21. L. D. Bergelson, V. A. Vaver, N. V. Prokazova, A. N. Ushakov and G. A. Popkova, *Biochim. biophys. Acta* **116**, 511 (1966).
22. P. V. Taberner, J. T. Rick and G. A. Kerkut, *Life Sci.* **11**, 335 (1972).
23. S. P. Bessman and E. R. B. McCabe, *Biochem. Pharmac.* **21**, 1135 (1972).
24. R. Pietruszko, K. Voightlander and D. Lester, *Biochem. Pharmac.* **27**, 1296 (1978).
25. F. Poldrugo and O. C. Snead, *Neuropharmacology* **23**, 109 (1984).
26. O. C. Snead, *Life Sci.* **20**, 1935 (1977).
27. R. H. Roth, J. D. Doherty and J. R. Walters, *Brain Res.* **189**, 556 (1980).

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Anti-inflammatory drug inhibition of transport of cystine and glutamate in cultured human fibroblasts

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In mammalian cells, several transport systems for amino acids with different specificity have been characterized [1]. Recent studies have shown that nonsteroidal anti-inflammatory drugs (NSAID) inhibit the transport of amino acids through system A in a variety of cell lines [2, 3]. System A is a Na^+-dependent, neutral amino acid transport system characterized by the transport of methylaminoisobutyric acid. Transport of amino acids through system N or system L is unaffected or slightly stimulated by NSAID [2, 3]. Inhibition by NSAID of system A amino acid transport develops slowly and probably is not the initial action of NSAID [2]. Maximum inhibition by indomethacin was observed after a 2-hr incubation with the drug. In previous papers we have shown that there exists an anionic amino acid transport system responsible for the uptake of cystine and glutamate in cultured human diploid fibroblasts [4, 5]. Similar transport systems have been found in rat hepatoma cells [6] and in isolated rat hepatocytes [7]. This communication describes our observations on the immediate and potent inhibition by NSAID of cystine and glutamate transport in human fibroblasts.

Materials and methods

Chemicals. L-[3,3'-^{3}H]Cystine was obtained from Amersham. Indomethacin and phenylbutazone were purchased from Sigma. 5-Methoxy-2-methylindole-3-acetic acid was from Aldrich. Mefenamic acid and piroxicam were gifts from the Sankyo Pharmaceutical Co. and the Pfizer Taito Co. respectively.

Cells. Experiments were performed with a strain of human diploid fibroblasts derived from fetal lung (IMR-90). The cells were cultured in Eagle's basal medium supplemented with 10% fetal calf serum.

Uptake method. L-Cystine uptake was measured as described previously [4]. Cells grown in a 35-mm diameter dish were rinsed three times in warmed phosphate-buffered saline, pH 7.4, and then were incubated in 0.5 ml of the uptake medium at 37°. The uptake medium consisted of the same buffer used to rinse the cells plus labeled amino acid (1 μCi/0.5 ml). NSAID were dissolved in dimethyl sulfoxide and added to the uptake medium (less than 0.2% dimethyl sulfoxide at final concentration). pH was adjusted when necessary. The incubation was terminated by rinsing the cells three times in ice-cold phosphate-buffered saline, and the radioactivity taken up by the cells was determined as described before.

Results and discussion

Indomethacin inhibited the uptake of cystine in human diploid fibroblasts (Fig. 1). Inhibition was immediate and reversible; uptake was inhibited within 10 sec and upon removal of the drug the rate of uptake was restored without a detectable delay (Fig. 1A). Since cystine is rapidly metabolized (i.e. reduced to cysteine) in the cells, it is possible that an inhibition of the metabolism was responsible for the decreased net rate of the cystine uptake. It has been shown that *N*-ethylmaleimide almost completely inhibits the intracellular metabolism of cystine with only a slight effect on uptake of cystine [4]. Therefore, uptake of [^{3}H]-cystine was measured in the presence of 0.1 mM *N*-ethylmaleimide, where about 95% of ^{3}H-labeled compounds recovered from the cells was identified as cystine. Figure 1B demonstrates apparent uphill transport of cystine and the direct inhibition of transport by indomethacin. Indomethacin decreased the influx V_{max} with little change in the influx K_m for cystine (Fig. 2). In the presence of 0.5 mM indomethacin, the influx V_{max} decreased to about one-fifth that of the control cells. The effects of indomethacin on the uptake of other amino acids were examined. The uptake of alanine, cysteine and leucine was inhibited only weakly by indomethacin at 0.5 mM, whereas the uptake of glutamate, which is largely mediated by the same transport system as cystine, was inhibited to the same extent as that of cystine (data not shown).

Figure 3 shows the effects of various NSAID on cystine uptake. All of these agents inhibited the uptake in a concentration-dependent manner. Mefenamic acid appears to be the most potent inhibitor. Several compounds, which are structurally related to indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] but do not possess anti-inflammatory activity, were also tested. Indole-3-acetic acid and 5-methoxy-2-methylindole-3-acetic acid at 1 mM were ineffective, and *p*-chlorobenzoic acid was weakly inhibitory at 1 mM (about 30% inhibition). Correlation between the inhibition of cystine uptake and the anti-inflammatory action is not clear. However, the order of potency in the inhibition of cystine uptake was roughly similar to that reported for their anti-inflammatory activity.

The amino acid transport system for cystine and glutamate in human diploid fibroblasts is Na^+-independent and shared by anionic amino acids such as homocysteate and α-aminoadipate [4, 5]. Indomethacin has been shown to affect Na^+ influx in human fibroblasts [9]. It is possible that the inhibition of A system amino acid transport may be linked with the inhibition of Na^+ influx, because the inhibition of Na^+ influx by indomethacin occurs more rapidly than the inhibition of A system amino acid transport [9] which is Na^+-dependent. However, it is unlikely that the inhibition of cystine transport by NSAID is related to the inhibition of Na^+ influx. Cystine transport is Na^+-independent and the inhibition of cystine uptake occurs more rapidly than the inhibition of Na^+ influx. In fact, indomethacin inhibited cystine uptake in Na^+-free medium to the same extent as that in the normal, Na^+-containing medium (data not shown). Correlates with other biochemical variables, e.g. cellular ATP levels, might be considered. However, it has been shown that ATP levels do

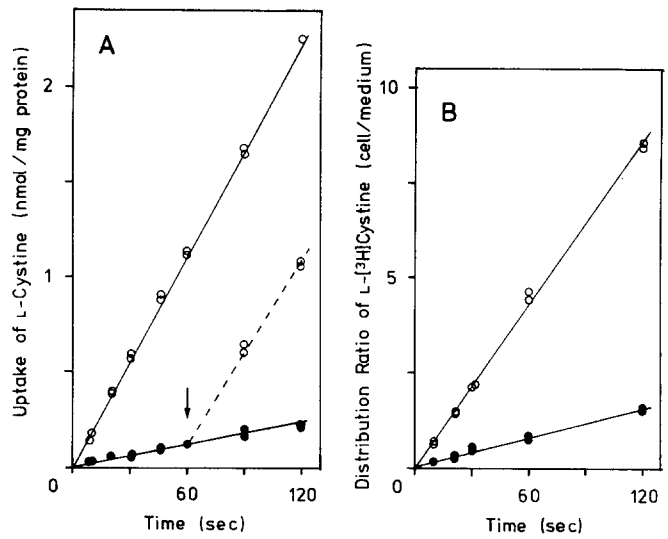

Fig. 1. Time course for the inhibition of L-[^{3}H]cystine uptake by indomethacin. (A) The uptake of L-cystine was measured with (●—●) and without (○—○) 0.5 mM indomethacin; (○---○) incubated for 60 sec in the presence of 0.5 mM indomethacin and then, as indicated by the arrow, the uptake medium was changed to that containing no indomethacin. The concentration of L-cystine in the uptake medium was 0.05 mM. (B) The cells were preincubated for 2 min with 0.1 mM *N*-ethylmaleimide, and then the uptake of L-[^{3}H]cystine was measured in the presence of 0.1 mM *N*-ethylmaleimide with (●—●) and without (○—○) 0.5 mM indomethacin. The concentration of L-cystine in the uptake medium was 0.05 mM. After 2 min of uptake with and without indomethacin, about 95% of intracellular radiolabel was found in cystine. From this and the assumption that 1 mg of cell protein is equivalent to 4 μl of cell water in human fibroblasts [8], intracellular concentrations of L-cystine were calculated.

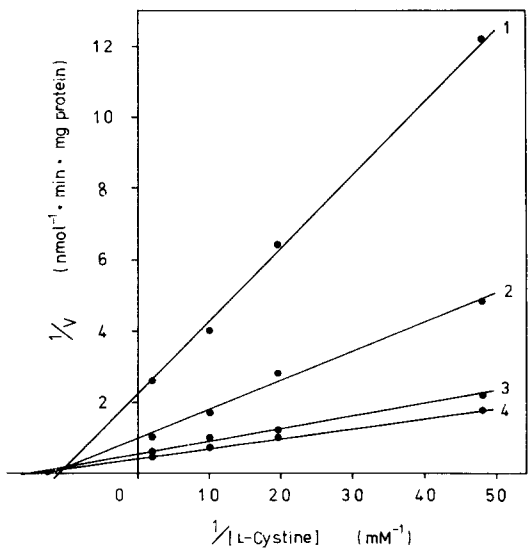

Fig. 2. Double-reciprocal plots of the inhibition of L-[^{3}H]-cystine uptake by indomethacin. Indomethacin was added at 0.5 mM (Curve 1), 0.2 mM (Curve 2), 0.05 mM (Curve 3), and 0 mM (Curve 4). The rate of uptake was measured by taking the values for the 2-min uptake.

not change when cultured cells are exposed to 0.4 mM indomethacin for 15 min [2]. The immediate inhibition by NSAID of cystine uptake suggests that NSAID interact with a cell constituent directly associated with the transport mechanism.

We showed in a previous paper that the inhibition of cystine uptake lowers intracellular levels of glutathione, a tripeptide containing cysteine, and leads to a decline in cell growth [10]. There is convincing evidence that NSAID, at concentrations higher than 0.2 mM, have antiproliferative activity on cultured cells [11, 12]. Presumably the ability of NSAID to inhibit cystine uptake may contribute in part to their antiproliferative action.

In summary, indomethacin and some other NSAID were found to inhibit selectively the uptake of cystine and glutamate in human fibroblasts. The inhibition was immediate, reversible, and noncompetitive.

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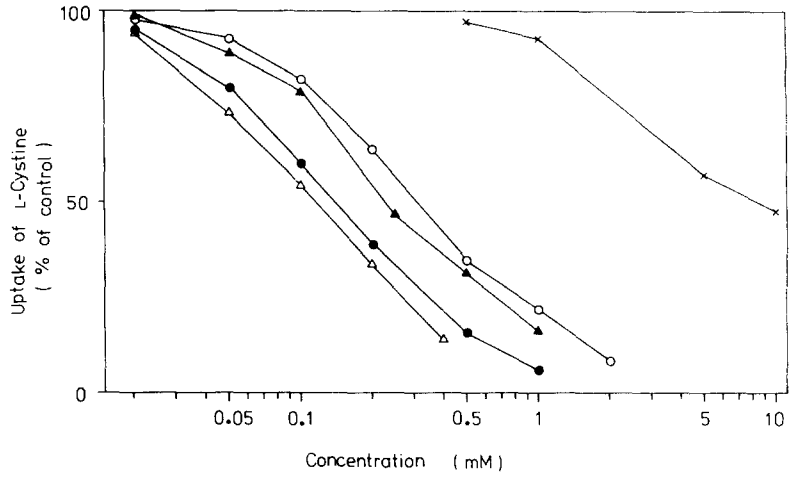

Fig. 3. Inhibition of L-[^{3}H]cystine uptake by some NSAID. NSAID added were indomethacin (●—●), mefenamic acid (△—△), piroxicam (▲—▲), phenylbutazone (○—○), and aspirin (×—×). The concentration of L-cystine in the uptake medium was 0.05 mM, and the rate of uptake was measured by taking the values for the 2-min uptake.

REFERENCES

1. H. N. Christensen, *Physiol. Rev.* **62**, 1193 (1982).
2. B. M. Bayer, T. N. Lo and M. A. Beaven, *J. biol. Chem.* **255**, 8784 (1980).
3. B. M. Bayer, A. P. Almeida and M. A. Beaven, *J. Pharmac. exp. Ther.* **219**, 752 (1981).
4. S. Bannai and E. Kitamura, *J. biol. Chem.* **255**, 2372 (1980).
5. S. Bannai and E. Kitamura, *J. biol. Chem.* **256**, 5770 (1981).
6. M. Makowske and H. N. Christensen, *J. biol. Chem.* **257**, 5663 (1982).
7. A. Takada and S. Bannai, *J. biol. Chem.* **259**, 2441 (1984).
8. M. F. White, G. C. Gazzola and H. N. Christensen, *J. biol. Chem.* **257**, 4443 (1982).
9. N. E. Owen and M. L. Villereal, *Expl. Cell Res.* **143**, 37 (1983).
10. S. Bannai and T. Ishii, *J. cell. Physiol.* **112**, 265 (1982).
11. V. Hial, M. C. F. DeMello, Z. Horakova and M. A. Beaven, *J. Pharmac. exp. Ther.* **202**, 446 (1977).
12. B. M. Bayer and M. A. Beaven, *Biochem. Pharmac.* **30**, 807 (1981).

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Identification of a second binding isotherm for actinomycin D–deoxyribonucleic acid at low drug concentrations

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Actinomycin D is a heterocyclic compound consisting of a phenoxazinone ring linked to two cyclic pentapeptides. The interaction of the drug with DNA has been studied for many years because of its ability to inhibit RNA synthesis [1], its use as a chemotherapeutic agent in treatment of certain cancers [2], and as a system for studying protein-nucleic acid interactions [3–5]. The models proposed for the mechanism by which actinomycin D binds to DNA are based upon intercalation of the planar chromophore ring at GC base pairs in the double helix [4] or pseudointercalation between helical DNA chains at GC base pairs [6]. Wells and Larson [5], however, have reported that an analysis of actinomycin D binding to synthetic polydeoxyribonucleotides indicated that binding was not always dependent on the presence of GC base pairs. It is important to note, however, that few studies of actinomycin D–DNA interaction have been performed with concentrations of the drug which are relevant to biological (0.001 to 0.1 μg/ml or 0.8 to 80 nM) or therapeutic (0.5 to 2.0 mg/sq. m and <80 nM serum concentration) use. A study was undertaken to analyze the interaction of this drug with DNA at concentrations that fall within the range of biological or therapeutic use. A unique site (or sites) was found on rat liver DNA which could be observed when actinomycin D binding was assayed at concentrations of drug at <80 nM or 0.1 μg/ml. This site (or sites) appeared to be distinct from those previously observed in the presence of higher concentrations of actinomycin D [3–5, 7].

Methods and results

DNA was extracted as described by Maniatis *et al.* [8] from rat liver nuclei isolated by a modification [9] of the method of Hewish and Burgoyne [10] and subsequently digested with EcoRI restriction endonuclease [8]. The DNA was dialyzed under sterile conditions in 50 mM Tris–HCl, pH 7.6, 0.1 M NaCl. EcoRI cleaves DNA on the