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# VINCULIN PHOSPHORYLATION BY THE SRC KINASE: INHIBITON BY CHLORPROMAZINE, IMIPRAMINE AND LOCAL ANESTHETICS

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The phosphorylation of vinculin by a highly purified src kinase was stimulated by anionic phospholipids and inhibited to varying degrees, by chlorpromazine, imipramine, dibucaine and tetracaine. The drug effects are ascribed to a competitive inhibition of the activation process by their ability to interact with phospholipid.

## INTRODUCTION

The src gene of Rous sarcoma virus (RSV)[1] encodes a 60,000-dalton protein that phosphorylates tyrosine in a variety of protein substrates (1-7). Vinculin, a 130,000-dalton cytoskeletal protein located at focal adhesion plaques (8, 9), is one of the intracellular targets of the src kinase (10). The intracellular distribution of vinculin is altered following the transformation of cells by RSV (11). Recently, using a highly purified preparation of the src kinase (12), we have found that phospholipids markedly stimulate the phosphorylation of vinculin and inhibit the phosphorylation of non-physiological substrates by the purified enzyme (13). This is of particular interest because a substantial portion of the src kinase is associated with the plasma membrane (14, 15, 16). These results imply that the phosphorylation of vinculin by the src kinase may occur at the plasma membrane.

Local anesthetics and antipsychotic drugs have been shown to interact with membrane phospholipids and affect cellular activities (17, 18). These drugs also affect the activity of some membrane-bound enzymes (17). One recent find-

[1]The abbreviations used are: RSV, Rous sarcoma virus; PG, phosphatidylglycerol; MES, 2-(N-morpholino) ethane sulfonic acid.

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ing of particular interest is the ability of these drugs to inhibit the activity of a $Ca^{2+}$-activated, phospholipid-dependent protein kinase (19, 20).

Here, we examined the effect of these drugs on vinculin phosphorylation by the src kinase. The results indicate that the drugs appear to interact with phospholipid and thereby strongly inhibit the stimulation of vinculin phosphorylation.

## EXPERIMENTAL PROCEDURES

### Materials and Chemicals.

The src kinase was prepared from rat tumors and stored at -70°C as described previously (12). The preparation used contained ~1 μg/ml of protein. Vinculin was prepared from frozen chicken gizzards by the method of Feramisco and Burridge (21) except that DEAE-Sephacel was used instead of DEAE-cellulose. The fractions from the DEAE column were used in this study. Casein purified powder (Sigma #C5890) was prepared using the method of Ashby and Walsh (22).

Phosphatidylglycerol (egg) and phosphatidylinositol (bovine brain) were purchased from Calbiochem-Behring (La Jolla, California); chlorpromazine hydrochloride, from Smith Kline and French Labs (Philadelphia, Pennsylvania); dibucaine hydrochloride, imipramine hydrochloride, and tetracaine, from Sigma. [$\gamma$-$^{32}$P]ATP (~3,000 Ci/mmol) was obtained from New England Nuclear. All other chemicals were reagent grade.

### Enzyme Assay.

Vinculin phosphorylation was assayed using the $Mn^{2+}$-assay system previously described (13). The standand reaction mixture (25 μl) contained 2.5 μl of purified enzyme, 5 μg vinculin, 1 μM [$\gamma$-$^{32}$P]ATP (specific activity, 227 Ci/mmol), 0.5 mM $MnCl_2$ and 5 μg PG in 25 mM MES buffer, pH 6.5. Various drugs were added as indicated in each experiment. The reaction was carried out at 22°C for 20 min. For the assay of casein phosphorylation, 5 μg of casein was used instead of vinculin.

## RESULTS AND DISCUSSION

The effect of PG, an anionic phospholipid, on the phosphorylation of vinculin and casein is shown in Figure 1. As previously reported (13), the phosphorylation of vinculin was stimulated and that of casein inhibited. Figure 2 demonstrates that the stimulation of vinculin phosphorylation by PG was blocked by chlorpromazine, imipramine, dibucaine and tetracaine. Chlorpromazine was the most potent inhibitor with an $ID_{50}$ of about 40 μM. When phosphatidylinositol was used instead of PG, the $ID_{50}$ for chlorpromazine was 27 μM.

As shown in Table 1, the phosphorylation of casein was not inhibited by any of the drugs tested at a concentration of 0.1 mM, whereas vinculin phos-

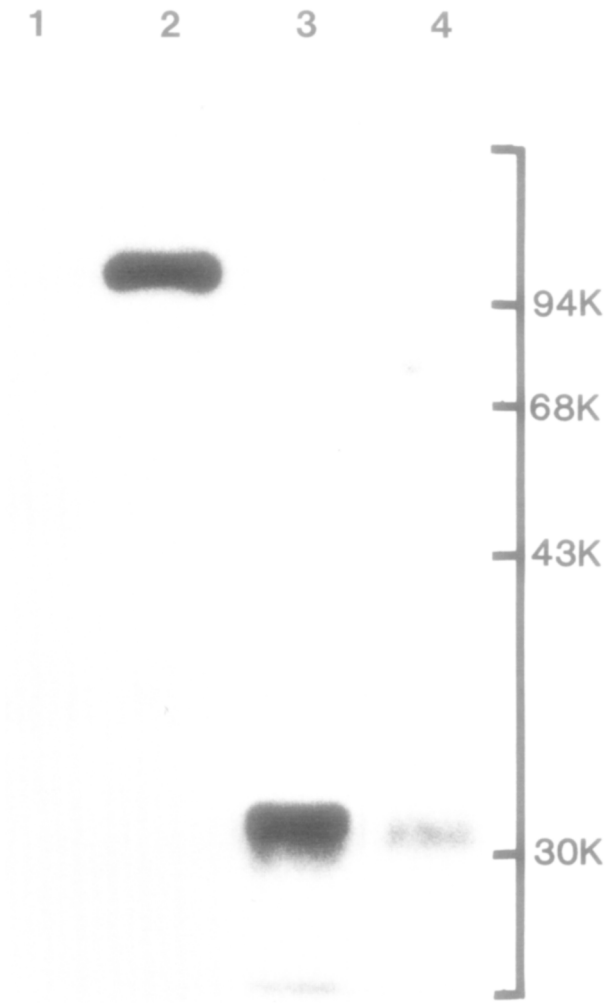
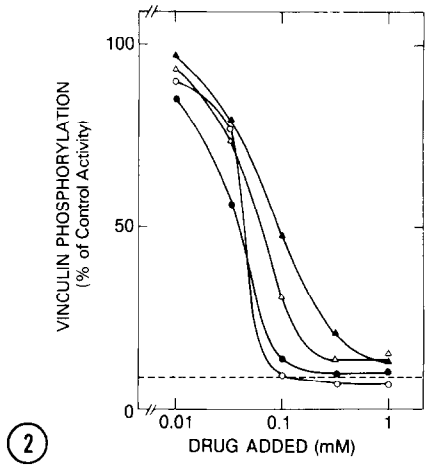



Figure 1. Effect of Phospholipid on Vinculin and Casein Phosphorylation by the src Kinase.

After incubation for 20 min at 22°C, the reaction products were analyzed on a 12.5% SDS polyacrylamide gel followed by autoradiography. Lanes 1-4 represent, vinculin without PG, vinculin with PG, casein without PG, and casein with PG, respectively.

phorylation was markedly inhibited by all the agents. To inhibit casein phosphorylation, the concentration of chlorpromazine had to be raised more than 10-fold; the $ID_{50}$ for chlorpromazine was 550 μM (data not shown). It is unlikely, therefore, that chlorpromazine interacts directly with the enzyme to inhibit the reaction.

Next, experiments were carried out to investigate the mechanism by which these drugs exert their inhibitory effect. The concentration of PG was titrated



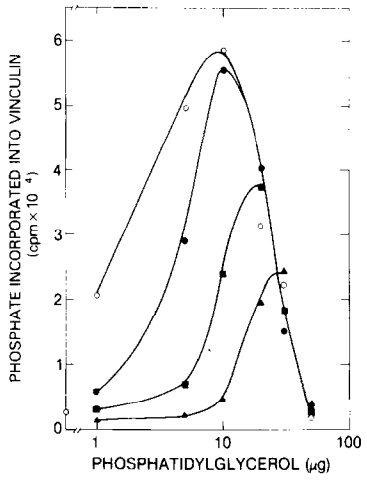




Figure 2. Inhibition of Vinculin Phosphorylation by Various Drugs.

The assay conditions were as described under "Experimental Procedures." Each assay (25 μl) contained 2.5 μl of purified enzyme, 5 μg PG, 5 μg vinculin, 0.5 mM $MnCl_2$, 1 μM [$\gamma$-$^{32}$P]ATP and indicated concentration of the drug in 25 mM MES buffer, pH 6.5. ●——●, with chlorpromazine; ○——○, with imipramine; △——△, with dibucaine; ▲——▲, with tetracaine. The dotted line indicates phosphate incorporation into vinculin without PG and drug.

Figure 3. Effect of Chlorpromazine on Vinculin Phosphorylation at Various Concentrations of Phospholipid.

Vinculin phosphorylation was measured as described under "Experimental Procedures," except that various concentrations of PG and chlorpromazine were added as indicated. The concentrations of chlorpromazine used were zero (○), 33 μM (●), 100 μM (■), and 333 μM (▲).

Table 1. Effect of various drugs on vinculin and casein phosphorylation by the src kinase.

| Drug added (0.1 mM) | Phosphate incorporated into | | | |
|---|---|---|---|---|
| | Vinculin | | Casein | |
| | cpm | % | cmp | % |
| None | 85,278 | 100 | 81,529 | 100 |
| Chlorpromazine | 11,700 | 14 | 80,944 | 99 |
| Imipramine | 8,103 | 10 | 77,049 | 95 |
| Dibucaine | 26,250 | 31 | 80,481 | 99 |
| Tetracaine | 40,713 | 48 | 80,969 | 99 |

Assay conditions were as described under "Experimental Procedures." Each drug was added at a final concentration of 0.1 mM.

Table 2. Effect of chlorpromazine on inhibition of casein phosphorylation by phosphatidylglycerol.

| Addition | Chlorpromazine (mM) | | |
|---|---|---|---|
| | 0 | 0.1 | 0.3 |
| | cpm | cpm | cpm |
| None | 81,529 | 80,944 | 69,493 |
| Phosphatidylglycerol, 5 μg | 64,548 | 88,048 | 85,020 |
| Phosphatidylglycerol, 10 μg | 35,185 | 63,322 | 57,879 |

Assay conditions were described under "Experimental Procedures" except that indicated concentrations of chlorpromazine and phosphatidylglycerol were added.

at four different concentrations of chlorpromazine (Fig. 3). In the absence of chlorpromazine, maximal stimulation by PG was observed at 5-10 μg per assay as reported in the preceding paper (13). At 5 μg of PG, the effect of chlorpromazine is most pronounced; 33 μM chlorpromazine caused about 50% inhibition and 333 μM totally inhibited the phosphorylation of vinculin. At 10 μg of PG, higher concentrations of chlorpromazine were required to inhibit the reaction. 33 μM chlorpromazine produced only 5% inhibition, 100 μM chlorpromazine inhibited by 60% and 333 μM inhibited by 95%. At 50 μg of PG, the phosphorylation reaction was inhibited by the phospholipid itself. The inhibitory action of chlorpromazine was not blocked by increasing the concentration of vinculin (data not shown). These data suggest that the inhibitory action of chlorpromazine was due to competition with phospholipid. Similar results have been reported with the $Ca^{2+}$-activated, phospholipid-dependent kinase (19, 20).

Further evidence for chlorpromazine interaction with phospholipid is shown in Table II. PG inhibited casein phosphorylation by 20% and 57% at 5 μg and 10 μg, respectively. Chlorpromazine, which has no effect on casein phosphorylation at 0.1 mM, was able to completely overcome the inhibition by 5 μg of PG and partially overcome the inhibition by 10 μg PG. Reversal of the inhibition was also observed with 0.3 mM chlorpromazine.

The present studies demonstrate that the primary effect of chlorpromazine and the other drugs is to inhibit vinculin phosphorylation via a competitive interaction with phospholipid. The results provide further evidence that phospholipids in the membrane bilayer can influence vinculin phosphorylation by the src kinase. However, the studies described here do not clarify the mechanism by which vinculin phosphorylation by the src kinase is stimulated.

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