3. H. Okuda, A. Hiratsuka, H. Nojima and T. Watabe, *Biochem. Pharmac.* **35**, 535 (1986).
4. B. N. Ames, J. McCann and E. Yamasaki, *Mutat. Res.* **31**, 347 (1975).
5. T. Watabe, T. Ishizuka, N. Ozawa and M. Isobe, *Biochem. Pharmac.* **31**, 2542 (1982).
6. C. C. Irving and R. A. Veazey, *Biochim. biophys. Acta* **166**, 246 (1968).
7. T. Watabe, T. Ishizuka, T. Fujieda, A. Hiratsuka and K. Ogura, *Jpn. J. Cancer Res. (Gann)* **76**, 684 (1985).
8. T. Watabe, Y. Hakamata, A. Hiratsuka and K. Ogura, *Carcinogenesis* **7**, 207 (1986).
9. T. Watabe, A. Hiratsuka and K. Ogura, *Biochem. biophys. Res. Commun.* **134**, 100 (1986).

Biochemical Pharmacology, Vol. 35, No. 24, pp. 4576–4579, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

Uptake of opioid drugs by rat cerebrocortical brain slices

(*Received* 24 *January* 1986; *accepted* 1 *July* 1986)

It is widely accepted that opioid drugs elicit analgesia by binding to specific cell-surface receptors in the CNS [1, 2]. The uptake of morphine into brain slices has been reported by previous workers [3, 4], but the extent to which these drugs can penetrate into the intracellular compartment of neural cells has been little investigated. It is known that the endogenous opioid peptides may be internalized by neurons as a result of receptor recycling [5, 6], and it is possible that opioid drugs may also be translocated across neuronal membranes in this fashion. In some cases their action may involve an intracellular receptor. The present study was designed to establish the extent to which opioid drugs are able to penetrate the intracellular compartment of brain cerebrocortical slices. For this purpose radiolabelled morphine, etorphine (μ-receptor agonists) and ethylketocyclazocine (EKC*; K-agonist/μ-antagonist) were employed as the opioid drugs.

Materials and methods

Brain slices (approx. 1.0 × 0.5 cm and 0.3 mm thick), were prepared from the cerebral cortex of adult Sprague–Dawley rats using a blade and recessed guide [7]. Individual slices were preincubated in 4.1 ml of Krebs–Tris medium of composition (mM); NaCl 138, KCl 5.0, $MgSO_4$ 1.0, $CaCl_2$ 1.2, NaH_2PO_4 1.2, Tris–HCl 20, glucose 10, pH 7.4. The incubation was carried out at 37° in 5 ml glass beakers. Each beaker carried a gassing line which delivered pure oxygen into the medium. After 30 min preincubation, 200 μl [^{3}H]opioid compound was added to each beaker (final concentration see results, final sp. act. 0.9 Ci/mol). Also, 200 μl [^{14}C]inulin, dissolved in Krebs–Tris medium (final concentration 0.05 mg/ml, final sp. act. 10.9 Ci/mol) was added 10 sec later. The period of incubation was terminated by the removal of the slices from the incubation beakers. After draining well the final wet weight of tissue was measured. Each slice was either dissolved in 1 ml of 1 M NaOH and an aliquot taken for radioactivity counting to determine total uptake, or homogenized by hand in 1 ml of 0.32 M sucrose, centrifuged at 10,000 *g* in a microcentrifuge for 2 min, and an aliquot of the supernatant taken for radioactivity counting. In the latter case, the pellet obtained was resuspended in 500 μl trichloroacetic acid (TCA) (10%) and recentrifuged. An aliquot of this supernatant was also taken for radioactivity measurement. The radioactivity in an aliquot of the incubation medium from each beaker was also determined by liquid scintillation counting.

The Inulin Space (I.S.) (μl/100 mg final wet weight) was calculated as follows:

$$\text{I.S.} = \frac{\text{dpm [}^{14}\text{C]inulin/100 mg final wet weight}}{\text{dpm [}^{14}\text{C]inulin/}\mu\text{l incubation medium}}$$

The total volume of 100 mg final wet weight slice was taken as 80 ± 1 [8]. The "non-inulin" space was the difference between the total volume of the tissue and the I.S.

The tissue/medium (T/M) ratio for the drug was taken as the ratio:

$$\frac{\text{dpm in "non-inulin" space}}{\text{dpm in same volume of incubation medium}}$$

Using the specific radioactivity of the drug in the medium it was possible to estimate the concentration of the drug in the intracellular space.

Ethylketocyclazocine methane sulphonate and etorphine HCl were the kind gifts of Sterling-Winthrop and C-Vet Ltd. respectively. Morphine sulphate was purchased from Macfarlan Smith Ltd. The radioactive compounds were all obtained from Amersham International.

Results and discussion

As shown in previous detailed studies, the equilibration of inulin between the incubation medium and the I.S. (inulin space) occurred within 15 min [8, 9]. The half-time for inulin uptake was close to 5 min, which is in good accordance with another report [9]. The mean value for the I.S. was calculated from all measurements after equilibration of the inulin within the slices. The I.S. was 46.3 ± 1.2 μl/100 mg final wet weight (mean ± SEM, N = 30). The "non-inulin" space was calculated to be 33.7 μl/100 mg final wet weight, assuming that 80% of the slice weight was water and 20% solids [8]. This was identical to the value for the non-inulin space calculated using the total volume of tissue determined by employing ^3H_2O (0.4 μCi/incubation), following the same experimental procedure.

The accumulation of EKC (10 μM) was maximal after 45 min, when the T/M for the drug was 38. The maximal uptake was 12.9 nmol/100 mg final wet weight of slice. Morphine uptake at 10 μM, was maximal after 20 to 30 min, at a final T/M ratio of 2.9. Etorphine accumulation at 10 μM followed a similar time scale to that of morphine uptake but the final T/M was 15. The maximal uptakes of morphine and etorphine were 1.0 and 5.1 nmol/100 mg final wet weight, respectively (Fig. 1). In experiments where cerebrocortical slices were incubated under similar conditions and exposed to 10 μM of these opioid drugs for 15 min, inhibitory effects were observed on Ca^{2+} uptake and amino acid neurotransmitter release [10]. The intracellular concentration of morphine, etorphine and EKC would be 21, 122 and 228 μM respectively in these conditions. This suggests that sufficient drug would be able to translocate the cell membrane in order to have an effect at putative intracellular site(s) of action.

The time course of the accumulation of these opioid drugs is incompatible with the characteristics of binding to high-affinity sites on the plasma membrane surface. It is likely that entry of EKC, morphine and etorphine to the

*Abbreviations used: EKC, ethylketocyclazocine; I.S., inulin space; TCA, trichloroacetic acid.

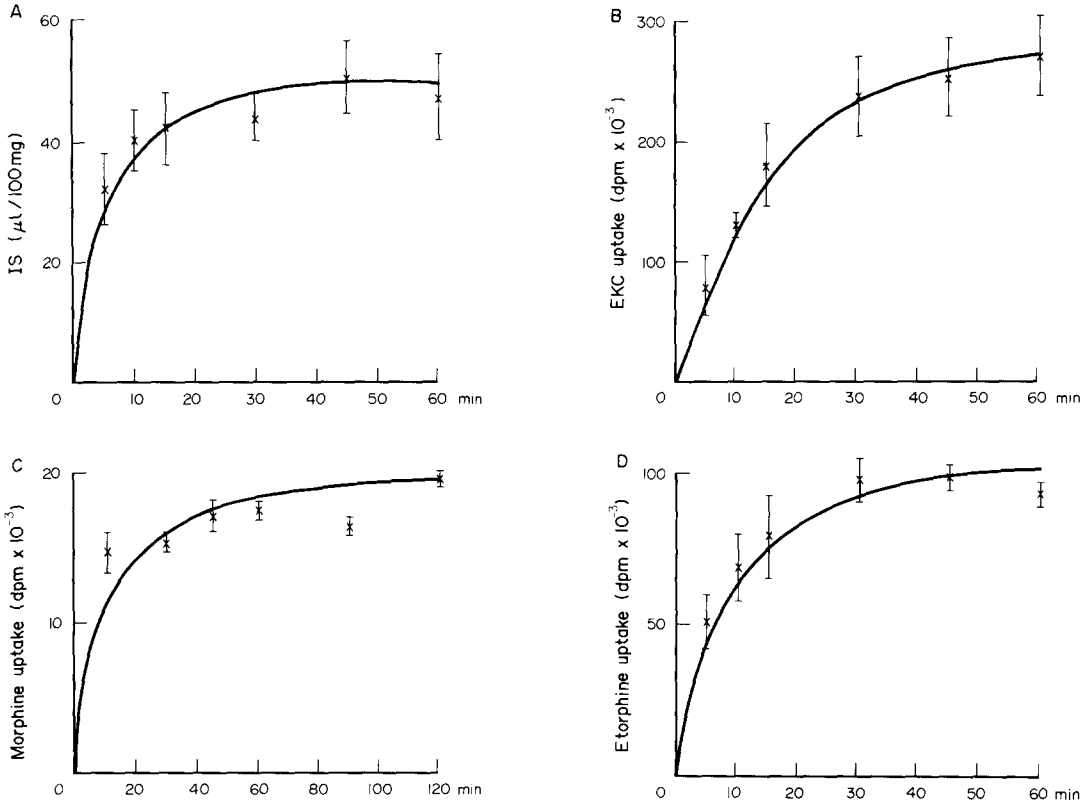

Fig. 1. The time course of uptake of inulin and opioid drugs by cerebrocortical brain slices: (A) [^{14}C]inulin accumulation. Inulin space (I.S.) values (μl/100 mg final wet weight) are mean of 5 experiments, N = 10. Bars show SD values. (B–D) [^{3}H]EKC, [^{3}H]morphine and [^{3}H]etorphine uptake respectively. EKC was dissolved in H_2O, morphine in incubation medium and etorphine in ethanol (final concentration of ethanol was 0.3%). The concentration of each opioid drug was 10 μM. The mean values from 2 experiments were corrected for accumulation of the opioid drugs in I.S. and are expressed as dpm/100 mg final wet weight slice. SD values are shown by the bars, N = 4.

intracellular space of the slices over this time scale is largely due to passage across cell membranes since there would not be enough drug binding sites on the surface membranes to account for the large T/M ratios measured.

The uptakes of morphine and etorphine showed similar temporal characteristics. The structures of these drugs are also similar, and both are μ-receptor agonists, therefore, in further studies, the results with morphine were taken as typical of both of these drugs. The uptakes of morphine and EKC were measured over a concentration range from 10 nM to 10 μM (Fig. 2). It can be seen that for morphine (10 nM to 10 μM) uptake was already maximal after 10 min, whereas for EKC maximal uptake was not reached even at 60 min over this concentration range (Fig. 2). The fact that 10 nM morphine gave essentially the same result as 10 μM morphine indicates that this drug could be crossing cell membranes at pharmacologically relevant concentrations as judged by the K_D values for receptor binding and by the drug concentrations evoking *in vivo* pharmacological responses, all of which are in the nanomolar range [11, 12].

The total uptake minus that accumulated in the inulin space was not saturable at these concentrations as revealed by the T/M ratio. Thus the average T/M at equilibrium, 60 min after the addition of morphine or EKC (10 nM to 10 μM), was 2.57 ± 0.08 and 42.2 ± 2.3 respectively (means $\pm$ SEM, N = 12). These results are in good agreement with those of Teller *et al.* [4] employing morphine. The accumulation of these opioid compounds into the intracellular compartment of the slices, as assessed by homogenization in sucrose and by further treatment with trichloroacetic acid (TCA), are also not saturated at the concentrations employed. These observations suggest that the translocation of the opioid drugs into the intracellular compartment of neural cells is not facilitated by a specific receptor protein, but is more likely to be due to the lipophilicity of these drugs.

It is likely that the lipid soluble drugs employed were also sequestered into the membrane lipids, therefore slices that had been incubated with varying concentrations of morphine or EKC were homogenized in 0.32 M sucrose, to lyse the cells and release non-sequestered opioid compounds. Up to 50% of the drugs at 10 nM, 100 nM and 10 μM were released in this way. A further treatment of the homogenized tissue with 10% TCA released an additional 25–30% of the drugs. This may be due to lysis of cells, nerve terminals and any lipid vesicles undisrupted by homogenization in sucrose. These values are an underestimate of the released opioid compounds, due to loss of material during the experimental protocol. Opioid drugs that are sequestered into membrane lipids are unlikely to be released by homogenization of the slices in sucrose. TCA treatment may allow release of some of the sequestered drugs by changing the properties of the membrane lipids. However, there is a substantial amount of morphine

and EKC accumulated in the intracellular compartment of cerebrocortical brain slices during these experiments.

In summary, the concentration of opioid drugs associated with the cerebrocortical brain slices at steady state was much higher than the drug concentration in the incubation medium. Compartmentation analysis with [^{14}C]inulin indicated that the majority of each of these drugs was in the intracellular compartment of the slice. This was confirmed by the release of over 75–80% of these drugs by cell lysis. The uptake of morphine and EKC was not saturable over a concentration range from 10 nM to 10 μM. It was concluded that the opioid compounds employed could penetrate the cell membranes in the following order: EKC > etorphine > morphine.

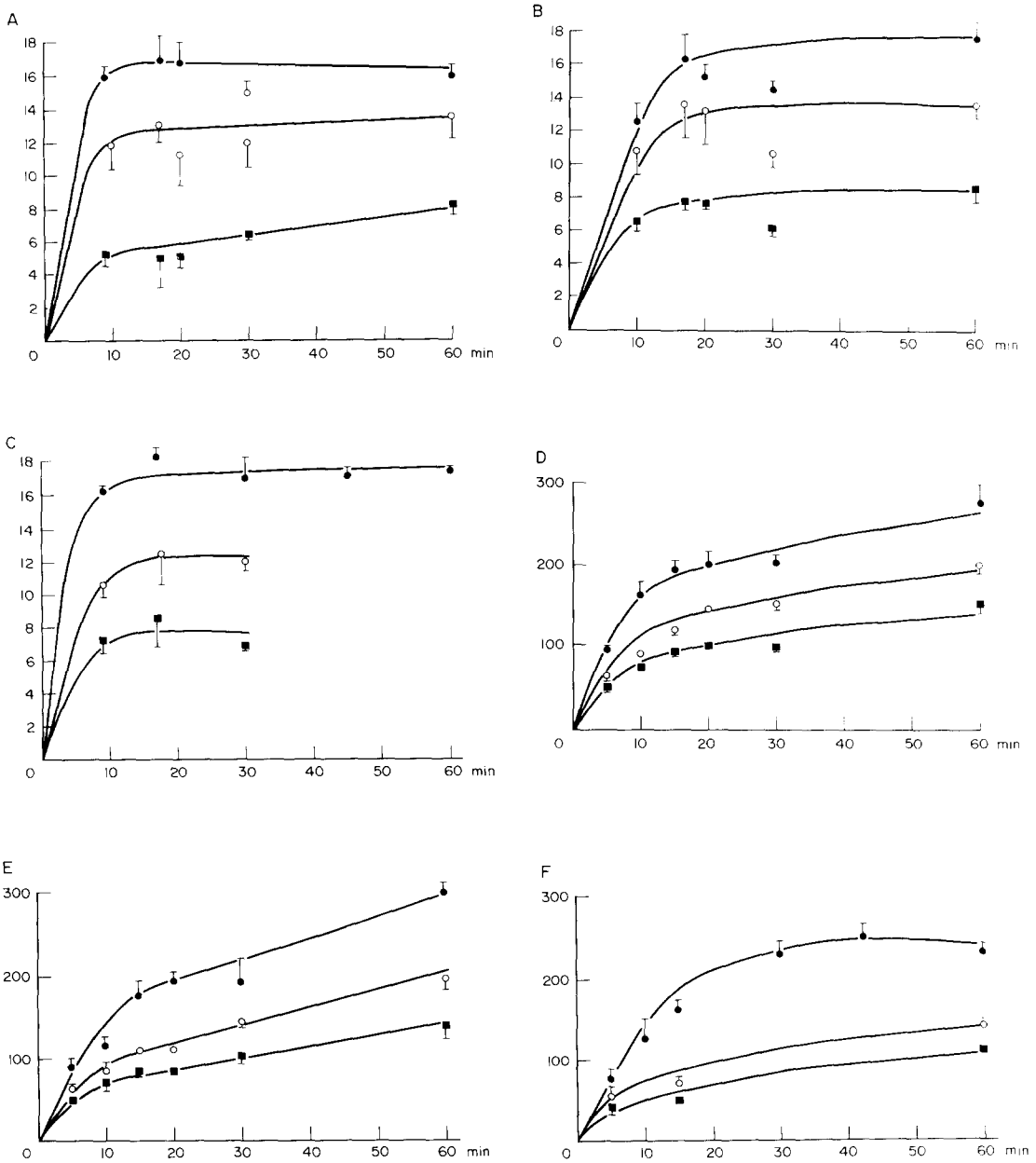

Fig. 2. The effect of concentration on the uptake of opioid drugs into the intracellular compartment of cerebrocortical brain slices. (A–C) 10 nM, 100 nM and 10 μM [^{3}H]morphine respectively. (D–F) 10 nM, 100 nM and 10 μM [^{3}H]EKC respectively: ordinate, uptake is in dpm $\times$ 10^{-3}/100 mg final wet weight; abscissa, time in min. The values are mean from two experiments, corrected for accumulation of the drugs in the inulin space I.S. SEM values are shown by the bars where they are greater than the dimension of the symbol, N = 4. ●, Total uptake into slice; ■, uptake into compartment released by homogenization of slice in 0.32 M sucrose; ○, uptake into compartment released by TCA treatment added to uptake into compartment released by disruption in sucrose.

Acknowledgements—E. J. W. was in receipt of an MRC postgraduate training award. We thank Sterling-Winthrop for financial assistance.

Department of Biochemistry ELIZABETH J. WHITE
Imperial College of Science and Technology H. F. BRADFORD*
London SW7 2AZ, U.K.

REFERENCES

1. C. B. Pert and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2243 (1973).
2. L. Terenius, *Acta. pharmac. Toxic.* **32**, 317 (1973).
3. J. T. Scrafani and C. C. Hug, Jr., *Biochem Pharmac.* **17**, 1557 (1968).
4. D. N. Teller, T. De Guzman and A. Lajtha, *Brain Res.* **77**, 121 (1974).
5. S. G. Blanchard, K-J. Chang and P. Cuatrecasas, *J. biol. Chem.* **258**, 1092 (1983).
6. B. L. Ruth and C. J. Coscia, *J. Neurochem.* **42**, 1677 (1984).
7. H. McIlwain, *Biochem. J.* **78**, 213 (1961).
8. S. Varon and H. McIlwain, *J. Neurochem.* **8**, 262 (1961).
9. M. Moller, K. Mollgard, H. Lund-Anderson and L. Hertz, *Exp. Brain Res.* **22**, 299 (1974).
10. H. F. Bradford, J. M. Crowder and E. J. White, *Br. J. Pharmac.* **88**, 87 (1986).
11. J. Mangan, S. J. Patterson, A. Tavani and H. W. Kosterlitz, *Naun. Schmiedberg's Archs Pharmak.* **319**, 197 (1982).
12. W. K. Schmidt, S. W. Tam, G. S. Schotzberger, D. H. Smith, R. Clark and V. G. Vernier, *Drug Alc. Dependence* **114**, 339 (1985).

* To whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 35, No. 24, pp. 4579–4581, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

High plasma protein binding as a parameter in the selection of betablockers for lactating women

(*Received* 20 *March* 1986; *accepted* 30 *June* 1986)

Many studies have been performed to determine, simultaneously, plasma and breastmilk concentrations of drugs after single or continuous doses in lactating women [1–5]. This has been the most frequently used approach in previous studies of the transfer of drugs from blood to breastmilk. It is generally considered that the main mechanism involved in drug transport from plasma to breastmilk is passive diffusion. Most weak acids and bases enter milk as the non-protein-bound, unionized forms to achieve concentrations that depend on the pH gradient between plasma and milk, the protein binding and the partition coefficient [1].

All these parameters are either known or may be estimated by *in vitro* experiments, thus it should be possible to forecast transfer of drugs into milk. The aim of this work therefore was to investigate whether *in vitro* studies can assist in the choice of drugs in lactating women, in order to avoid the cost and complexity of clinical studies.

Betablockers are frequently prescribed as hypotensive agents for young patients and are therefore often chosen for the treatment of hypertension during lactation. Consequently this class of compounds was selected for study.

Materials and methods

The milk binding of adrenergic beta-receptor antagonists was studied by equilibrium dialysis using the Dianorn® apparatus. The experimental conditions were 37°, pH = 6, constant stirring at 20 r.p.m. for 3.5 hr, at which time equilibrium was reached. Concentrations ranging from 0.25 to 250 μg/ml were used for each of the beta-blockers in 0.066 M phosphate buffer. They were prepared by isotopic dilution of a constant amount of labelled compound mixed with increasing amounts of unlabelled drug.

All labelled drugs [^{14}C]acebutolol (6.4 Ci/mole, Specia), [^{14}C]bornaprolol (44 Ci/mole, Pharmuka), [^{14}C]butofilolol (12 Ci/mole, CEA), [^{14}C]pindolol (22.5 Ci/mole, Sandoz), [^{3}H]propranolol (25,000 Ci/mole, Amersham), [^{3}H]sotalol (500 Ci/mole, CEA) have a chemical purity greater than 98%.

R_F values of the beta-adrenoceptor blocking drugs were determined by thin layer chromatography (TLC), using the following system of solvants: $CHCl_3/CH_3OH/NH_4OH$, 80:20:1.5, v:v.

Pooled human breastmilk was used (pH 6, 20 mM triglycerides). Milk fat was obtained by centrifugation for 30 min at 5000 *g* and resuspended in phosphate buffer at pH 6, then ultrasonicated.

At equilibrium the bound (B) and free (F) concentrations were measured and a curve B = f(F) was plotted. Since the binding was not saturable in the range of drug concentrations studied, no association constant *(K)* or binding sites concentration *(N)* could be derived from the data. The only binding parameter that could be estimated was a partition coefficient, *N*K, which relates the bound to free drug concentrations,

$$B = (NK).F \quad (1)$$

The data were fitted to equation (1) by a least squares regression program.

Results

The six drugs differ significantly in lipophilicity, as indicated by their R_f values upon TLC (Table 1). Their plasma binding is also related to the R_f values, i.e. the degree of lipophilicity.

Table 1 also summarizes the binding percentages obtained for these drugs in whole milk, whey and fat. The results indicate that the sum of binding percentages in whey and fat at an equivalent concentration of triglycerides, relative to milk, is superimposable to the percentage of overall binding in milk. The binding to milk is noticeably lower for the less lipophilic drugs, although they are highly concentrated in milk. Furthermore it is apparent that the bindings to both plasma and milk are positively correlated with the degree of lipophilicity.

Figure 1 illustrates the effect of pH on the binding of propranolol to milk. The milk binding, expressed in terms of the binding ratio B/F is correlated positively with the pH ($r_1 = 0.986$, $\alpha_1 < 0.01$) and negatively with the log value of the ratio of ionized to unionized drug, I/NI, assuming a pK_a of 9.5 ($r_2 = 0.983$, $\alpha_2 < 0.01$). As shown in Fig. 2, there are linear relationships between the R_f value and the log value of *NK* product of the drug, both in serum and in breast milk. The slopes of the respective curves, which exhibit this relationship in serum and in breastmilk, were not significantly different ($P > 0.20$).