lowing cessation of treatment, being decreased by 50% ($P < 0.01$) compared with the control group. Nevertheless there was an increase in the levels of renal cytochrome P-450 of 40% ($P < 0.05$, week 2) and of 135% ($P < 0.01$, week 4) after cessation of AD treatment. The activity of hepatic NADPH-cytochrome *c* reductase was decreased by 20% ($P < 0.01$) at week 4 and in kidney increased by 440% ($P < 0.01$) and 354% ($P < 0.01$) at weeks 2 and 4 respectively.

Using a pharmacological model (AD) of decreased hepatic cytochrome P-450 and NADPH cytochrome *c* reductase, our data further suggest a role for hepatic cytochrome P-450 levels in the regulation of renal cytochrome P-450. Of interest, the renal enzymatic activity is increased two weeks prior to a significant decrease in hepatic enzymes, perhaps due to the early detection of some endogenous inducer(s) by the kidney [1].

The inhibitory effect of AD on the hepatic drug metabolizing enzymes did not reach statistical significance until four weeks after discontinuing treatment. This may be explained by the complex pharmacokinetic behaviour of the drug, i.e. long elimination half-life, capacity of fat to act as reservoir, or even a delayed hepatotoxic effect.

The kidneys represent about 3–7% of the total cytochrome P-450 and NADPH cytochrome *c* reductase content in the rat [14]. The induction pattern of different enzyme systems in the same organ may vary and this may account for the disproportionate increase in NADPH cytochrome *c* reductase activity and cytochrome P-450 levels in the kidney following amiodarone treatment [15]. This disproportion may, however, be due to the existence of multiple forms of cytochrome P-450 where selective induction of some isoenzymes would result in a smaller increase in total cytochrome P-450 levels. Selective induction of some mono oxygenase activities in kidney microsomes following a reduction in hepatic cytochrome P-450 levels has been recently reported [1].

The pharmacological agent used in this study (AD) produces enzyme inhibition in association with direct hepatotoxicity; whether an increase in renal enzyme activity follows administration of a non hepatotoxic enzyme inhibitor remains to be seen.

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REFERENCES

1. G. Babany, V. Descatoire, M. Corbic, S. Gendre, C. Degott, D. Larrey, P. Letteron, J. C. Wandscheer, C. Funck-Brentano and D. Pessayre, *Biochem. Pharmac.* **34**, 311 (1985).
2. D. M. Holt, G. T. Tucker and P. R. Jackson, *Am. Heart J.* **106**, 840 (1983).
3. D. W. Holt, P. C. Adams, R. W. F. Cambell, A. R. Morley, J. Callaghan and G. C. A. Storey, *Br. J. clin. Pharmac.* **17**, 195P (1984).
4. D. M. Paton, D. R. Webner and M. Neutze, *Meth. Find. Exptl. Clin. Pharmac.* **6**, 41 (1984).
5. T. A. Plomp, W. M. Weirsinga and R. A. A. Maes, *Arzne-Forsch./Drug Res.* **35**, 122 (1985).
6. J. B. Simon, P. N. Manley, J. F. Brien, and P. W. Armstrong, *N. Engl. J. Med.* **311**, 167 (1984).
7. O. Grech-Belanger, *Res. Commun. Chem. Pathol. Pharmacol* **44**, 15 (1984).
8. J. R. Means, G. P. Carlson and R. C. Schnell, *Toxicol. Appl. Pharmacol* **80**, 293 (1979).
9. S. V. Jakobsson and D. L. Cinti, *J. Pharmac. exp. Ther.* **185**, 226 (1973).
10. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2370 (1964).
11. P. Mazel, in *Fundamentals of Drug Metabolism and Drug Disposition* (Eds. B. N. La Du, H. G. Mandel and F. L. Way), p. 546. Williams & Wilkins, Baltimore (1971).
12. A. R. Boobis, M. J. Brodie, G. C. Kahn, D. R. Fletcher, J. H. Saunders and D. S. Davies, *Br. J. clin. Pharmac.* **9**, 11 (1980).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
14. W. Hietanen, H. Bartsch, M. Ahotupa, S. S. Park and H. V. Gelboinon, *Drug Metabolism* (Ed. G. Siest), p. 141. Pergamon Press, Oxford (1985).
15. P. Jenner and B. Testa, in *Drug Metabolism: Chemical and Biochemical Aspects* (Ed. J. Swarbrick), p. 445. Marcel Dekker, New York (1976).

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Inhibition of epidermal growth factor binding to HeLa cells by auranofin*

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Auranofin (AF; 2,3,4,6-tetra-O-acetyl-1-thio-beta-D-glucopyranosato-*S*-[triethylphosphine] gold) is a novel, antirheumatic, lipophilic gold complex widely used in the treatment of rheumatoid arthritis [1].

The compound has inhibitory effects on several activation responses in human leukocytes which involve the Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C; PK-C). For example AF inhibits platelet aggregation [1] and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide anion release from human neutrophil polymorphs (PMN) [2] and monocytes [3]. The drug has little effect on superoxide release from PMN and monocytes [3] elicited by the Ca^{2+} ionophore A23187, which acts via Ca^{2+}/calmodulin-dependent pathways independently of PK-C [4].

In view of the apparent selective effect of AF on PK-C-dependent pathways, we are investigating the effect of AF on other cellular events modified by TPA and which involve activation of PK-C. One such event, well characterised in cultured cell lines, is the TPA inhibition of epidermal growth factor (EGF) binding [5]. TPA increases a PK-C mediated phosphorylation of the EGF receptor, a decrease in EGF binding and an inhibition of the intrinsic tyrosine kinase activity of the receptor [6]. In the present paper we show that AF does not interfere with TPA inhibition of EGF binding to HeLa cells, and is itself a powerful inhibitor of binding.

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Materials and methods

AF was a gift from Dr Michael Whitehouse, Queen Elizabeth Hospital and TPA was from CRC Inc., Eden Prairie, MN. [^{125}I]EGF was prepared as previously described [5], and [γ-^{32}P]ATP was prepared as described [7].

HeLa cells were grown in Eagle's minimal essential medium supplemented with 10 mM Hepes and 10% fetal calf serum. Cultures were maintained in a humidified atmosphere of 5% carbon dioxide at 37°. The cells were routinely plated at 2×10^5 cells per 30 mm dish and used at a density of approx. 10^6 cells per dish.

Cells were washed twice with Dulbecco's phosphate buffered saline containing 1 mg/ml bovine serum albumin (PBS-BSA) at 37°. Compounds were dissolved in dimethylsulphoxide (DMSO) resulting in a final solvent concentration of 1%. The same concentration of DMSO was added to control cultures. After incubation with the test compound, cultures were washed in PBS-BSA at 2°, and incubated in 1 ml of cold PBS-BSA containing [^{125}I]EGF. A final concentration of 1 nM [^{125}I]EGF was used unless otherwise stated. After incubation for 4 hr at 2°, the cultures were washed with cold PBS-BSA, the cells dissolved in 0.5 N NaOH and radioactivity measured using a gamma counter. In all experiments, non-specific binding (determined in the presence of 160 nM EGF) was subtracted from the total [^{125}I]EGF binding.

To determine the effect of prolonged incubation of HeLa cells with PDBu, whole cell homogenates were extracted with buffer containing 0.2% Triton X-100 and the extracts chromatographed on DEAE-cellulose [7]. PK-C assays were carried out as described before [7].

Results and discussion

The effects of AF and TPA on the binding of [^{125}I]EGF to HeLa cells are shown in Figs 1 and 2. In these experiments the cells were preincubated with AF or TPA at 37° for varying times before determination of [^{125}I]EGF binding at 2°. Measurement of binding at 2° eliminates any possible effects of the pretreatments on internalization of the growth factor [8]. It is clear that pretreatment with both TPA and AF resulted in a time- and concentration-dependent decrease in [^{125}I]EGF binding. Incubation with 10 μg/ml AF for about 60 min was required to achieve maximal inhibition, presumably reflecting the time to partition into the HeLa cell membranes. The binding assays shown in Figs 1 and 2 contained 1 nM [^{125}I] and, under these conditions, the concentration of AF required for 50% inhibition of binding was approximately 3 μg/ml. Addition of TPA and AF together always resulted in greater inhibition of EGF binding that either compound alone (Fig. 2), but no evidence for synergism was found. No toxicity was observed at concentrations up to 40 μg/ml AF as determined by release of lactate dehydrogenase into the medium and by trypan blue exclusion (data not shown). This apparent mimicry of the effect of TPA by AF was unexpected, given the antagonistic effect of AF on some responses to the phorbol ester.

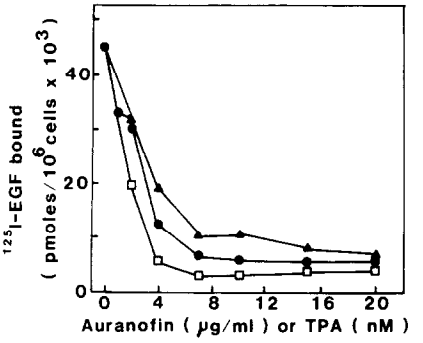

Fig. 2. Dose response for AF- and TPA-induced inhibition of [^{125}I]EGF binding. HeLa cells were incubated for 60 min in PBS-BSA at 37° containing the indicated concentrations of TPA (▲), AF (●) or AF plus TPA (□). [^{125}I]EGF binding was determined at 2° as described in Materials and Methods. Each point is the mean of duplicate determinations.

Figure 3 shows a Scatchard analysis of [^{125}I]EGF-binding data obtained in the presence and absence of 10 μg/ml AF. Over the EGF concentration range used (0.1–25 nM) no evidence for a curvilinear plot was obtained. Non-linear Scatchard plots have been reported for [^{125}I]EGF binding to several cultured cell lines [5, 9]. The presence of AF increased the K_d from 2.6 to 11.9 nM and decreased the apparent number of receptors from 3.7 to 1.8×10^4 per cell.

The TPA-inhibition of EGF-binding is almost certainly a consequence of PK-C mediated phosphorylation of the EGF receptor. Thus a similar pattern of receptor phosphorylation is induced *in vivo* by TPA and *in vitro* by

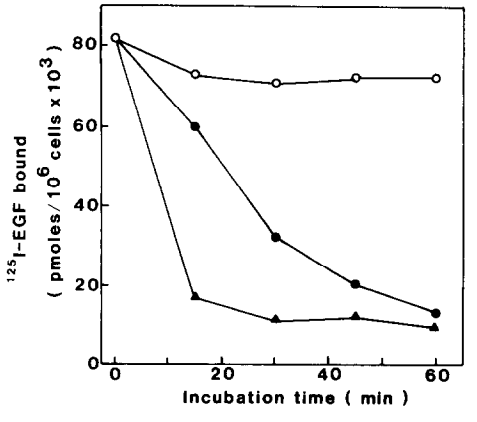

Fig. 1. Time course of inhibition of [^{125}I]EGF binding by AF and TPA. HeLa cells were incubated for the indicated times in PBS-BSA at 37° containing 1% DMSO (○), 10 μg/ml AF (●) or 10^{-7} M TPA (▲). [^{125}I]EGF binding was then determined at 2° as described in Materials and Methods. Each point is the mean of duplicate determinations.

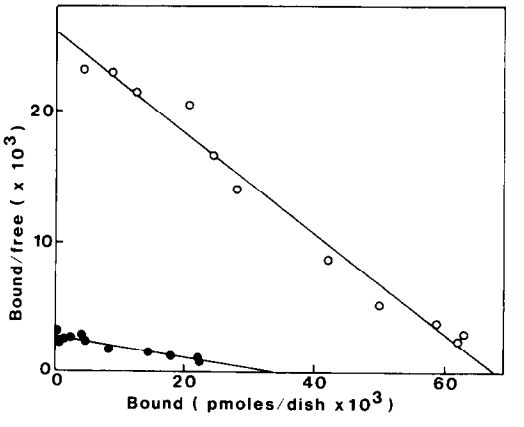

Fig. 3. Scatchard analysis of [^{125}I]EGF binding in the presence and absence of AF. HeLa cells were incubated for 60 min at 37° in PBS-BSA containing 1% DMSO (○) or 10 μg/ml AF (●). [^{125}I]EGF binding was determined at 2° over the concentration range 0.1–25 nM as described in Materials and Methods. Each point is the mean of duplicate determinations.

purified PK-C [6]. In addition, the TPA-sensitivity of EGF binding to isolated membranes is restored by incubating membranes with PK-C [10]. TPA is believed to activate PK-C by initiating its translocation to cellular membranes [11, 12]. Consequently, it was of interest to determine whether the effect of AF on EGF binding was also mediated via PK-C. Several reports have shown that prolonged exposure of cells to phorbol esters induces a loss of PK-C activity and a loss of biological responsiveness to phorbol esters [13, 14]. Pre-treatment of HeLa cells with phorbol-12,13-dibutyrate for 40 hr resulted in a marked loss of PK-C activity (Fig. 4), although a small portion of the total cellular PK-C activity persisted (Fig. 4, Panel B).

Binding of [^{125}I]EGF to pre-treated cells was refactory to inhibition by TPA, but remained sensitive to inhibition by AF (Fig. 5). One interpretation of this result is that AF does not require PK-C for its effect on EGF binding. However, we do not believe that this can be stated with certainty. TPA results in the massive translocation of PK-C to cellular membranes and individual biological responses requiring localized membrane-association of the enzyme may be particularly sensitive to declining PK-C levels. For example, we have observed that the effects of TPA on phospholipid metabolism are more sensitive to depletion of cellular PK-C than the effects of bombesin, even though the responses to both agents are mediated through PK-C [15]. In the present experiments cells pretreated with the dibutyrate still retained significant PK-C activity (Fig. 4) and an effect of AF on this enzyme remains a possibility.

The mechanism for the unexpected inhibitory effect of AF on EGF binding is quite unknown. As reviewed elsewhere [16], AF interacts with low molecular weight thiols as well as with sulfhydryl groups in proteins and would be expected to perturb membrane structure. Experiments are in progress to determine whether the inhibitory effect is due to a direct interaction with the EGF receptor, or an indirect consequence of, for example, altered receptor phosphorylation.

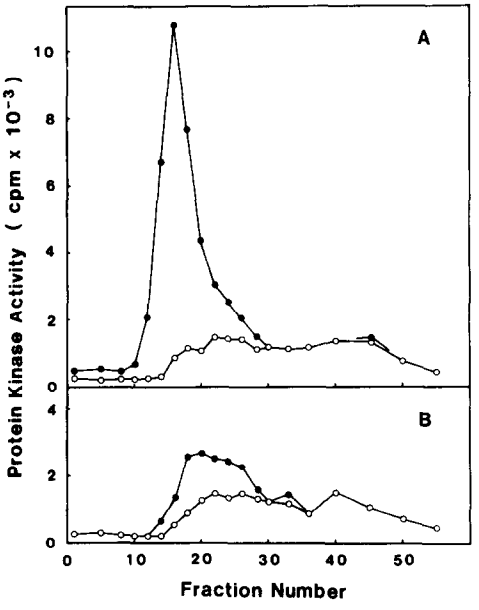

Fig. 4. Effect of pretreatment with phorbol-12,13-dibutyrate on cellular protein kinase C activity. HeLa cells were incubated with DMSO (Panel A) or 400 nM phorbol-12,13-dibutyrate (Panel B) for 24 hr; a further 400 nM phorbol ester was added and incubation continued for a further 16 hr. The cells were then homogenised and extracts chromatographed on DEAE cellulose as described in Materials and Methods. Protein kinase C activity determined in the absence (○) or presence (●) of Ca^{2+} and phospholipid.

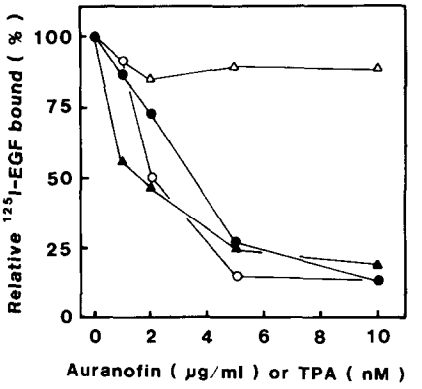

Fig. 5. Effect of pretreatment with phorbol-12,13-dibutyrate on inhibition of [^{125}I]EGF binding by AF and TPA. HeLa cells were incubated with 400 nM phorbol-12,13-dibutyrate or DMSO for 24 hr; a further 400 nM phorbol ester was added and incubation contained for a further 16 hr. The cells were then incubated with varying concentrations of AF or TPA for 60 min before measuring [^{125}I]EGF binding at 2°. ▲, ●; preincubation with DMSO followed by incubation with TPA or AF respectively. △, ○; preincubation with phorbol-12,13-dibutyrate followed by incubation with TPA or AF respectively. Each point is the mean of duplicate assays.

In summary, AF inhibited the binding of [^{125}I]EGF to HeLa cells in a time- and concentration-dependent manner. Pre-treatment of cells for 60 min with AF (10 μg/ml) increased the Kd from 2.6 to 11.9 nM and decreased the apparent number of receptors per cell from 3.7×10^4 to 1.8×10^4. AF-sensitivity of EGF binding was retained in cells pre-treated with PDBu. Such treatment caused a marked loss of protein kinase C activity, and was associated with refractoriness of EGF binding to inhibition by TPA.

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REFERENCES

1. I. Nathan, A. E. Finkelstein, D. T. Walz and A. Dvilansky, *Inflammation* **6**, 79 (1982).
2. I. Hafstrom, B. E. Seligmann, M. M. Freidman and J. I. Gallin, *J. Immunol.* **132**, 2007 (1984).
3. N. P. Hurst, manuscript in preparation.
4. H. Rasmussen and P. Q. Barret, *Physiol. Rev.* **64**, 938 (1984).
5. A. W. Murray and M. Froscio, *Carcinogenesis* **1**, 681 (1980).
6. C. Cochet, G. N. Gill, J. Meisenhelder, J. A. Cooper and T. Hunter, *J. biol. Chem.* **259**, 2553 (1984).
7. P. M. Tapley and A. W. Murray, *Biochem. biophys. Res. Commun.* **118**, 835 (1984).
8. J. Schlessinger, Y. Schechter, M. C. Willingham and I. Pastan, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2659 (1978).
9. M. Shoyab, J. E. De Larco and G. J. Todaro, *Nature, Lond.* **279**, 387 (1979).
10. J. C. Fearn and A. C. King, *Cell* **40**, 991 (1985).

11. A. S. Kraft and W. B. Anderson, *Nature, Lond.* **301**, 621 (1983).
12. P. M. Tapley and A. W. Murray, *Biochem. biophys. Res. Commun.* **122**, 158 (1984).
13. H. St. C. Gainer and A. W. Murray, *Biochem. biophys. Res. Commun.* **126**, 1109 (1985).
14. A. Rodriguez-Pena and E. Rozengurt, *Biochem. biophys. Res. Commun.* **120**, 1053 (1984).
15. J. G. Muir and A. W. Murray, *J. Cell. Physiol.* in press.
16. S. T. Crooke, R. M. Snyder, T. R. Butt, D. Ecker, H. S. Allandeen, B. Monia and C. K. Mirabelli, *Biochem. Pharmac.* **35**, 3423 (1986).

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Comparative study of [^{3}H]glutamate binding sites in rat retina and cerebral cortex

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It has been believed that the high affinity and Na^+-independent binding of radiolabeled L-glutamic acid (Glu*), a potential candidate for a central excitatory neurotransmitter, is indeed a biochemical measure for the association of this acidic amino acid with its physiologically relevant synaptic receptors [1–3]. The binding is affected significantly by the inclusion of some physiological inorganic ions such as Cl^-, Ca^{2+} and Na^+ [4]. These biochemical binding studies together with electrophysiological investigations have revealed the multiplicity of receptors for central excitatory acidic amino acid neurotransmitters: *N*-methyl-D-aspartic acid (NMDA) sensitive receptors (A1), quisqualic acid (QA) sensitive receptors (A2), kainic acid (KA) sensitive receptors (A3), and L-2-amino-4-phosphonobutyric acid (AP4) sensitive receptors (A4) [4, 5].

On the other hand, relatively little attention has been paid to the binding of [^{3}H]Glu in the retina which is supposed to contain Glu-ergic synapses in its structure [6, 7]. It has been demonstrated that [^{3}H]Glu really binds to membrane fractions from the chick retina with a high affinity [8]. In the present study, we have attempted to analyze the retinal [^{3}H]Glu binding in comparison with cerebral [^{3}H]Glu binding in terms of the modulation by various ions.

Materials and methods

Materials. QA, NMDA, KA, DL-AP4 and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]Glu (L-[3,4-^{3}H]glutamic acid, 46.6 Ci/mmole) was obtained from New England Nuclear (Boston, MA, U.S.A.). Other chemicals used were all of commercially guaranteed grade.

Membrane preparation. Groups of three male albino Wistar rats weighing 200–250 g were housed together in a metallic breeding-cage at a room temperature of 25 ± 2° and a humidity of 55 ± 5%, in a room with a 12-hr light–dark cycle. Animals were decapitated between 10:00 and 11:00 a.m. in the light cycle. Retinas were dissected out and placed into ice-cold deionized distilled water for homogenization within 1 min after the animals were killed. Cerebral cortex was dissected out on a chilled plastic plate according to the procedures described by Glowinski and Iversen [9]. The retina and cerebral cortex were homogenized individually in 50 vol. glass-distilled deionized water using a Polytron homogenizer at setting No. 6 for 30 sec at 4°. The homogenates were centrifuged at 50,000 *g* for 30 min, and the resultant pellets were suspended in 50 mM Tris-acetate buffer (pH 7.4). The suspensions were again centrifuged as above. These washing procedures were repeated three times. The final pellets thus obtained were suspended in 0.32 M sucrose and the suspensions were frozen at −80° for 19–20 hr [10]. The frozen suspensions were thawed at room temperature and washed twice by centrifuging at 50,000 *g* for 30 min with 50 mM Tris-acetate buffer (pH 7.4) before each use.

Binding assay for [^{3}H]*Glu.* Each membranous homogenate suspension was incubated with 10 nM [^{3}H]Glu in 500 μl of 50 mM Tris-acetate buffer (pH 7.4) at 2° or 30° for 60 min in the presence and absence of various compounds. Incubation was terminated by the addition of 3 ml of ice-cold buffer and subsequent filtration through a Whatman GF/B glass filter under a constant vacuum of 15 mm Hg. After washing the filter four times with 3 ml of ice-cold buffer, the radioactivity trapped on the filter was measured by a liquid scintillation spectrometer (LSC 900, Aloka, Japan) using 5 ml of modified Triton–toluene scintillant [11] at a counting efficiency of 40–42%. The radioactivity found in the presence of 1 mM nonradioactive Glu was subtracted from each experimental value to obtain the specific binding of [^{3}H]Glu [12]. The specific binding increased linearly with incubation time and reached a plateau within 30 min independently of the incubation temperature. Thin-layer chromatography on cellulose-coated plates with phenol–H_2O (75:25, at 22 ± 2°) as a solvent system revealed that no significant degradation of the radioactive ligand occurred during the incubation with cerebral and retinal preparations at 30° for 60 min.

Binding assays were always carried out in triplicate with a variation of less than 10%. Protein content was measured by the method of Lowry *et al.* [13]. The protein concentration usually employed was between 200 and 250 μg per assay. Results were usually expressed as the mean ± SE, and the statistical significance was determined by Student's *t*-test.

Results and discussion

As shown in Fig. 1, neither Cl^- nor Cl^-/Ca^{2+} elicited a significant alteration in the retinal binding despite the occurrence of a profound augmentation of the cerebral binding by these ions. Chloride as well as Cl^-/Ca^{2+} induced a significant increment of the density of the binding sites without altering their affinities in the cerebral synaptic membranous preparations [12]. In addition, Na^+ remarkably facilitated the binding of [^{3}H]Glu to cerebral preparations with a concomitant suppression of the retinal binding. A 100 mM concentration of sodium acetate exerted about a 5-fold elevation of the cerebral binding, while inducing a 50% reduction of the retinal binding (Fig. 1). These results suggest the possible difference in the ionic modulatory mechanisms of the binding between the retina and cerebral cortex. Since sodium ions are known to affect differentially the opiate receptor binding of agonists and antagonists [14], it seems possible that Na^+ may provide a useful tool for the differentiation and/or subclassification of the Glu binding sites. Similarly significant suppression of [^{3}H]Glu binding occurs in the peripheral endocrine organs such as the pituitary [10] and adrenal [15].

* Abbreviations: AP4, L-2-amino-4-phosphonobutyric acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; Glu, L-glutamic acid; KA, kainic acid; NMDA, *N*-methyl-D-aspartic acid; and QA, quisqualic acid.