit alveolar macrophages during 2 min incubation with 0.1 μM or 5 min incubation with 100 μM L-[<sup>3</sup>H]arginine were  $8714 \pm 1221$  and  $12400 \pm 930$  d.p.m. ( $3 \times 10^6$  cells)<sup>-1</sup>, respectively, corresponding to  $0.56 \pm 0.08$  and  $793 \pm 59$  pmol L-arginine ( $3 \times 10^6$  cells)<sup>-1</sup>. Generally, accumulated radioactivity showed much smaller variations within one cell preparation than between different cell preparations. Therefore, data were mostly expressed as a percentage of controls or of a reference treatment group within an individual cell preparation. If not stated otherwise, the tracer concentration of L-[<sup>3</sup>H]arginine (0.1 μM) was used as the standard condition to study L-arginine uptake.

Time course experiments showed that the L-[<sup>3</sup>H]arginine uptake in alveolar macrophages changed depending on the

time period in culture. After the first hours in culture L-[<sup>3</sup>H]arginine uptake was increased, maximally by about 180% after 6–8 h (data not shown). Thereafter, it declined continuously reaching a level of about 20% after 20 h in culture (Table 1).

The protein kinase C-activating phorbol ester PMA (Blumberg, 1980; Ashendal, 1985), present during the 20 h culture period, caused a concentration-dependent increase in L-[<sup>3</sup>H]arginine uptake. At 100 nM, the highest concentration of PMA tested, a large increase was observed (Fig. 2, Table 1). Thus, the presence of PMA not only opposed the culture-dependent decline in L-[<sup>3</sup>H]arginine uptake, but enhanced it above the levels observed in non-cultured alveolar macrophages (Table 1). It should be noted that the concentration response for this effect of PMA was rather steep, since 10 nM PMA caused an increase by only about 200% (Fig. 2). It should be emphasized that in Fig. 2 a large

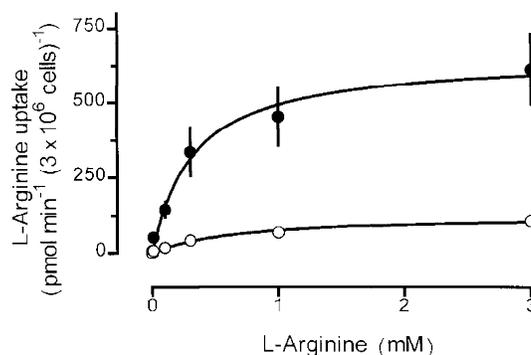


**Figure 2.** Effects of PMA and α-PMA on L-[<sup>3</sup>H]arginine uptake in rabbit alveolar macrophages

Alveolar macrophages were cultured for 20 h in the absence (Ctr.) or presence of PMA or α-PMA (at the concentrations indicated). Thereafter, L-[<sup>3</sup>H]arginine uptake was studied during a 2 min incubation period in amino acid-free Krebs–Hepes solution to which L-[<sup>3</sup>H]arginine (37 kBq, 0.1 μM) was added. Height of columns: L-[<sup>3</sup>H]arginine uptake expressed as a percentage of mean uptake observed in the controls of the respective cell preparation (Ctr.). Values are means ± s.e.m. of 6–10 experiments. \**P* < 0.05; \*\**P* < 0.01, compared with Ctr.

### Figure 3. Effect of PMA on the kinetics of L-arginine uptake in rabbit alveolar macrophages

Alveolar macrophages were cultured for 20 h in the absence (○) or presence of 100 nM PMA (●). Thereafter, L-[<sup>3</sup>H]arginine uptake was studied during a 2 min incubation period in amino acid-free Krebs–Hepes solution to which L-[<sup>3</sup>H]arginine, at the concentrations indicated by the abscissa, was added. L-Arginine uptake was calculated from the accumulated radioactivity taking into account the specific activity of the tracer. Values are means  $\pm$  s.e.m. of 9–14 experiments of 7 separate cell preparations.



number of experiments were pooled and that the magnitude of the effect of PMA showed considerable variance between different series of experiments ranging from an increase of about 5-fold to a more than 15-fold increase. Therefore, when the effect of PMA was further characterized (see below), the effect of PMA on the actual cell preparation had to be used as a reference.  $\alpha$ -PMA (100 nM), a phorbol ester which does not activate protein kinase C (Blumberg, 1980), did not cause an increase, but rather reduced L-[<sup>3</sup>H]arginine uptake by 40% (Fig. 2).

Analysis of the kinetics of L-[<sup>3</sup>H]arginine uptake in rabbit alveolar macrophages (Fig. 3) revealed that  $V_{\max}$  was increased from  $127 \pm 8.9$  pmol min<sup>-1</sup> ( $3 \times 10^6$  cells)<sup>-1</sup> (mean  $\pm$  s.d.) in control alveolar macrophages to  $655 \pm 32$  pmol min<sup>-1</sup> ( $3 \times 10^6$  cells)<sup>-1</sup> (mean  $\pm$  s.d.) after culture in the presence of 100 nM PMA ( $P < 0.01$ ), whereas the  $K_m$  was reduced from  $0.65 \pm 0.13$  mM (mean  $\pm$  s.d.) in controls to  $0.32 \pm 0.05$  mM (mean  $\pm$  s.d.) after culture in the presence of PMA ( $P < 0.05$ ).

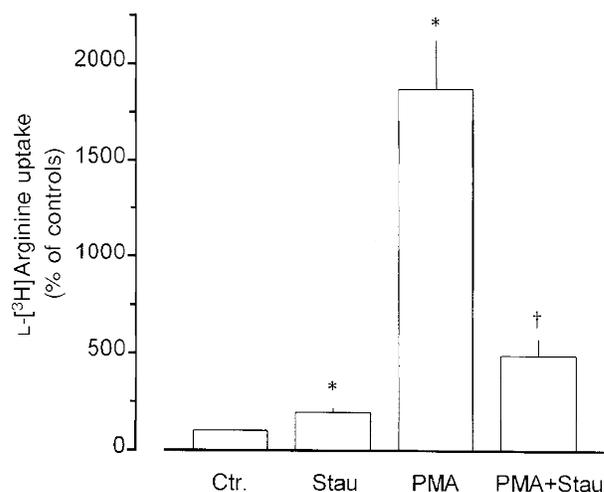
Staurosporine (100 nM, present during the 20 h culture period), although causing a small, but significant increase in L-[<sup>3</sup>H]arginine uptake by its own, largely inhibited the stimulatory effect of 100 nM PMA (Fig. 4). Chelerythrine alone had no effect, but when applied during the culture period at 10 and 30  $\mu$ M it inhibited the stimulatory effect of

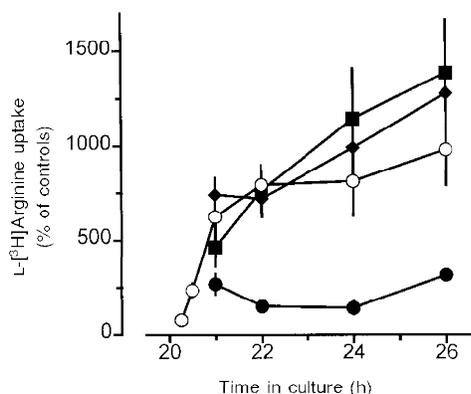
100 nM PMA by  $49 \pm 8$  and  $62 \pm 7\%$ , respectively (each  $n = 8$ ).

When PMA (100 nM) was added after the 20 h culture period for 0.25–6 h, it caused a rapid increase in L-[<sup>3</sup>H]arginine uptake, which was significantly different from control levels by 0.5 h ( $149 \pm 44\%$ ,  $n = 6$ ;  $P < 0.05$  vs. respective time controls) and almost maximal after about 1–2 h (Fig. 5). The magnitude of this short-term stimulatory effect of PMA was in the range of that observed after PMA had been present for 20 h (compare Fig. 5 vs. Figs 2 and 6; Fig. 8). This short-term effect of 100 nM PMA was largely inhibited by 100 nM staurosporine (Fig. 5). Likewise, chelerythrine present at 10 and 30  $\mu$ M together with 100 nM PMA for 2 h inhibited the short-term stimulatory effect of PMA by  $56 \pm 7$  and  $72 \pm 6\%$ , respectively (each  $n = 8$ ). However, this short-term effect of 100 nM PMA was not affected by cycloheximide or by actinomycin D (Fig. 5). Neither drug, when present alone for up to 6 h, affected L-[<sup>3</sup>H]arginine uptake significantly (data not shown). On the other hand, when cycloheximide (30  $\mu$ M) or actinomycin D (5  $\mu$ g ml<sup>-1</sup>) had been present during the 20 h culture period, L-[<sup>3</sup>H]arginine uptake was reduced by  $54 \pm 13\%$  ( $n = 6$ ) and  $79 \pm 6\%$  ( $n = 8$ ), respectively. When present for 20 h together with 100 nM PMA, cycloheximide and actinomycin D reduced L-[<sup>3</sup>H]arginine uptake by  $68 \pm 14\%$  ( $n = 10$ ) and  $98 \pm 2\%$

### Figure 4. Effects of PMA and staurosporine on L-[<sup>3</sup>H]arginine uptake in rabbit alveolar macrophages

Alveolar macrophages were cultured for 20 h in the absence (Ctr) or presence of staurosporine (Stau, 100 nM) or PMA (100 nM) alone or in combination, as indicated. Thereafter, L-[<sup>3</sup>H]arginine uptake was studied during a 2 min incubation period in amino acid-free Krebs–Hepes solution to which L-[<sup>3</sup>H]arginine (37 kBq, 0.1  $\mu$ M) was added. Height of columns: L-[<sup>3</sup>H]arginine uptake expressed as a percentage of the mean uptake observed in the controls of the respective cell preparation (Ctr). Results are means  $\pm$  s.e.m. of 6–22 experiments. \* $P < 0.01$ , compared with Ctr.; † $P < 0.01$ , compared with PMA alone.



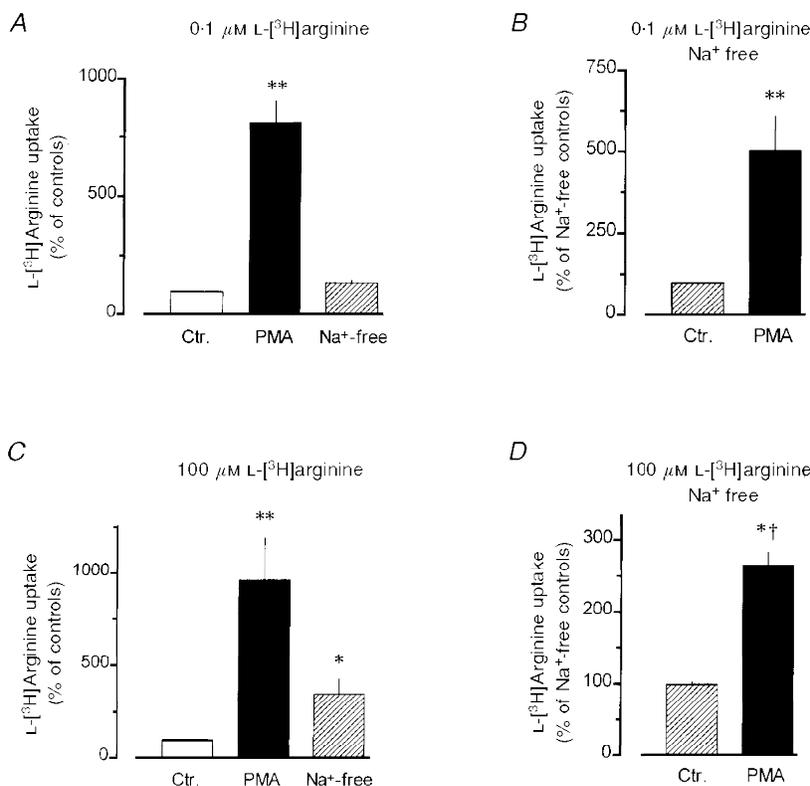


**Figure 5. Characterization of short-term effects of PMA on L-[<sup>3</sup>H]arginine uptake in rabbit alveolar macrophages**

After alveolar macrophages had been cultured for 20 h in the absence of test substances, PMA alone (100 nM, ○) or in combination with cycloheximide (30 μM, ◆), actinomycin D (5 μg ml<sup>-1</sup>, ■) or staurosporine (100 nM, ●) was added to the medium and the cells were cultured for an additional 0.25–6 h as indicated by the abscissa. Thereafter, L-[<sup>3</sup>H]arginine uptake was studied during a 2 min incubation period in amino acid-free Krebs–Hepes solution to which L-[<sup>3</sup>H]arginine (37 kBq, 0.1 μM) was added. Ordinate: L-[<sup>3</sup>H]arginine uptake expressed as percentage of the mean uptake observed in the respective time controls of the individual cell preparation. Values are means ± s.e.m. of 3–13 experiments.

(*n* = 4), respectively, of the PMA-stimulated controls. Considering the about 10-fold elevated levels in the PMA-stimulated controls, it is evident that PMA still caused a significant (about 7-fold) increase in the presence of cycloheximide, but failed to enhance L-[<sup>3</sup>H]arginine uptake in the presence of actinomycin D.

L-Lysine (10 mM) inhibited the uptake of 0.1 and 100 μM L-[<sup>3</sup>H]arginine by 96 ± 1 and 91 ± 2%, respectively, in alveolar macrophages cultured in the absence of PMA and by 97 ± 1% and 89 ± 3%, respectively, in alveolar macrophages cultured in the presence of PMA (*n* = 6–9). Replacing the sodium ions in the incubation medium by



**Figure 6. Effect of removal of sodium ions on L-[<sup>3</sup>H]arginine uptake in rabbit alveolar macrophages and its modulation by PMA**

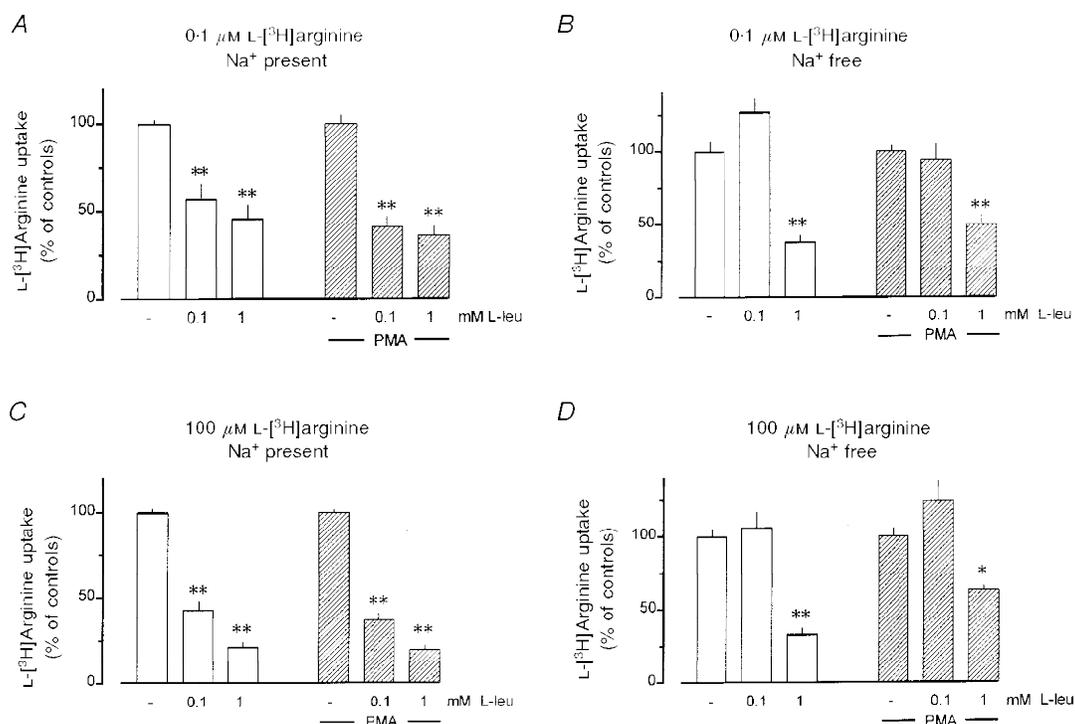
Alveolar macrophages were cultured for 20 h in the absence (Ctr.) or presence of 100 nM PMA, as indicated. Thereafter, L-[<sup>3</sup>H]arginine uptake was studied during a 2 min (*A* and *B*) or 5 min (*C* and *D*) period of incubation in amino acid-free Krebs–Hepes solution to which 0.1 μM (*A* and *B*) or 100 μM (*C* and *D*) L-[<sup>3</sup>H]arginine (37 kBq) was added. In the experiments shown in parts *B* and *D* as well as in the ones given by the 'Na<sup>+</sup>-free' columns of parts *A* and *C*, sodium ions of the incubation medium were replaced by equimolar choline. Height of columns: L-[<sup>3</sup>H]arginine uptake expressed as a percentage of the mean uptake observed in the controls of the respective cell preparation. Note the different scales. Results are means ± s.e.m. of 9–27 experiments. \**P* < 0.05, \*\**P* < 0.01 compared with respective Ctr.; †*P* < 0.05, compared with the respective observations in sodium-containing medium.

choline had no significant effect on uptake of  $0.1 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine (Fig. 6A), but enhanced uptake of  $100 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine by about 280% (Fig. 6C). After treatment with PMA L-[ $^3\text{H}$ ]arginine uptake was also enhanced under sodium-free conditions (Fig. 6B and D), but at  $100 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine the magnitude of the stimulatory effect of PMA was significantly smaller than that observed in sodium-containing medium (Fig. 6C and D). However, the uptake in sodium-free controls was already markedly elevated so that the maximum level of L-arginine uptake reached after PMA treatment appeared to be similar in sodium-free and sodium-containing media. This is also indicated by the absolute amounts of L-arginine taken up in these experiments ( $169 \pm 21 \text{ pmol } (3 \times 10^6 \text{ cells})^{-1} (5 \text{ min})^{-1}$  ( $n = 18$ ) and  $215 \pm 39 \text{ pmol } (3 \times 10^6 \text{ cells})^{-1} (5 \text{ min})^{-1}$  ( $n = 27$ ) after PMA treatment in sodium-containing and sodium-free medium, respectively).

In order to further characterize L-arginine transport, the effect of the neutral amino acid L-leucine on the uptake of  $0.1$  and  $100 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine was investigated (Fig. 7). Inhibition of L-[ $^3\text{H}$ ]arginine by L-leucine was sodium

dependent. Thus, in the absence of sodium ions,  $0.1 \text{ mM}$  L-leucine failed to affect L-[ $^3\text{H}$ ]arginine uptake under all conditions studied ( $0.1$  and  $100 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine, each in macrophages cultured in the absence and presence of PMA, Fig. 7B and D). However, in the presence of sodium ions,  $0.1 \text{ mM}$  L-leucine inhibited the uptake of  $0.1 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine by 43% in control alveolar macrophages and by 59% in cells cultured in the presence of PMA (Fig. 7A). Likewise, in the presence of sodium ions,  $0.1 \text{ mM}$  L-leucine inhibited the uptake of  $100 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine by 56 and 63% in control and PMA-treated macrophages, respectively (Fig. 7C). At the higher concentration of  $1 \text{ mM}$ , the inhibitory effect of L-leucine in the presence of sodium ions was somewhat greater, particularly in the experiments with  $100 \mu\text{M}$  L-[ $^3\text{H}$ ]arginine (Fig. 7C), but, in addition,  $1 \text{ mM}$  L-leucine also caused a clear inhibition in the absence of sodium ions, under all conditions studied (Fig. 7B and D).

When NEM ( $200 \mu\text{M}$ ) was added for the last 10 min of the culture period, subsequent uptake of L-[ $^3\text{H}$ ]arginine was reduced by 71% (Fig. 8). On the other hand, pretreatment with NEM failed to affect the uptake of L-[ $^3\text{H}$ ]arginine in



**Figure 7.** Sodium-dependent effects of L-leucine on L-[ $^3\text{H}$ ]arginine uptake in rabbit alveolar macrophages

Alveolar macrophages were cultured for 20 h in the absence (Ctr.) or presence of  $100 \text{ nM}$  PMA, as indicated. Thereafter, L-[ $^3\text{H}$ ]arginine uptake was studied during a 2 min (A, B) or 5 min (C, D) period of incubation in amino acid-free Krebs-Hepes solution to which  $0.1 \mu\text{M}$  (A, B) or  $100 \mu\text{M}$  (C, D) L-[ $^3\text{H}$ ]arginine (37 kBq) was added. In the experiments shown in B and D, sodium ions of the incubation medium were replaced by equimolar choline. The values below the columns indicate the millimolar concentrations of L-leucine (L-leu) added to incubation medium. Height of columns: L-[ $^3\text{H}$ ]arginine uptake expressed as a percentage of the mean uptake observed in the controls of the respective cell preparation. Results are means + s.e.m. of 9–27 experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared with respective experiments in the absence of L-leucine.

alveolar macrophages which had been exposed for either 20 or 2 h to PMA (Fig. 8).

Finally, L-arginine transport into alveolar macrophages showed a marked temperature dependency. Thus, in control alveolar macrophages L-[<sup>3</sup>H]arginine uptake was reduced by  $58 \pm 3$  and  $87 \pm 1\%$  (each  $n=9$ ), when the incubation temperature was reduced from 37 °C to 30 and 23 °C, respectively. In alveolar macrophages cultured in the presence of PMA the temperature dependency of L-arginine transport was significantly attenuated. Thus, after reduction of the incubation temperature to 30 and 23 °C, L-[<sup>3</sup>H]arginine uptake was reduced by only  $33 \pm 5\%$  and  $76 \pm 3\%$  (each  $n=9$ ), respectively (each  $P < 0.01$  vs. controls). The activation energy (determined by performing Arrhenius plots), was reduced from  $110.4 \text{ kJ mol}^{-1}$  in control alveolar macrophages to  $81.9 \text{ kJ mol}^{-1}$  in PMA-treated alveolar macrophages, resulting in  $Q_{10}$  values of 4.16 and 2.88, respectively.

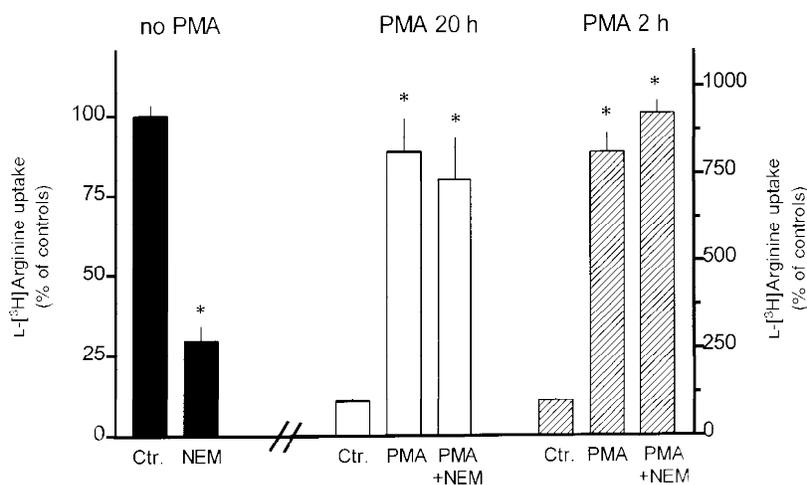
In order to test whether microfilament-associated intracellular transport mechanisms might be involved in the PMA-mediated activation of L-arginine transport the effect of cytochalasin B was studied. When cytochalasin B ( $30 \mu\text{M}$ ) was present between 19.5 and 21 h of culture L-[<sup>3</sup>H]arginine uptake was reduced by  $26 \pm 6\%$  ( $n=6$ ,  $P < 0.05$ ). When L-[<sup>3</sup>H]arginine uptake was enhanced by PMA (100 nM, present from 20–21 h of culture), cytochalasin B ( $30 \mu\text{M}$ , present 30 min before PMA, i.e. from 19.5 to 21 h of culture) caused a reduction by a similar fraction ( $30 \pm 2\%$ ,  $n=5$ ;  $P < 0.01$ ).

### L-[<sup>3</sup>H]Arginine uptake in rat and mouse alveolar macrophages

Since the number of alveolar macrophages recovered from lungs of rats, and particularly of mice, is rather small (compared with rabbit lungs) only some selected experiments were performed on alveolar macrophages of these species. In comparison to rabbit alveolar macrophages, L-[<sup>3</sup>H]arginine uptake in freshly prepared rat alveolar macrophages was about twice as high and it declined less when the cells were cultured for 20 h (Table 1). L-[<sup>3</sup>H]Arginine uptake in freshly prepared mouse alveolar macrophages was even higher and further increased when the cells were cultured for 20 h (Table 1).

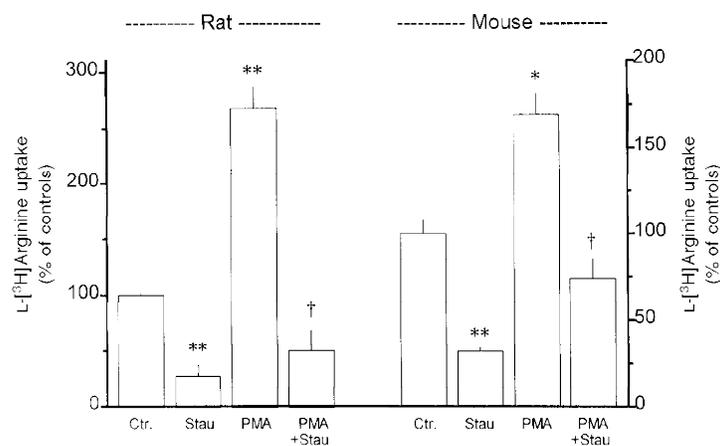
As in rabbit alveolar macrophages, PMA (100 nM, present during the 20 h culture period) caused a significant increase in L-[<sup>3</sup>H]arginine uptake, both in rat and mouse alveolar macrophages. However, due to the higher control levels in rat and mouse alveolar macrophages, the relative increases were considerably smaller (Table 1). However, the absolute level of L-[<sup>3</sup>H]arginine uptake reached in PMA-treated rat alveolar macrophages was about three times higher than in rabbit alveolar macrophages, and in PMA-treated mouse alveolar macrophages it was markedly higher.

In contrast to observations in rabbit alveolar macrophages, staurosporine (100 nM, present during the 20 h culture period) caused a reduction in L-[<sup>3</sup>H]arginine uptake by about 60% in rat alveolar macrophages and by 70% in mouse alveolar macrophages (Fig. 9). Likewise, chelerythrine (10 and  $30 \mu\text{M}$ , present during the 20 h culture period) caused a reduction in L-[<sup>3</sup>H]arginine uptake in rat alveolar



**Figure 8.** Effects of *N*-ethylmaleimide (NEM) on L-[<sup>3</sup>H]arginine uptake in rabbit alveolar macrophages

Alveolar macrophages were cultured for 20 h in the absence or presence of 100 nM PMA (PMA 20 h) or cultured for 20 h in the absence of PMA followed by 2 h culture in the presence of 100 nM PMA (PMA 2 h). In the respective experiments NEM ( $200 \mu\text{M}$ ) was added for the last 10 min of the culture period. Ctr., control. Thereafter, L-[<sup>3</sup>H]arginine uptake was studied during a 2 min period of incubation in amino acid-free Krebs–Hepes solution to which  $0.1 \mu\text{M}$  L-[<sup>3</sup>H]arginine (37 kBq) was added. Height of columns: L-[<sup>3</sup>H]arginine uptake expressed as percentage of the mean uptake observed in the controls of the respective cell preparation. Results are means + s.e.m. of 9–27 experiments. \* $P < 0.01$  compared with respective Ctr.



**Figure 9. Role of protein kinase C in the regulation of L-[<sup>3</sup>H]arginine uptake in rat and mouse alveolar macrophages**

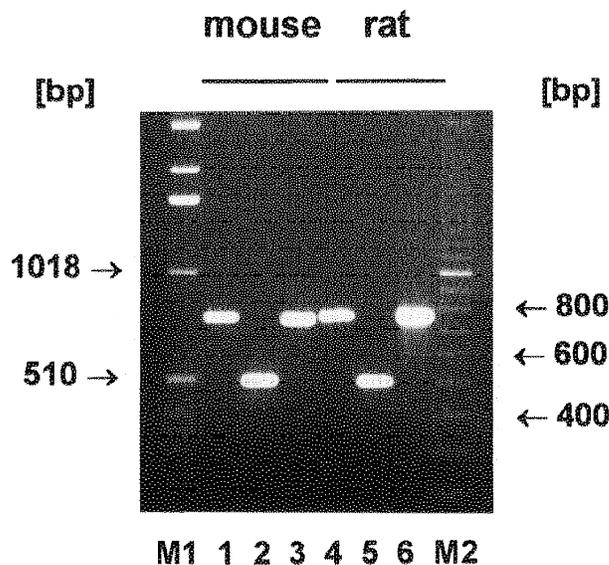
Alveolar macrophages were cultured for 20 h in the absence (Ctr.) or presence of staurosporine (Stau, 100 nM) or PMA (100 nM) alone or in combination, as indicated. Thereafter, L-[<sup>3</sup>H]arginine uptake was studied during a 2 min incubation period in amino acid-free Krebs–Hepes solution to which L-[<sup>3</sup>H]arginine (37 kBq, 0.1 μM) was added. Height of columns: L-[<sup>3</sup>H]arginine uptake expressed as a percentage of the mean uptake observed in the controls of the respective cell preparation (Ctr.). Results are means + s.e.m. of 6–22 experiments. \**P* < 0.05, \*\**P* < 0.01, compared with respective controls (Ctr.); †*P* < 0.01, compared with respective value in PMA alone.

macrophages by 36 ± 5 and 56 ± 6%, respectively (each *n* = 9). Furthermore, 100 nM staurosporine (present together with 100 nM PMA for 20 h) almost abolished the stimulatory effect of PMA in both rat and mouse alveolar macrophages (Fig. 9).

Finally, significant effects on L-[<sup>3</sup>H]arginine uptake in rat alveolar macrophages could also be demonstrated after short-term exposure to PMA or staurosporine. When PMA (100 nM) or staurosporine (100 nM) had been present for only 1 h (from 20–21 h of culture), L-[<sup>3</sup>H]arginine uptake was increased by 67 ± 9% (*n* = 4, *P* < 0.01) and inhibited by 52 ± 9% (*n* = 7, *P* < 0.01), respectively.

**Expression of mRNA for CAT proteins in rat and mouse alveolar macrophages**

Using specific primers based on the published sequences for rCAT-1 and mCAT-2B (the primers for mCAT-2B showed 100% homology to the corresponding rat sequences whereas that of the sense primer of the primer pair for rCAT-1 showed a single mismatch to the corresponding sequence of mCAT-1; see Methods and Discussion) RT-PCRs were carried out with RNA isolated from mouse and rat alveolar macrophages which had been cultured for 20 h (i.e. the conditions under which the uptake studies had been performed). Figure 10 shows that PCR products of the expected size were obtained for CAT-1 and even slightly



**Figure 10. Detection of mRNA for CAT proteins by RT-PCR in mouse and rat alveolar macrophages**

Alveolar macrophages of mouse and rats were cultured for 20 h in 60 mm culture dishes (6 × 10<sup>6</sup> cells dish<sup>-1</sup>) followed by RNA isolation. RT-PCR was performed as described in Methods using RNA from mouse (lanes 1–3) or rat (lanes 4–6) alveolar macrophages and primers specific for CAT-1 (lanes 1 + 4); CAT-2B (lanes 2 + 5); m-β-actin (lane 3) and r-β-actin (lane 6). M1 and M2 represent markers for numbers of base pairs (bp).

stronger signals for CAT-2B from both rat and mouse alveolar macrophages. Similar results were obtained in three independent cell preparations for each species. Although RT-PCR is only a semiquantitative technique it may be mentioned that neither the expression of mRNA for CAT-1 nor that for CAT-2B appeared to be reduced in three different cell preparations of rat alveolar macrophages cultured for 20 h in the presence of 100 nM staurosporine.

## DISCUSSION

In the present study uptake of L-arginine into alveolar macrophages was characterized and a regulatory role of protein kinase C demonstrated. Most of the present experiments were carried out on cells obtained from rabbits, but some key experiments were repeated on alveolar macrophages of rats and mice in order to allow more general conclusions.

In macrophages of all three species arginase appears to be expressed constitutively (see Introduction), whereas marked species differences exist with regard to the expression of NO synthase. Thus, under all the present experimental conditions, no NO synthase activity was detectable in alveolar macrophages of rabbits (Hey *et al.* 1995, 1996). On the other hand, rat alveolar macrophages showed some basal NO synthase activity when the cells had been cultured in the absence of any particular inductive stimulus, and showed a substantial further increase in NO synthase activity after exposure to LPS (Hey *et al.* 1995, 1997). Likewise, in several murine macrophage cell types (bone marrow, different cell lines) the induction of marked NO synthase activity was demonstrated (e.g. Bogle *et al.* 1992; Baydoun *et al.* 1993; Modolell *et al.* 1995), although we are not aware of any study on mouse alveolar macrophages.

### L-Arginine uptake in rabbit alveolar macrophages

In mammalian cells several systems mediating the transport of cationic amino acids (L-arginine, L-lysine and L-ornithine) have been described (for review see Closs, 1996). They have been differentiated by their affinity for the cationic amino acids, by their dependence on sodium ions and their ability to also transport neutral amino acids. In the present study, L-arginine uptake in rabbit alveolar macrophages was saturable and showed a  $K_m$  value in the higher micromolar range (Fig. 3). It was blockable by L-lysine, another cationic amino acid, indicating that it was mediated via cationic amino acid transport systems. The involvement of specific transport mechanisms is further indicated by the marked temperature dependency. Finally, L-arginine uptake did not depend on the presence of extracellular sodium ions (Fig. 6) excluding a role of the system  $B^{0,+}$  which has been described as the only system to transport cationic amino acids in a sodium-dependent manner (van Winkle *et al.* 1985). In contrast, sodium ions appear to exert some inhibitory effect on L-arginine uptake, an effect which was, however, only seen at the higher concentrations of L-arginine (100  $\mu M$ ), but not at the low concentration of 0.1  $\mu M$  (Fig. 6). An

inhibitory effect of sodium ions on cationic amino acid transport has been described for the system  $b^{0,+}$  (van Winkle *et al.* 1988). System  $b^{0,+}$  is additionally characterized by the ability to transport large neutral amino acids in a sodium-independent manner, and in the present experiments L-leucine (1 mM) inhibited L-arginine uptake in the absence of sodium ions by about 50% (Fig. 7B and D). However, the situation is more complex, since in the presence of sodium ions, L-leucine markedly inhibited L-arginine uptake even at the low concentration of 100  $\mu M$  (Fig. 7A and C). This suggests the additional involvement of system  $y^+L$  which has been reported to transport cationic amino acids independently of sodium ions and concomitantly to show high affinity for large neutral amino acids in a sodium-dependent manner (Devés *et al.* 1993). Finally, NEM has been shown to inactivate system  $y^+$  without affecting system  $y^+L$  (Devés *et al.* 1993). In the present experiments, pretreatment of the alveolar macrophages for 10 min with NEM resulted in a marked reduction of L-arginine uptake (Fig. 8). Altogether it becomes evident that in rabbit alveolar macrophages a rather heterogeneous population of transport systems appears to be involved in the transport of cationic amino acids. However, it is not clear at present whether the different transporter characteristics reflect separate transporter entities or different functional states of the same transporter. The fact that pretreatment with PMA caused a rapid change in some of the characteristics of L-arginine transport (see below) may support the latter possibility.

The activity of L-arginine transport in alveolar macrophages markedly changed depending on the time in culture. Compared with freshly prepared cells, there was a transient increase followed by a reduction by about 80% below the initial values after 20 h in culture. It remains unclear which of the activation states may represent the basic situation *in vivo*, as 'freshly isolated cells' are not naive cells, because they had just experienced the isolation and adherence protocol which is certainly a particular cellular stress.

The presence of the phorbol ester PMA during the 20 h culture period not only prevented the decline in the activity of L-arginine transport, but enhanced it substantially above initial levels (Table 1, Fig. 2). Two additional observations indicate that this effect of PMA was mediated via specific activation of protein kinase C. (i) The phorbol ester  $\alpha$ -PMA, which does not activate protein kinase C, had no effect on L-arginine transport. (ii) Staurosporine and chelerythrine, inhibitors of protein kinase C (Tamaoki *et al.* 1986; Herbert *et al.* 1990), opposed the effect of PMA. Following activation by phorbol esters, protein kinase C often shows rapid downregulation, but in the present study the effect of PMA did not vanish after 20 h of exposure to the phorbol ester. However, there exist several isoenzymes of protein kinase C and only some of them show rapid downregulation whereas others can maintain sustained responses to phorbol esters or diacylglycerol (for review see Dekker & Parker, 1994). A role for protein kinase C as a stimulatory pathway in the control of cationic amino acid transporters has already been

demonstrated in human lymphocytes (Yoshimoto *et al.* 1992), human endothelial cells (Pan *et al.* 1995) and Caco-2 cells, an intestinal epithelial cell line (Pan & Stevens, 1995), whereas in mouse astrocytes staurosporine failed to affect L-arginine transport upregulated by LPS (Schmidlin & Wiesinger, 1995).

The mechanism by which staurosporine on its own caused a slight activation of L-arginine transport (Fig. 4) is unclear at present. However, it is unlikely that this effect involves protein kinase C, as chelerythrine was without effect. Likewise, staurosporine had stimulatory effects on the expression of NO synthase in endothelial cells (Li *et al.* 1996) and vascular smooth muscle (Hecker *et al.* 1997) by mechanisms unlikely to involve inhibition of protein kinase C.

Analysis of the kinetics of L-arginine uptake revealed that the stimulatory effect of PMA on L-arginine transport was mainly caused by an increase in  $V_{\max}$  (Fig. 3), indicating that activation of protein kinase C caused an increase in the amount of active transporter and/or enhanced the transporter activity. The fact that an effect of PMA was already evident after 30 min and was almost maximal after about 2 h (Figs 5 and 8) suggests that protein kinase C can cause an activation of pre-existing silent transporters. Alternatively, activation of protein kinase C may increase the activity of already functional transporters. The following observations support these assumptions.

(i) The short term effects of PMA were also seen in the presence of actinomycin D or cycloheximide, i.e. when the *de novo* synthesis of proteins was inhibited (Fig. 5).

(ii) Some characteristics of the L-arginine transport changed after exposure to PMA. (a) The inhibitory effect of NEM was lost after pretreatment with PMA, and this effect was identical independently of whether the cells were treated with PMA for only 2 or for 20 h (Fig. 8). Since NEM has been shown to inactivate system  $y^+$ , but not system  $y^+L$  (Debés *et al.* 1993), it may be concluded that transporters with properties of system  $y^+$  lost their significance in PMA-treated cells. One explanation could be that system  $y^+$  contributes significantly to the L-arginine uptake in untreated cells but the large increase in transport activity after PMA is caused by activation of transporters with characteristics of system  $y^+L$ . However, in conflict with such an interpretation are the observations that sensitivity to the inhibitory effects of L-leucine (another discriminator between system  $y^+$  and  $y^+L$ ) was similar in PMA-treated and untreated cells. An alternative explanation for the loss of NEM sensitivity could be that the transporters representing system  $y^+$  may have changed some of their characteristics following activation by PMA. Thus, PMA could have caused structural changes in the transporter proteins with the consequence that either alkylation of sulfhydryl groups lost the functional significance or critical sulfhydryl groups may be less accessible for NEM. Whether this is caused by direct protein kinase C-mediated phosphorylation of transporter proteins (see also below) or whether protein kinase C affects

modulatory proteins remains an open question which has to be addressed in future studies. (b) The  $K_m$  was reduced after treatment with PMA, although only by a factor of two. Again, this could have been caused by changes in already active transporters or by the activation of a different transporter entity which then accounted for most of the L-arginine transport after PMA stimulation. (c) The observation that the  $Q_{10}$  value of the L-arginine uptake was reduced (i.e. the activation energy of the transporter was lower) after treatment with PMA further supports the assumption that protein kinase C can mediate alterations in the transporter proteins which may facilitate the translocation of L-arginine across the cell membrane.

Inhibition of the *de novo* synthesis of transporter proteins during the 20 h culture period caused a large reduction in L-arginine uptake, both in the absence and presence of PMA, indicating that a continuous protein synthesis is required for the maintenance of L-arginine transport and its stimulation by PMA. However, stimulation of *de novo* synthesis of transporter proteins or of modulatory proteins appears not to be the primary effect of PMA in alveolar macrophages, whereas in the above-mentioned studies on lymphocytes, endothelial cells and Caco-2 cells (Yoshimoto *et al.* 1992; Pan *et al.* 1995; Pan & Stevens, 1995) an enhanced expression of transporter proteins was caused by activation of protein kinase C.

Since the cytoskeleton is known to be a particular target for protein kinase C (see Kiley *et al.* 1992; Dekker & Parker, 1994), it appeared to be an interesting idea to test whether the protein kinase C-mediated acute activation of L-arginine transport might involve a translocation of 'silent' transporter proteins to the cell surface. However, cytochalasin B, a toxin which inhibits microfilament-associated intracellular transport processes by inhibiting actin polymerization (Wessells *et al.* 1971) did not prevent the acute stimulatory effect of PMA. After 90 min exposure to cytochalasin B, L-arginine transport in the absence and presence of PMA was inhibited by about the same fraction (25–30%), indicating that a cytochalasin B-sensitive trafficking of transporter proteins may be of significance in alveolar macrophages. However, the relatively similar reduction by cytochalasin B of L-arginine transport, independent of the state of activation of protein kinase C, suggests that the acute activation of L-arginine transport via protein kinase C may not involve a cytochalasin B-sensitive mechanism, such as translocation. Therefore, it may be concluded that protein kinase C may cause activation of L-arginine transport by an action on transporter proteins (or modulators) already incorporated into the cell surface membrane.

#### L-Arginine uptake in rat and mouse alveolar macrophages

In rat and mouse alveolar macrophages, protein kinase C appears to play a similar role in the control of L-arginine transport to that in rabbit alveolar macrophages. However,

the activity of L-arginine transport in alveolar macrophages of rat and mouse was higher than that in rabbit alveolar macrophages and this appeared to be caused mainly by the fact that protein kinase C was already considerably activated under basal culture conditions. This is indicated by the marked inhibitory effect of staurosporine and chelerythrine alone. The reason for these different states of activation of protein kinase C and of the L-arginine transport in alveolar macrophages of rabbits on the one side and of rat and mouse on the other side remains unclear at present, particularly as there were no differences in the way the cells were treated. Nonetheless, it can be seen that the overall magnitude of the modulatory effects of protein kinase C-mediated mechanisms on L-arginine transport is similar in alveolar macrophages from all three species. Though the effect of PMA alone is relatively smaller in rat and mice alveolar macrophages, for the estimation of the whole range of protein kinase C-mediated modulation of L-arginine transport, the difference between the reduced levels after staurosporine and the enhanced levels after PMA has to be considered (Fig. 9), and this difference is similar to what is seen in rabbit alveolar macrophages (compare with Fig. 4). Finally, it is interesting to note that a marked inhibitory effect of staurosporine on L-arginine uptake in rat alveolar macrophages was already seen after 1 h, indicating that in this species also protein kinase C is involved in the acute modulation of the activity of cationic amino acid transporters.

Although the present experiments do not allow a final conclusion about the molecular mechanisms by which protein kinase C may activate L-arginine transport in alveolar macrophages, it is worth noting that currently several proteins with properties of a cationic amino acid transporter (CAT) have been cloned, initially from murine cells (see Closs, 1996). They were originally named MCAT-1, MCAT-2A and MCAT-2B, the latter being a splicing variant of MCAT-2A. MCAT-2A is a low affinity transporter ( $K_m > 2$  mM) which appears to be expressed only in the liver and may therefore not be of significance for the present observations. On the other hand, the kinetic properties of MCAT-1 and MCAT-2B are indistinguishable and their  $K_m$  values (between 0.14 and 0.38 mM) are comparable to those observed in the present study (between 0.32 and 0.65 mM). It should be mentioned that murine macrophages (RAW 264.7 cell line) expressed the MCAT-2B (Closs *et al.* 1993) and, in the present study, both in mouse and rat alveolar macrophages, mRNA for both CAT-1 and CAT-2B could be demonstrated by RT-PCR (Fig. 10). Although the limitation of RT-PCR with regard to quantitative statements should be kept in mind, the expression of mRNA for CAT-1 and CAT-2B appeared not to be reduced in rat alveolar macrophages cultured in the presence of staurosporine. This is at least in line with the above conclusions that protein kinase C may modulate the activity of pre-existing transporters. It is worth noting that the sequences of all CAT proteins possess several putative protein kinase C

phosphorylation sites. Therefore, it will be an interesting task for future studies to characterize in more detail the CATs in alveolar macrophages, in particular to elucidate whether the large activation of the L-arginine transport is related to a direct phosphorylation of CAT proteins.

In conclusion, the present experiments showed multiple transport systems are involved in the uptake of L-arginine in alveolar macrophages and that protein kinase C represents an important stimulatory pathway in the acute control of the activity of L-arginine transport in these cells, probably by modulation of the activity of already expressed cationic amino acid transporters.

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