RESEARCH COMMUNICATION

Uptake of nitric oxide synthase inhibitors by macrophage RAW 264.7 cells

Kurt SCHMIDT,* Peter KLATT and Bernd MAYER

Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria

Uptake of the nitric oxide synthase inhibitors N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA) by macrophages is mediated by two different mechanisms. Activation of the cells with cytokines resulted in an up-regulation of L-NMA uptake but did not affect L-NNA transport. Characterization of the transport sites revealed that uptake of L-NMA is mediated by a cationic amino acid transporter (system y^+) whereas a neutral amino acid transporter (system L) accounts for the uptake of L-NNA.

INTRODUCTION

Nitric oxide (NO) mediates cytostatic and cytotoxic effects of activated macrophages [1]. Upon stimulation with cytokines and bacterial lipopolysaccharides, macrophages express a Ca^{2+}/calmodulin-independent NO synthase, which converts L-arginine in an NADPH-dependent reaction into L-citrulline and NO [2]. Consistent with the identification of the guanidino group of L-arginine as the precursor of NO [3–5], N^G-derivatized analogues of L-arginine, such as N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA) (Figure 1) are inhibitors of NO biosynthesis in both intact macrophages [6–9] and cell-free systems [7,9–13]. Only little is known, however, about the transport systems mediating uptake of NO synthase inhibitors by macrophages. There is evidence that L-arginine is transported via a basic amino acid carrier [14–17], but the role of this transporter in the uptake of NO synthase inhibitors has not been investigated so far. We have addressed this issue and used radiolabelled L-NMA and L-NNA to measure the uptake of these compounds by RAW 264.7 macrophages and to characterize the respective transport system(s).

EXPERIMENTAL

Materials

Tissue-culture media and ingredients, except foetal calf serum (SEBAK GmbH, Aidenbach, Germany), were from GIBCO, Life Technology GmbH (Eggenstein, Germany). Plates and Petri dishes were from Costar Europe Ltd. (Badhoevedorp, The Netherlands). L-[2,3,4,5-^{3}H]Arginine, L-[4,5-^{3}H]leucine, N^G-nitro-L-[2,3,4,5-^{3}H]arginine and N^G-methyl-L-[5-^{14}C]arginine were from MedPro (Amersham International, U.K.). Recombinant murine interferon γ (IFN-γ) was a gift from Bender (Vienna, Austria). All other biochemicals, including lipopolysaccharide (LPS) from *Salmonella typhosa*, were purchased from Sigma Chemical GmbH (Deisenhofen, Germany).

Cell culture

RAW 264.7 macrophages were cultured in Petri dishes in Opti-MEM (GIBCO) supplemented with 10% (v/v) foetal calf serum, penicillin (100 i.u./ml), streptomycin (100 i.u./ml) and amphotericin B (1.25 μg/ml). Before the experiments, cells were subcultured in 24-well plates and, where indicated, incubated for 24 h with 2 μg/ml LPS and 50 i.u./ml IFN-γ.

Measurement of amino acid transport

Confluent monolayers (~150 μg of protein/well) were washed with isotonic Hepes buffer, pH 7.4, containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$ (incubation buffer) and equilibrated for 15 min at 37 °C in 0.4 ml of the same buffer. Amino acid uptake was initiated by addition of 50 μl of a solution of the respective radiolabelled compound (200000–700000 d.p.m.) and 50 μl of a solution of the amino acids to be tested. After incubation (0.5–15 min), cells were washed three times with 2 ml of ice-cold incubation buffer and digested with 1 ml of 0.1 M NaOH. Radioactivity was measured by liquid scintillation counting. Uptake of L-NMA and L-NNA was linear over at least 15 min, but to ensure the determination of initial uptake rates incubation was routinely performed for 30 s.

Data analysis

Data obtained from saturation or inhibition experiments were analysed with a Hewlett–Packard work station (series 9000, model 310) using the GIPMAX non-linear least-squares computer curve-fitting program [18]. All K_m and K_i values were derived from individual concentration–response curves and are expressed as geometric means with 95% confidence limits,

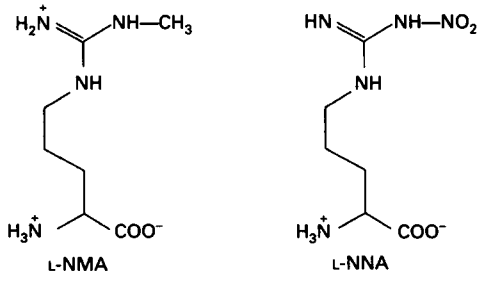

Figure 1 Structures of L-NMA and L-NNA

Abbreviations used: IFN-γ, interferon γ; LPS, lipopolysaccharide; L-NMA, N^G-methyl-L-arginine; NO, nitric oxide; L-NNA, N^G-nitro-L-arginine.

* To whom correspondence should be addressed.

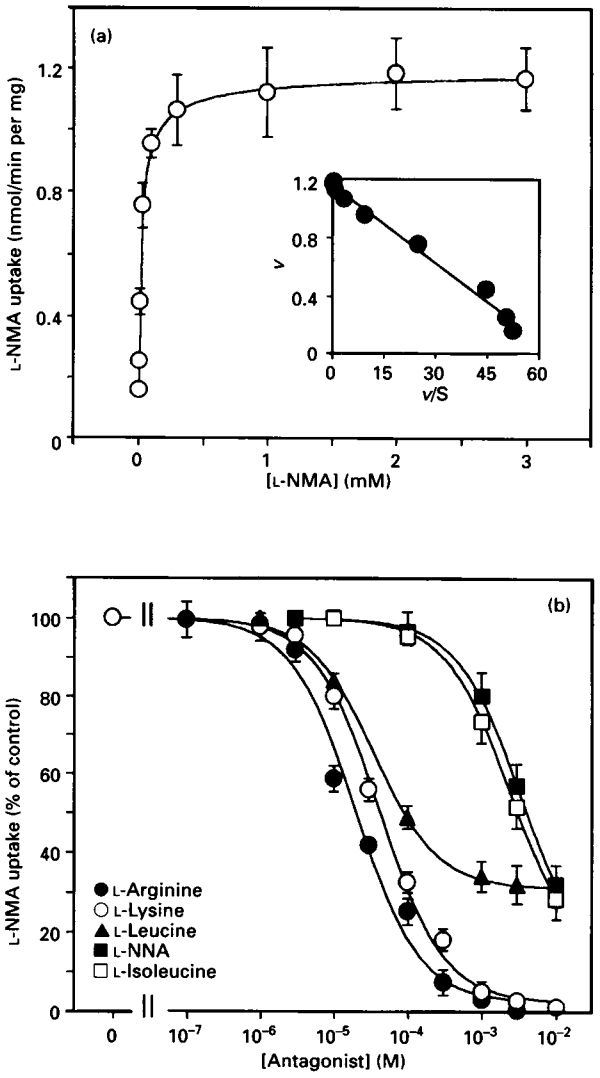

Figure 2 Characteristics of L-NMA transport

(**a**) Kinetics: uptake of L-[^{14}C]NMA was measured at concentrations ranging from 3 μM to 3 mM (200000 d.p.m. each). Data represent means $\pm$ S.E.M. ($n = 4$). Inset: Eadie–Hofstee replot. (**b**) Inhibition of L-[^{14}C]NMA uptake by amino acids: macrophages were incubated with 3 μM L-[^{14}C]NMA (200000 d.p.m.) in the absence and presence of increasing concentrations of unlabelled amino acids. Data are means $\pm$ S.E.M. ($n = 3$) and are expressed as percentages of control uptake.

calculated as the product of S.E.M. $\times$ Student's t value; $V_{max.}$ values were calculated as arithmetic means $\pm$ S.E.M. The statistical significances were evaluated by analysis of variance using Scheffe's F test [19].

RESULTS

Uptake of L-NMA by macrophages was saturable with a K_m of 23 (12–47) μM and a $V_{max.}$ of 1.16 $\pm$ 0.20 nmol/min per mg of protein (Figure 2a). The values of these parameters were not affected when NaCl was substituted by 145 mM choline chloride in the incubation medium. Upon activation of macrophages with LPS/IFN-γ, $V_{max.}$ was enhanced 3.6-fold (Table 1) whereas K_m remained unaffected (results not shown). Figure 2(b) shows the concentration-dependent inhibition of L-[^{14}C]NMA uptake by

Table 1 Maximal uptake rates for various amino acids in control and LPS/IFN-γ-pretreated macrophages

Uptake of L-[^{14}C]NMA, L-[^{3}H]NNA, L-[^{3}H]arginine and L-[^{3}H]leucine was measured at concentrations ranging from 3 μM to 3 mM (600000 d.p.m. each) in control and LPS/IFN-γ-pretreated macrophages (see the Experimental section). Maximal uptake rates ($V_{max.}$) are expressed as means $\pm$ S.E.M. ($n = 3$–5) (*$P < 0.01$ versus control). K_m values are listed in Table 2 and were identical in both control and activated macrophages.

	$V_{max.}$ (nmol/min per mg of protein)	
Amino acid	Control macrophages	Activated macrophages
L-NMA	1.16 $\pm$ 0.20	4.17 $\pm$ 0.38*
L-NNA	0.68 $\pm$ 0.14	0.62 $\pm$ 0.17
L-Arginine	1.40 $\pm$ 0.28	4.76 $\pm$ 0.37*
L-Leucine	0.48 $\pm$ 0.04	0.44 $\pm$ 0.09

some selected amino acids. Basic amino acids, such as L-arginine and L-lysine, were potent inhibitors, whereas L-NNA and branched-chain amino acids, such as L-leucine and L-isoleucine, were much less active. Interestingly, L-leucine only partially inhibited L-[^{14}C]NMA uptake, indicating a non-competitive interaction with the transport site. To confirm this hypothesis we measured substrate kinetics of L-NMA transport in the presence of 1 mM L-leucine and found that $V_{max.}$ was reduced from 1.16 to 0.38 nmol/min per mg of protein whereas K_m remained unaffected. These data demonstrate that L-leucine inhibits uptake of L-NMA in a non-competitive manner, whereas all other investigated amino acids compete with L-NMA for the active site of the transporter.

Similar to L-NMA uptake, transport of L-NNA also occurred in a Na^+-independent manner, but the kinetic parameters [K_m 205 (160–262) μM, $V_{max.}$ 0.68 $\pm$ 0.14 nmol/min per mg of protein] clearly differed from those of L-NMA (Figure 3a). Furthermore, uptake of L-NNA was not enhanced in activated macrophages (Table 1), indicating that different transport systems may mediate the uptake of L-NMA and L-NNA. In support of this hypothesis we found that basic amino acids, which were potent inhibitors of L-[^{14}C]NMA uptake, did not compete for transport of L-[^{3}H]NNA (Figure 3b) whereas L-leucine and L-isoleucine, which exhibited only low affinity for the L-NMA transporter, potently antagonized uptake of L-[^{3}H]NNA. These data indicate that uptake of L-NMA is mediated by the L-arginine transporter, whereas L-NNA utilizes the L-leucine transporter.

To further substantiate this hypothesis we investigated uptake of L-[^{3}H]arginine and L-[^{3}H]leucine by macrophages. The characteristics of L-arginine uptake were similar to those of L-NMA uptake. Transport occurred in a Na^+-independent manner [K_m 15 (6–37) μM, $V_{max.}$ 1.40 $\pm$ 0.28 nmol/min per mg of protein] and was stimulated 3.4-fold upon activation of macrophages with LPS/IFN-γ (Table 1). Basic amino acids were potent inhibitors of L-[^{3}H]arginine uptake (K_i 15–142 μM) whereas neutral amino acids were much less active ($K_i > 1$ mM). As shown in Table 2, no substantial differences in the K_i values were observed when either L-[^{14}C]NMA or L-[^{3}H]arginine was used as radiolabelled substrate, indicating that uptake of L-arginine and L-NMA is mediated by system y^+.

In contrast to L-arginine transport, uptake of L-leucine closely resembled that of L-NNA, as it was Na^+-independent [K_m 10 (5–21) μM, $V_{max.}$ 0.48 $\pm$ 0.04 nmol/min per mg of protein] and not enhanced in activated macrophages (Table 1). Furthermore, uptake of L-[^{3}H]leucine was inhibited by branched-chain and aromatic amino acids (L-leucine, L-isoleucine and L-phenyl-

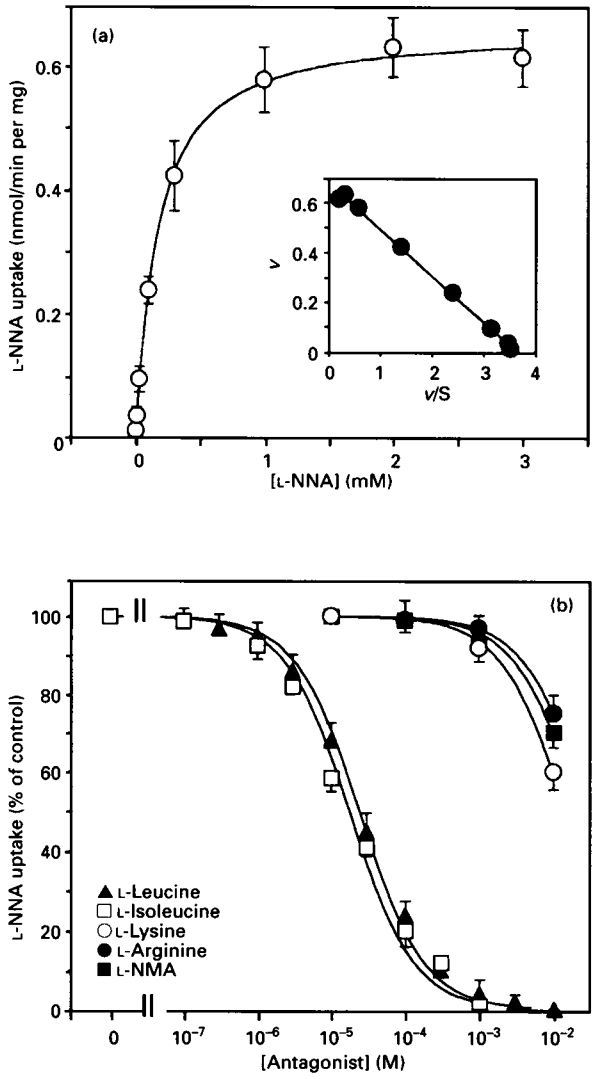

Figure 3 Characteristics of L-NNA transport

(**a**) Kinetics: uptake of L-[^{3}H]NNA was measured at concentrations ranging from 3 μM to 3 mM (600000 d.p.m. each). Data represent means ± S.E.M. ($n = 4$). Inset: Eadie–Hofstee replot. (**b**) Inhibition of L-[^{3}H]NNA uptake by amino acids: macrophages were incubated with 10 nM L-[^{3}H]NNA (600000 d.p.m.) in the absence and presence of increasing concentrations of unlabelled amino acids. Data are means ± S.E.M. ($n = 3$) and are expressed as percentages of control uptake.

alanine), whereas other neutral amino acids or basic amino acids were completely inactive (Table 2). This substrate specificity indicates that both L-leucine and L-NNA are transported by system L.

DISCUSSION

In the present study we have demonstrated that uptake of L-NMA is mediated by system y^+, whereas system L accounts for the transport of L-NNA. System y^+ is a well characterized Na^+-independent transporter for basic amino acids and has been found in a variety of cells, including murine peritoneal macrophages [14,15] and the macrophage cell line J774 [16,17]. Although L-leucine is no substrate for system y^+, we observed an inhibitory effect of this amino acid on the uptake of L-NMA and L-arginine. Kinetic studies revealed that transport activity of system y^+ was inhibited by L-leucine in a non-competitive manner. A similar inhibitory effect was described for the uptake of L-lysine by vascular smooth muscle cells [20] but has not been detected in other tissues so far.

The Na^+-independent transport of neutral amino acids either is mediated by system L, which prefers branched-chain and aromatic amino acids, such as L-leucine, L-isoleucine and L-phenylalanine, or by system ASC with alanine, serine and cysteine as substrates (for review, see [21]). Interestingly, in murine peritoneal macrophages only a rather unselective neutral amino acid transporter has been identified so far, which does not discriminate between the typical substrates of systems L and ASC [22]. In our experiments performed with RAW 264.7 macrophages we did not observe such unselective transport of neutral amino acids, since uptake of L-[^{3}H]leucine and L-[^{3}H]NNA was inhibited by L-leucine, L-isoleucine and L-phenylalanine but not by L-alanine, L-serine and L-cysteine. Furthermore, we did not observe any inhibitory effect of L-NNA on L-[^{14}C]cysteine uptake (K. Schmidt, P. Klatt and B. Mayer, unpublished work), clearly demonstrating that transport of L-NNA is not mediated by system ASC. Differences in the electrical charge of the guanidino group of L-NMA and L-NNA may account for the different transporters involved in their uptake. At physiological pH, the guanidino moiety of L-NMA is, similarly to that of L-arginine, positively charged and therefore recognized by basic amino acid transporters, whereas L-NNA resembles neutral amino acids because the guanidino group is not protonated at neutral pH.

The mechanisms for uptake of L-arginine analogues studied here with macrophages may be similar in other tissues. In cultured porcine aortic endothelial cells, uptake of L-NMA and L-NNA is also mediated by the transport systems y^+ and L respectively [23]. While the kinetic parameters for L-NNA (K_m 617 μM) and L-leucine (K_m 5 μM) were comparable with those obtained with macrophages, the uptakes of L-NMA and L-arginine were biphasic with K_m values of 6 and 600 μM for the high- and low-affinity sites respectively, indicating that the endothelial basic amino acid transporter differs from that expressed in macrophages. Inhibition of L-arginine uptake by L-NMA but not by L-NNA indicates that these transport systems may also account for uptake of NO synthase inhibitors by cultured neurons [24] and different tumour cell lines [25].

Pretreatment of macrophages with LPS/IFN-γ results in an enhanced transport activity of system y^+, although the fold increases differ among different types of macrophages. While activation of peritoneal macrophages leads to a 10-fold increase in the uptake rate for L-arginine [15] and L-lysine [14], the transport activity of system y^+ is stimulated 3-fold in activated J774 [16,17] and RAW 264.7 cells (this paper). We now demonstrate that, in contrast to the enhanced transport activity of system y^+, uptake via system L is not stimulated under these conditions. Thus, activation of macrophages leads to an enhanced uptake of L-NMA, whereas transport of L-NNA remains unaffected. In addition to these differences in $V_{max.}$(7-fold), the affinity of L-NMA for system y^+(K_m 23 μM) was 10-fold higher than that of L-NNA to system L (K_m 205 μM), indicating that the availability and thus the activity of NO synthase inhibitors transported by system L may be limited.

The reported K_i values for inhibition of inducible macrophage NO synthase by L-NMA and L-NNA are 2.7 and 4.4 μM respectively [26,27]. Accordingly, L-arginine analogues exhibit at least one order of magnitude lower affinities for their transporters than for NO synthase, indicating that the biological effects of L-NMA and L-NNA may be limited by their rates of uptake.

Table 2 Affinities (K_m or K_i values) of various amino acids for systems y^+ and L

Uptake of L-[^{14}C]NMA (3 μM) and L-[^{3}H]arginine, L-[^{3}H]NNA and L-[^{3}H]leucine (10 nM each) was measured in the presence of increasing concentrations of various unlabelled amino acids. K_m values are marked with asterisks and were derived from saturation curves. For competitive inhibition, K_i values were calculated according to the equation $K_i = IC_{50}/[1 + ([S]/K_m)]$, and for the non-competitive inhibition observed with L-leucine (see Figure 2b) it was assumed that $IC_{50} = K_i$. All values are geometric means ($n = 3$–5) with 95% confidence limits in parentheses.

	K_m or K_i values (μM)			
	System y^+		System L	
Amino acid	L-[^{14}C]NMA	L-[^{3}H]Arginine	L-[^{3}H]NNA	L-[^{3}H]Leucine
L-Arginine	24 (8-76)	15 (6–37)*	> 10000	> 10000
L-NMA	23 (12–47)*	90 (36–221)	> 10000	> 10000
L-Lysine	45 (17–120)	91 (28–294)	> 10000	> 10000
L-Ornithine	50 (34–74)	142 (70–289)	> 10000	> 10000
L-NNA	~2000	~3000	205 (160–262)*	290 (103–817)
L-Leucine	35 (15–81)	50 (24–108)	33 (10–114)	10 (5–21)*
L-Isoleucine	~2000	~3000	25 (10–63)	39 (13–116)
L-Phenylalanine	~1500	~2000	43 (17–108)	50 (18–138)
L-Alanine	~7000	~7000	> 10000	> 10000
L-Serine	~2500	~3000	> 10000	> 10000
L-Cysteine	~2000	~2500	~6000	~6000

These results may have important practical implications, because they suggest that circulating plasma amino acids may interfere with inhibition of NO biosynthesis by L-arginine analogues *in vivo*.

This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (P 8836). We thank Dr. P. Towers (Amersham International, U.K.) for a generous gift of L-[^{3}H]arginine, N^G-nitro-L-[^{3}H]arginine and N^G-methyl-L-[^{14}C]arginine. The excellent technical assistance of Mrs. Karin Gollner and Mrs. Margit Rehn is gratefully acknowledged.

REFERENCES

1 Nathan, C. F. and Hibbs, J. B. (1991) Curr. Opin. Immunol. **3**, 65–70
2 Nathan, C. (1992) FASEB J. **6**, 3051–3064
3 Palmer, R. M. J., Ashton, D. S. and Moncada, S. (1988) Nature (London) **333**, 664–666
4 Schmidt, H. H. H. W., Nau, H., Wittfoht, W., Gerlach, J., Prescher, K.-E., Klein, M. M., Niroomand, F. and Böhme, E. (1988) Eur. J. Pharmacol. **154**, 213–216
5 Sakuma, I., Stuehr, D. J., Gross, S. S., Nathan, C. and Levi, R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8664–8667
6 Hibbs, J. B., Taintor, R. R., Vavrin, Z. and Rachlin, E. M. (1988) Biochem. Biophys. Res. Commun. **157**, 87–94
7 Gross, S., Stuehr, D., Aisaka, K., Jaffe, E., Levi, R. and Griffith, O. (1990) Biochem. Biophys. Res. Commun. **170**, 96–103
8 Lambert, L. E., Whitten, J. P., Baron, B. M., Cheng, H. C., Doherty, N. S. and McDonald, I. A. (1991) Life Sci. **48**, 69–75
9 McCall, T. B., Feelisch, M., Palmer, R. M. J. and Moncada, S. (1991) Br. J. Pharmacol. **102**, 234–238
10 Stuehr, D. J., Kwon, N. S., Gross, S. S., Thiel, B. A., Levi, R. and Nathan, C. F. (1989) Biochem. Biophys. Res. Commun. **161**, 420–426
11 Olken, N. M. and Marletta, M. A. (1993) Biochemistry **32**, 9677–9685
12 Förstermann, U., Schmidt, H. H. H. W., Pollock, J. S., Sheng, H., Mitchell, J. A., Warner, T. D., Nakane, M. and Murad, F. (1991) Biochem. Pharmacol. **42**, 1849–1857
13 Feldman, P. L., Griffith, O. W., Hong, H. and Stuehr, D. J. (1993) J. Med. Chem. **36**, 491–496
14 Sato, H., Ishii, T., Sugita, Y. and Bannai, S. (1991) Biochim. Biophys. Acta **1069**, 46–52
15 Sato, H., Fujiwara, M. and Bannai, S. (1992) J. Leukocyte Biol. **52**, 161–164
16 Bogle, R. G., Baydoun, A. R., Pearson, J. D., Moncada, S. and Mann, G. E. (1992) Biochem. J. **284**, 15–18
17 Baydoun, A. R., Bogle, R. G., Pearson, J. D. and Mann, G. E. (1993) Br. J. Pharmacol. **110**, 1401–1406
18 Wiemer, G. A., Wellstein, A., Palm, D., von Hattingberg, H. M. and Brockmeier, D. (1982) Naunyn–Schmiedeberg's Arch. Pharmacol. **321**, 11–19
19 Scheffé, H. (1953) Biometrika **40**, 87–104
20 Low, B. C., Ross, I. K. and Grigor, M. R. (1993) J. Cell. Physiol. **156**, 626–634
21 Christensen, H. N. (1989) Methods Enzymol. **173**, 576–616
22 Sato, H., Watanabe, H., Ishii, T. and Bannai, S. (1987) J. Biol. Chem. **262**, 13015–13019
23 Schmidt, K., Klatt, P. and Mayer, B. (1993) Mol. Pharmacol. **44**, 615–621
24 Westergaard, N., Beart, P. M. and Schousboe, A. (1993) J. Neurochem. **61**, 364–367
25 Chénais, B., Yapo, A., Lepoivre, M. and Tenu, J. P. (1993) Biochem. Biophys. Res. Commun. **196**, 1558–1565
26 Olken, N. M., Rusche, K. M., Richards, M. K. and Marletta, M. A. (1991) Biochem. Biophys. Res. Commun. **177**, 828–833
27 Furfine, E. S., Harmon, M. F., Paith, J. E. and Garvey, E. P. (1993) Biochemistry **32**, 8512–8517

Received 18 April 1994/17 May 1994; accepted 18 May 1994