Biochemical Pharmacology, Vol. 36, No. 17, pp. 2821–2826, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

AMILORIDE INHIBITS THE PROTEIN TYROSINE KINASES ASSOCIATED WITH THE CELLULAR AND THE TRANSFORMING SRC-GENE PRODUCTS*

PETER PRESEK and CHRISTOPH REUTER

Rudolf-Buchheim-Institut für Pharmakologie der Justus-Liebig-Universität Giessen, Frankfurter Straße 107, D-6300 Giessen, Federal Republic of Germany

(*Received* 14 *August* 1986; *accepted* 20 *March* 1987)

Abstract—Amiloride inhibits a protein tyrosine kinase from rat brain extracts. The kinase activity is characterized by an anti-serum (TBR-serum) which immunoprecipitates $pp60^{c\text{-}src}$, the cellular counterpart of the transforming protein $pp60^{v\text{-}src}$ of Rous sarcoma virus. In immunocomplexes, TBR-IgG serves as an artificial but specific phosphate acceptor. The phosphate incorporation into TBR-IgG is a time- and temperature-dependent process.

In the presence of amiloride the TBR-IgG phosphorylation is reduced. The drug does not influence the immunocomplexes formed by TBR-IgG and $pp60^{src}$ and no amiloride-activated protein tyrosine phosphatase can be detected in the immunocomplex system. Half-maximal inhibition of the tyrosine kinase occurs at 300 μM amiloride and is competitive with respect to ATP. Viral $pp60^{src}$ kinase of transformed cells is more sensitive to amiloride (IC_{50}: 50–100 μM). Furthermore, normal cellular tyrosine kinases are to a lesser extent inhibited by amiloride as compared to the transforming viral $pp60^{src}$ kinase. These results may indicate different amiloride-sensitive forms of cellular $pp60^{src}$ kinases.

Early changes in ion fluxes have been supposed to play an important role in cell response to growth factors [1–4]. Therefore, inhibition of the amiloride-sensitive Na^+–H^+ exchange was thought to be one of the earliest important cellular events responsible for the blockade of mitogenic effects of growth factors caused by amiloride. The relevant mechanisms, however, of amiloride-sensitive processes during mitogenesis have again become obscure, as it has been demonstrated that amiloride also inhibits a variety of protein kinases [5–7].

Growth factor receptor associated tyrosine kinases are known to be inhibited by amiloride [8], but no studies have been reported about the effect of the drug on onc-gene product tyrosine kinases. Those data, however, seem to be of special interest since it has been shown that even very closely related tyrosine protein kinases such as viral and cellular $pp60^{src}$ are inhibited to an extremely different extent by di(adenosine-5′)tetraphosphate (Ap_4A)† [9]. $pp60^{v\text{-}src}$, a phosphoprotein of 60 kDa, is the product of the Rous sarcoma virus (RSV) src-gene and represents a prototypical retroviral transforming protein tyrosine kinase [10]. The cellular counterpart of the viral $pp60^{src}$ has been preserved phylogenetically in all metazoa tested so far [11, 12]. Nervous tissues especially revealed high tyrosine kinase activities associated with cellular $pp60^{src}$ [13, 14].

In this paper we provide data that amiloride also acts as an inhibitor of the type of a tyrosine-specific protein kinase which is associated with the cellular src-gene product in rat brain. $pp60^{src}$ kinase activities from RSV-transformed and normal cells were compared as to their inhibition by amiloride.

*This work is part of the thesis of Ch.R. to be presented to the Fachbereich Humanmedizin, JLU Giessen.

†Abbreviations used: RSV, Rous sarcoma virus; SR-A or SR-D, Rous sarcoma virus, strain Schmidt-Ruppin, subgroup A or D; viral or cellular $pp60^{src}$, the 60,000 dalton product of the viral (v-) or cellular (c-) src-gene; TBR-serum, serum from tumor bearing rabbits immunoprecipitating $pp60^{src}$; CEF, chicken embryo fibroblasts; PMSF, phenylmethylsulfonylfluoride; amiloride, N-amidino-3,5-diamino-6-chloropyrazine-carboxamide; pyrazinamide, pyrazinoic acid amide; triamterene, 6-phenyl-2,4,7-triaminopteridine; Ap_4A, P^1, P^4-di(adenosine-5′)tetraphosphate and Ap_5A, P^1,P^5-di(adenosine-5′)pentaphosphate.

MATERIALS AND METHODS

Materials

Amiloride hydrochloride, pyrazine, pyrazinamide, triamterene and sodium orthovanadate (Na_3VO_4) were obtained from Sigma, München. 2-Aminopyrazine, pyrazinecarboxylic acid and 2,3-pyrazinedicarboxylic acid are from Merck, Darmstadt. Heparin was obtained from Braun Melsungen, Melsungen.

^{32}P-orthophosphate (carrier free) and ^{32}P-γ-ATP (3000 Ci/mmol or 11.1 × 10^{13} bequerels/mmol) were purchased from Amersham-Buchler, Braunschweig, F.R.G.

Methods

Cells and viruses. RSV-SR-A was used to transform chicken embryo fibroblasts (CEF) which were prepared and maintained as previously described [15]. RSV-SR-D transformed STU-mouse cells, D_{17},

[16] were a gift of Dr. H. Bauer, Institut für Medizinische Virologie, Gießen, F.R.G.

Nearly confluent cell cultures were labeled for 4 hr with 1 mCi of ^{32}P-orthophosphate per ml in phosphate-free Dulbecco's modified Eagle's medium.

Preparation of extracts. Urethane-anesthetized adult Sprague–Dawley rats were treated with heparin (6000 units per animal) and perfused as described by Berry and Friend [17]. Brains were obtained by dissection and homogenized in ice-cold lysis buffer (10 mM Tris–HCl, pH 7.5; 5 mM EDTA; 2 mM 2-mercaptoethanol; 10 mM NaF; 0.2 mM PMSF; 1 mM ϵ-amino capronic acid; 0.5 mg/ml trypsin-kallikrein inhibitor; 0.1% (v/v) Triton X-100). For 1 g of wet tissue 3 ml of lysis buffer were used and for cells in culture 1 ml for about 10^7 cells. All subsequent steps were carried out at 4°. Cells or tissue were homogenized in a glass–Teflon homogenizer (10 strokes) and centrifuged at 50,000 *g* for 30 min (cells) or 60 min (brains). The resulting supernatants were used for experiments. As we found no significant differences in kinase activities between freshly prepared extracts or frozen materials (brains or extracts), we usually performed the experiments with extracts stored before at −30°. Protein determination was done by the Coomassie dye-binding assay according to Bradford [18].

Protein kinase assay in immunocomplexes. The activity of the protein tyrosine kinase was determined in a solid phase assay. Heat-inactivated rabbit antiserum against pp60^{src} (TBR-serum) was absorbed for 2 hr at 4° onto swollen Protein-A Sepharose (Deutsche Pharmacia, Freiburg). TBR-sera were obtained as originally described [19]. For 4 mg (dry weight) of the substituted Sepharose 5 μl of antiserum were used. The Protein-A-IgG complexes were washed three times with buffer A (100 mM sodium phosphate buffer, pH 7.0; 40 mM NaF; 10 mM EDTA; 0.05% (v/v) Triton X-100). Thirty microlitres of antigenic material were added and incubated for 50 min at 4°. The resulting immunocomplexes were subsequently washed, twice with ice-cold buffer A and twice with 25 mM Tris–HCl, pH 7.5 and blotted dry. Amiloride was added in a volume of 10 μl before the kinase assay was begun. The drug was dissolved in 25 mM Tris–HCl, pH 7.5 to a final concentration of 5 mM without any change of the pH-value of the buffer. The phosphotransferase reaction was started by addition of 10 μl of tracer (25 mM Tris–HCl, pH 7.5, divalent cations and 0.5–1 μCi ^{32}P-γ-ATP). Immunoprecipitated cellular pp60^{src} were incubated with tracer for 20 min at 30° and the viral pp60^{src} 3 min at 4°. The reaction was terminated by addition of 40 μl sample buffer. Samples were analysed on 11% SDS polyacrylamide slab gels as previously described [20]. The dried gels were exposed to Fuji X-ray films (Fuji Photo Film Co, Ltd.). The radioactivity incorporated into the heavy chain of IgG was quantitated by Cerenkov counting. The activity of pp60^{src} tyrosine kinase is given in femtomoles phosphate incorporated into the heavy chains of TBR-IgG.

Identification of phosphoamino acids. Two-dimensional separation and identification of ^{32}P-labeled phosphoamino acids from phosphorylated TBR-IgG heavy chains were performed according to Hunter and Sefton [21].

Immunoprecipitation of ^{32}P-labeled pp60^{src}. ^{32}P-orthophosphate labeled cells were lysed with RIPA buffer [22]. Preparation of extracts and immunoprecipitations were carried out as described above. We used 4 mg Protein-A Sepharose and 10 μl of TBR-serum or control serum per 120 μl of extract. After 8 washes with ice-cold buffer A to remove unbound material, 50 μl of sample buffer were added and the immunoprecipitated phosphoproteins were analysed on 11% SDS slab gels. Immunoprecipitation of cellular pp60^{src} was more efficient if the method was modified as follows: 120 μl of ^{32}P-labeled cell extract were incubated with 10 μl TBR-serum G_4 or control serum for 50 min at 4° in the presence or absence of 1.5 mM amiloride. Swollen protein-A Sepharose (4 mg, dry weight) was added and incubated for additional 2 hr at 4°. All other procedures were performed as mentioned above.

RESULTS

Characterization of the protein kinase activity in immunocomplexes

We examined extracts of rat brains with TBR-sera for protein tyrosine kinase activity in a solid-phase immuno assay which is the common method to assay pp60^{src} kinase [11, 23, 24]. As we found high activities of TBR-reacting tyrosine kinases in blood cells and serum [25], we perfused the brains *in situ* to minimize contaminations with tyrosine kinases originating from blood.

As expected, the TBR-IgG phosphorylation by the kinase is a temperature- and time-dependent process. Since the efficiency of phosphate incorporation into the immunoglobulin is low both at 4° and 20°, we performed all phosphorylation experiments at 30°. Under these conditions the phosphate incorporation was linear within the first 20 min (Fig. 1A). At least a 2-fold dilution of extract was necessary in order that the kinase activity was proportional to the amount of extract (Fig. 1B). We identified tyrosine residues as phosphate acceptors in immunoglobulin heavy chains (Fig. 1B, inset). Traces of phosphoserine (< 5%) were also found. An unidentified serine kinase seems to be responsible for this phenomenon. Similar observations have already been reported and discussed in detail for normal and tumor cells [24, 26, 27]. No tyrosine kinase activity was detected by normal rabbit sera or in the absence of antigenic material. An extensive increase in substrate phosphorylation by pp60^{src} was obtained using Mn^{2+} instead of Mg^{2+} as a divalent cation [24, 27]. To obtain the highest TBR-IgG phosphorylation rate we evaluated the divalent cation requirement for the rat brain tyrosine kinase. The kinase activity increased with increasing divalent cation concentrations up to 60 mM (not shown). As compared to Mg^{2+} alone, Mn^{2+} as well as a mixture of Mg^{2+} and Mn^{2+} increased the tyrosine phosphorylation of IgG about 2–3-fold. The mixture is more efficient than Mn^{2+} alone. To minimize unspecific effects of high salt concentrations we used 30 mM Mg^{2+} or 30 mM Mn^{2+}

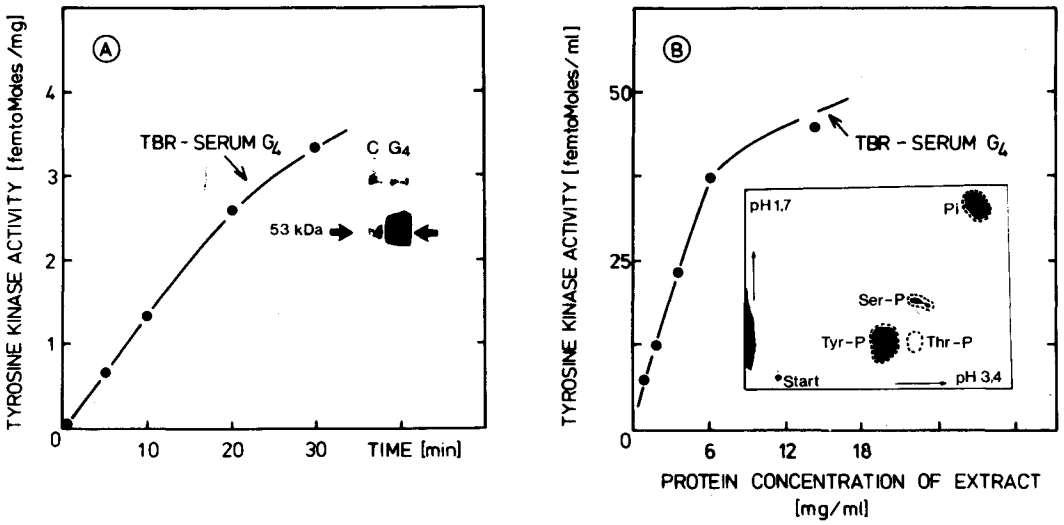

Fig. 1. Phosphorylation of TBR-IgG by a protein tyrosine kinase from rat brain extracts. Dependence of time (A) and of protein content of extract (B). For time dependence the protein concentration was 15.2 mg/ml. The activity of the protein tyrosine kinase is given in femtomoles phosphate incorporated into TBR-IgG. For both A and B, 30 mM Mg^{2+} was used in the kinase assay. Inset A: autoradiogram of the phosphorylated immunoglobulins analysed by SDS slab gel electrophoresis. Lane C: control rabbit serum; lane G_4: TBR-serum, G_4. The arrows indicate the heavy chain region of IgG. Inset B: phosphoamino acid analysis of the phosphorylated IgG of TBR-serum G_4. The positions of the standard phosphoamino acids, identified by ninhydrin, are shown with dotted lines on the autoradiogram: Tyr-P, phosphotyrosine; Thr-P, phosphothreonine; Ser-P, phosphoserine; P_i, orthophosphate.

or a mixture of 15 mM Mg^{2+} and 15 mM Mn^{2+} as final concentrations in the assays. The corresponding tyrosine kinase activities calculated were 2.8 ± 0.7, 4.7 ± 0.5 and 6.3 ± 1.4 fmoles/mg, respectively. The data are mean values and standard deviations from four independent experiments.

Effect of amiloride on pp60^{src} tyrosine kinase activity

Addition of amiloride to immunoprecipitated pp60$^{c\text{-}src}$ from rat brain extracts reduced the phosphate incorporation into the TBR-IgG. Half-maximal inhibition occurred at 300 μM amiloride and is formally competitive with respect to ATP (Fig. 2). To specify the inhibition by amiloride we also tested pyrazine and other pyrazine-derivatives such as 2-aminopyrazine, pyrazinecarboxylic acid, 2,3-pyrazinecarboxylic acid and pyrazinamide. These drugs did not inhibit the kinase even in final concentrations of more than 1.5 mM. Triamterene, which acts as a potassium-sparing diuretic like amiloride but

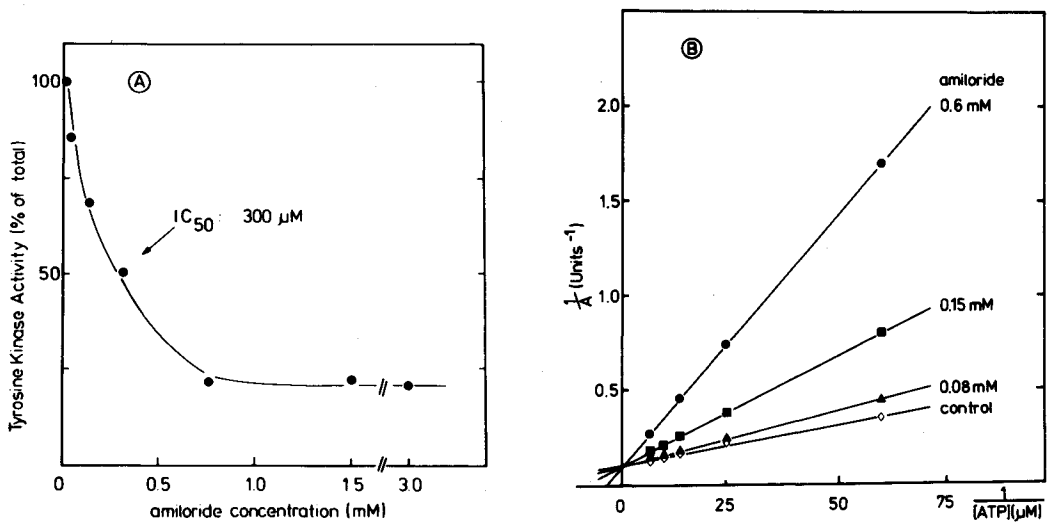

Fig. 2. Inhibition of tyrosine phosphorylation of TBR-IgG by amiloride (A) and the kinetics of inhibition presented graphically by the double reciprocal method (B). The experiments were performed with rat brain extracts. For both A and B, 30 mM Mg^{2+} was used in the kinase assay. 100% kinase activity in A was 6.3 femtomoles/ml.

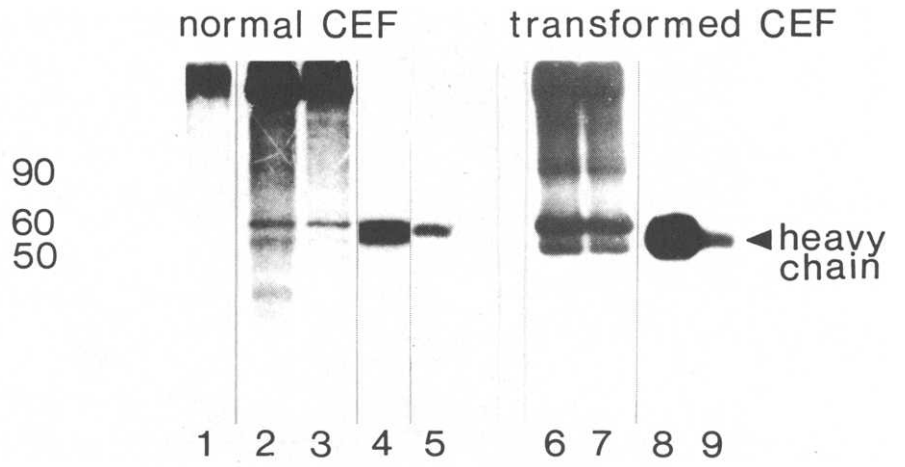

Fig. 3. Effect of amiloride on immunocomplexes formed with cellular or viral pp60^{src}. pp60^{src} of ^{32}P-labeled CEF or SR-A transformed CEF were immunoprecipitated with TBR-serum G_4. Phosphoproteins were visualized by autoradiography. The resulting autoradiogram is presented. Lanes 1–5: normal CEF; lanes 6–9: SR-A transformed CEF. Immunoprecipitates of ^{32}P-labeled pp60^{src}: lane 1: control; lanes 2 and 6: without amiloride; lanes 3 and 7: with 1.5 mM amiloride. The corresponding kinase assays: lanes 4 and 8: without amiloride; lanes 5 and 9: with 1.5 mM amiloride.

is no pyrazine-derivative, had no effect on pp60$^{c\text{-}src}$ kinase activity.

Besides a direct influence of amiloride on pp60^{src} kinase, other mechanisms responsible for the decreased phosphorylation rate had to be excluded. (1) In our system, amiloride had no effect on the rate of ATP degradation as shown by thin-layer chromatography with polyethyleneimine coated plates developed with 1 M LiCl. (2) The stability of the pp60^{src}–IgG complex was not disturbed by the drug. Even in the presence of 1.5 mM amiloride the amount of ^{32}P-labeled immunoprecipitated pp60^{src} was only marginally reduced (Fig. 3). (3) The inhibition of TBR-IgG phosphorylation by amiloride was reversible. Transient incubation of the immunocomplexes with amiloride before the kinase assay was performed did not affect the IgG-phosphorylation. (4) It has been reported that amiloride can stimulate protein phosphatase activity [28]. In our system, no reduction of IgG phosphorylation was observed after incubation of immobilized ^{32}P-phosphorylated TBR-IgG in the presence of 1.5 mM amiloride and 15 mM $MgCl_2$ at 30° for 20 min. Furthermore, vanadate (VO_4^{3-}) which inhibits protein tyrosine phosphatases [29] did not increase the TBR-IgG phosphorylation when present at a final concentration of 150 μM. These results indicate that amiloride directly inhibits the protein tyrosine kinase activity of pp60^{src} in the immunocomplex system. As can be seen in Fig. 4, amiloride inhibited both viral and cellular pp60^{src} tyrosine kinases. The transforming tyrosine kinase was more sensitive to amiloride. The IC_{50} values varied between 50–100 μM, while the amiloride concentration for half-maximal inhibition of the cellular

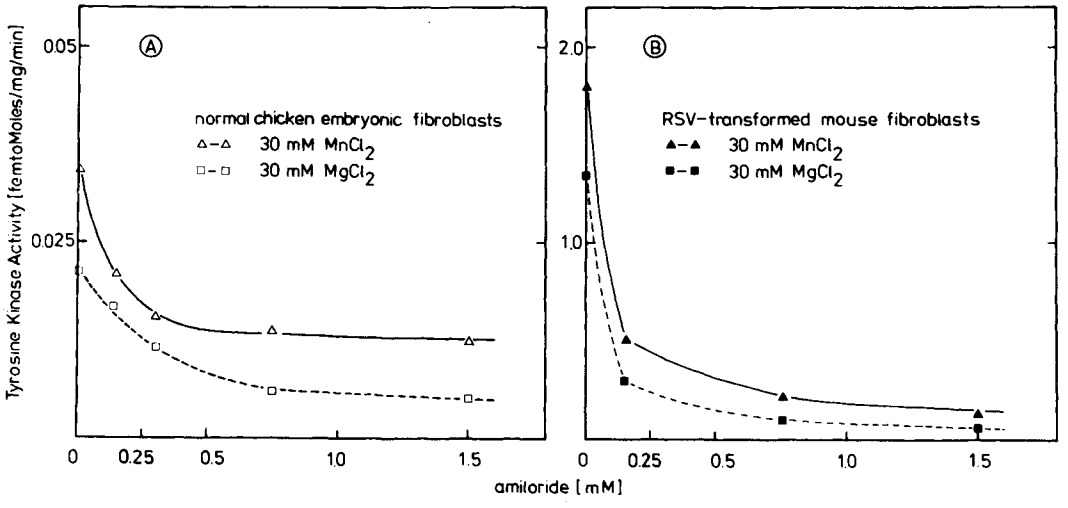

Fig. 4. Inhibition of pp60^{src} tyrosine kinase activity by amiloride using normal CEF (A) and SR-D transformed mouse fibroblasts (B). Protein concentration of extract was 3.5 mg/ml in A and 6.9 mg/ml in B.

Table 1. Maximal amiloride-dependent inhibition of pp60^{src} tyrosine kinase from normal and RSV-transformed cells

Divalent cations used	Amiloride-dependent inhibition in percent (%) of total kinase activity			
	Non-transformed		RSV-transformed	
	rat brain	CEF	SR-A CEF	D_{17} mouse fibroblasts
30 mM Mg^{2+}	73 ± 4 (5)*	77 ± 5 (4)	96	97
30 mM Mn^{2+}	67†	62 ± 4 (3)	93	94
15/15 mM Mg^{2+}/Mn^{2+}	64	68	96	ND

The tyrosine kinase activity was determined in the solid phase system with different divalent cations as described in Methods. Cations were present at the indicated concentrations. The final amiloride concentration was 1.5 mM, respectively.

* Means values ± SD; the numbers of experiments performed are given in parenthesis.

† These values represent mean values of two independent experiments.

ND: not done.

pp60^{src} kinase was about 300 μM. Viral pp60^{src} kinase was also inhibited by the above tested pyrazine-derivatives. But all IC_{50} values calculated ranged between 2 and 4 mM final drug concentrations. The cellular kinase was only incompletely inhibited by amiloride. Even in the presence of 1.5 mM amiloride only about 70% of the total pp60$^{c\text{-}src}$ kinase activity was blocked. Using the same drug concentrations, viral pp60^{src} tyrosine kinase, however, was inhibited more than 90% in every case. Similar results were obtained using chicken as well as mouse and rat cells. The data for pp60^{src} kinases from normal and RSV-transformed cells are shown in Table 1. Taken together, our data may indicate different amiloride-sensitive forms of cellular pp60^{src} kinases.

DISCUSSION

The data presented in this paper demonstrate that amiloride acts as an inhibitor of the protein tyrosine kinases associated with the cellular and viral scr-gene products (Figs 2 and 4; Table 1). pp60^{src} kinase activity was determined in a solid phase immuno system, by which anti-pp60^{src}-IgG of TBR-serum serves as an artificial but specific substrate for the immunoprecipitated tyrosine kinases. This assay provides information about fundamental properties of pp60^{src} such as kinase activity and tyrosine specificity. Inhibition of pp60$^{v\text{-}src}$ kinase activity by various substances such as quercetin [30, 31], Ap_5A [20], Ap_4A [9, 32] has also been studied with this system.

The IC_{50} values determined for pp60^{src} kinases (50–300 μM amiloride) are very similar to that determined for tyrosine kinases associated with growth factor receptors (100–500 μM) [8] and a number of other investigated protein kinases [5–7]. Viral pp60^{src} kinase is 3–6-fold more sensitive to amiloride and is almost completely inhibited as compared to pp60$^{c\text{-}src}$ kinase (Fig. 4, Table 1). Ap_4A has also been reported to be more effective in inhibiting viral pp60^{src} kinase than the cellular counterpart. An approximately 50-fold difference in IC_{50} values (1 and 46 μM Ap_4A) was found by Levy *et al.* [32], while no inhibition of pp60$^{c\text{-}src}$ kinase even at 100 μM Ap_4A was reported by Barnekow [9]. As can be seen in Table 1, cellular pp60^{src} kinase is also inhibited to a lesser extent by amiloride than the viral one. The decreased sensitivity and incomplete inhibition may indicate the existence of different amiloride-sensitive forms of cellular pp60^{src} kinases in rat brain tissue. Post-translational modifications of cellular pp60^{src} molecules affecting their ATP binding domains could be responsible for this phenomenon. Brugge and co-workers [14] have reported that increased pp60^{src} kinase activity in neurons is accompanied by structurally modified neuronal pp60^{src} molecules. pp60^{src} of glia cells appeared to be unaltered. The observation made by Shealy and Erikson [33] that differently phosphorylated forms of pp60$^{c\text{-}src}$ exist in human fibroblasts, may also explain the incomplete inhibition of pp60$^{c\text{-}src}$ from fibroblasts by amiloride (Fig. 4A). Viral pp60^{src}, however, may consist of one single, amiloride-sensitive population.

Our data indicate that the competition of amiloride with the binding of ATP seems to be the only inhibitory mechanism (Fig. 2). The observation that the drug competes with ATP may explain the various effects of amiloride to ATP-linked processes such as inhibition of the (Na^+–K^+)-ATPase [34], DNA- and protein biosynthesis [35, 36]. Another amiloride effect, pointed out by Pouysségur and co-workers [1], is the inhibition of ribosomal S 6 protein phosphorylation in intact cells. In a recent report, Blenis and Erikson [37] described stimulation of S 6 phosphorylation in response to phorbolesters, serum stimulation, and pp60^{src} expression in RSV-transformed CEF. S 6 is not phosphorylated at tyrosine residues. Since we do not know if the S 6 phosphorylating kinase is part of a protein kinase cascade or is directly inhibited by amiloride, we can only speculate that other amiloride-sensitive kinases like pp60^{src} kinase, growth factor receptor kinases or protein kinase C may be involved in the regulation of S 6 phosphorylation.

The multiple effects of amiloride make it difficult to interpret the molecular mechanisms involved in drug actions in intact cells. On the other hand the influence of drugs to the biological activity of oncgene products will be a very important field to further pharmacological investigations.

Acknowledgements—We gratefully acknowledge the technical assistance of I. Schneider and J. Schäfer. We would like to thank M. Leib for typing the manuscript.

This work was supported by Deutsche Forschungsgemeinschaft (SFB 47).

REFERENCES

1. J. Pouyssegur, J. C. Chambard, A. Franchi, S. Paris and E. van Obberghen-Schilling, *Proc. natn. Acad. Sci. U.S.A.* **79**, 3935 (1982).
2. W. H. Moolenaar, Y. Yarden, S. W. de Laat and J. Schlessinger, *J. biol. Chem.* **257**, 8502 (1982).
3. S. Schuldiner and E. Rozengurt, *Proc. natn. Acad. Sci. U.S.A.* **79**, 7778 (1982).
4. C. Frelin, P. Vigne and M. Lazdunski, *J. biol. Chem.* **258**, 6272 (1983).
5. R. K. Ralph, J. Smart, S. J. Wojcik and J. McQuillan, *Biochem. biophys. Res. Commun.* **104**, 1054 (1982).
6. R. Holland, J. R. Woodgett and D. G. Hardie, *FEBS Lett.* **154**, 269 (1983).
7. J. M. Besterman, W. S. May Jr., H. Le Vine III, E. J. Cragoe Jr. and P. Cuatrecasas, *J. biol. Chem.* **260**, 1155 (1985).
8. R. J. Davis and M. P. Czech, *J. biol. Chem.* **260**, 2543 (1985).
9. A. Barnekow, *Biosci. Rep.* **3**, 153 (1983).
10. T. Hunter and J. A. Cooper, *Ann. Rev. Biochem.* **54**, 897 (1985).
11. M. Schartl and A. Barnekow, *Differentiation* **23**, 109 (1982).
12. A. Barnekow and M. Schartl, *Molec. Cell. Biol.* **4**, 1179 (1984).
13. M. Gessler and A. Barnekow, *Biosci. Rep.* **4**, 757 (1984).
14. J. S. Brugge, P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner and R. W. Keane, *Nature, Lond.* **316**, 554 (1985).
15. D. Becker, R. Kurth, D. Critchley, R. R. Friis and H. Bauer, *J. Virol.* **21**, 1042 (1977).
16. W. Schäfer, *Z. Naturforsch.* **34C**, 306 (1979).
17. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
18. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
19. J. S. Brugge and R. L. Erikson, *Nature, Lond.* **269**, 346 (1977).
20. P. Presek, H. Glossmann, E. Eigenbrodt, W. Schoner, H. Rübsamen, R. R. Friis and H. Bauer, *Cancer Res.* **40**, 1733 (1980).
21. T. Hunter and B. W. Sefton, *Proc. natn. Acad. Sci. U.S.A.* **77**, 1311 (1980).
22. M. S. Collett and R. L. Erikson, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2021 (1978).
23. C. Jacobs and H. Rübsamen, *Cancer Res.* **43**, 1696 (1983).
24. P. M. Coussens, J. A. Cooper, T. Hunter and D. Shalloway, *Molec. Cell. Biol.* **5**, 2753 (1985).
25. A. Haas, I. Heller and P. Presek, *Biochem. biophys. Res. Commun.* **135**, 426 (1986).
26. Y. Fukami and F. Lipmann, *Proc. natn. Acad. Sci. U.S.A.* **82**, 321 (1985).
27. N. D. Richert, D. L. Blithe and I. Pastan, *J. biol. Chem.* **257**, 7143 (1982).
28. A. Le Cam, P. Auberger and M. Samson, *Biochem. biophys. Res. Commun.* **106**, 1062 (1982).
29. J. G. Foulkes, *Curr. Top. Microbiol. Immunol.* **107**, 163 (1983).
30. H. Glossmann, P. Presek and E. Eigenbrodt, *Naunyn-Schmiedeberg's Archs Pharmac.* **317**, 100 (1981).
31. Y. Graziani, E. Erikson and R. L. Erikson, *Eur. J. Biochem.* **135**, 583 (1983).
32. B. T. Levy, L. K. Sorge, C. C. Drum and P. F. Maness, *Molec. Cell. Biol.* **3**, 1718 (1983).
33. D. J. Shealy and R. L. Erikson, *Nature, Lond.* **293**, 666 (1981).
34. D. T. Yamaguchi, R. Sakai, L. Bahn, E. J. Cragoe Jr. and S. C. Jordan, *J. Immunol.* **137**, 1300 (1986).
35. S. P. Solthoff and L. J. Mandel, *Science* **220**, 957 (1983).
36. M. Lubin, F. Cahn and B. A. Coutermarsh, *J. Cell Physiol.* **113**, 247 (1982).
37. J. Blenis and R. L. Erikson, *Proc. natn. Acad. Sci. U.S.A.* **82**, 7621 (1985).