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Inhibition of T-cell invasion across cultured fibroblast monolayers by phenothiazine-related calmodulin inhibitors: impairment of lymphocyte motility by trifluoperazine and chlorpromazine, and alteration of the monolayer by pimozide

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Abstract

Phenothiazines inhibit the typical shape changes displayed by activated lymphocytes and thereby their migration through polycarbonate filters. The structure activity relationship of this effect is distinct from calmodulin inhibition. Our aim was to study this effect of phenothiazines on lymphocyte migration in an environment with living solid tissue cells. We assessed the effect of trifluoperazine and chlorpromazine (TFP and CP, two strong inhibitors of lymphocyte motility) and pimozide (PIM, a much weaker inhibitor of lymphocyte motility but a strong inhibitor of calmodulin) on invasion of human Molt-4 T-cells across precultured fibroblast monolayers. As expected invasion was inhibited by TFP and CP in the micromolar range that also inhibited motility. Surprisingly, PIM inhibited monolayer invasion at least as efficiently as TFP and CP (from 2.25 μM on). Preincubation of the monolayers or the lymphoid cells show that PIM exerted this novel invasion inhibiting effect on the monolayer. TFP and CP had a much weaker effect on the monolayer. Since these three compounds inhibit calmodulin in the same order, it is likely that this effect on the monolayer was caused by inhibition of a calmodulin-dependent pathway. KN-62, a specific inhibitor of calmodulin-dependent protein kinase II acted on the monolayer like PIM, whereas ML-7, a specific inhibitor of myosin regulatory light chain kinase, inhibited lymphoid cell motility like TFP and CP. In conclusion, invasion of T-cells across cellular monolayers is inhibited both by PIM and by phenothiazines like TFP and CP, but via distinct mechanisms: TFP and CP inhibit lymphocyte motility via a calmodulin independent pathway, whereas PIM impairs the monolayer's tolerance for invasion, most likely via a calmodulin and CamKII dependent pathway.

Keywords: T-cell; Migration; Pimozide; Trifluoperazine; Chlorpromazine; Calmodulin

1. Introduction

Phenothiazines such as TFP and CP and the structurally related diphenylbutylpiperidine PIM are used as antipsychotic drugs by virtue of their action as dopamine antagonists. In addition to their neuroleptic effect, phenothiazines display a wide variety of pharmacological activities, ranging from cytotoxicity [1] to immune suppression [2–4], inhibition of endocytosis [5], and hemolysis [6]. At the biochemical level, phenothiazines are potent inhibitors of calmodulin [7], and TFP in particular is widely used as the calmodulin inhibitor of choice in many cell culture studies. However, phenothiazines have also been shown to inhibit protein kinase C [8], inositol phosphate metabolism [9], potassium channels [10], and actin polymerization [11]. It is not always clear which of these mechanisms are responsible for the different effects of phenothiazines at the cellular

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Abbreviations: TFP, trifluoperazine; CP, chlorpromazine; PIM, pimozide; MLCK, myosin regulatory light chain kinase; CamKII, calmodulin-dependent protein kinase II.

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level. Thus, a few years ago, it was found that phenothiazines inhibit the typical shape changes displayed by activated lymphocytes and thereby inhibit their migration through polycarbonate filters, but the structure activity relationship of this effect was distinct from other effects including calmodulin and protein kinase C inhibition [12]. However, the inhibition of lymphocyte motility was accompanied by a reduction in F-actin, suggesting that the active phenothiazines interfered with mechanisms regulating actin polymerisation.

The previous studies had dealt with inhibition of lymphocyte motility by phenothiazines in an inert environment (e.g. tissue culture dishes and polycarbonate filters). Our aim was to extend those observations to a more elaborate *in vitro* model for tissue infiltration by lymphoid cells, where cell-cell interactions come also into play. We had previously developed a monolayer invasion assay in which the invasion of normal activated or malignant T-cells across cultured monolayers of fibroblastlike cells can be visualised and quantitated [13,14]. In this model, actin polymerisation-driven shape changes performed by the lymphocytes are a prerequisite for their invasion across the fibroblast monolayer [15–17]. Therefore we expected that phenothiazines would also inhibit monolayer invasion by T-cells. As tester cells we used the same human Molt-4 T-cell line as in the earlier study on lymphocyte motility. As test compounds we chose TFP and CP, two commonly used phenothiazines that strongly inhibited lymphocyte shape change ($IC_{50} = 4.5\ \mu M$ for TFP and 7 μM for CP [12]). As a negative control we included PIM, which has tenfold less activity as an inhibitor of lymphocyte motility ($IC_{50} = 46\ \mu M$ [12]). As a structurally unrelated calmodulin inhibitor we included W-7, and as an unrelated neuroleptic we included haloperidol.

2. Materials and methods

2.1. Cells, culture conditions, and chemicals

All test chemicals were from Sigma. Stock solutions of 50 mM were prepared in DMSO. The final concentration of DMSO in the treated cultures was always lower than 0.1%. C3H/10T½clone8 mouse embryo cells, further called 10T½ (American Type Culture Collection #CCL226) were grown to confluence in Eagle's basal medium with 10% fetal calf serum. The motile subline of the Molt-4 human T-cell lymphoma [12] was obtained from Dr. N. Matthews (Yamanouchi Research Institute, Oxford, UK). The cells were grown in a 1/1 mixture of Dulbecco's modified Eagle's medium and RPMI1640 medium, supplemented with 10% fetal calf serum.

2.2. Shape change analysis

The extension factor used to quantitate shape changes has been described before [18]. In brief, the perimeter and the area of 50 cell contours were measured in each condition. The extension factor ($1 - 4\pi \times \text{area} \times \text{perimeter}^{-2}$) is zero for a circle and increases to a theoretical maximum of 1 as the complexity of the shape increases. In practice, resting lymphoid cells yield a value of about 0.05, motile lymphoid cells a value of about 0.20.

2.3. Migration of Molt-4 cells through micropore filters

Molt-4 cells were treated with 9 μM of the test compounds for 1 hr before the assay. 5×10^5 Molt-4 cells were added in the upper well of a 6.5-mm Transwell (untreated polycarbonate filter, 8-μm pore size; Costar) in the presence of the drugs. Four hr later, the cells in the lower well were recovered and counted using a haemocytometer.

2.4. Monolayer invasion assay

A detailed description of the monolayer invasion assay has been published [13,14]. The monolayer invasion assay is based on the enumeration, in phase contrast microscopy of living cultures, of T-lymphoma cells that have crawled across a precultured monolayer of 10T½ cells (an immortalized murine fibroblast-like line). In brief, confluent 10T½ cultures were changed to lymphoma cell medium with 2% foetal calf serum, and then the Molt-4 cells were added at a density of 5×10^4 per cm^2. Three hr later, the cells lying on top of the monolayer were washed off, and the underlying lymphoma cells were then counted in 10 microscope fields of 0.6 mm^2 per culture dish, in duplicate dishes for each condition in each experiment. Unless otherwise stated, the Molt-4 cells were preincubated for 1 hr and then inoculated on the monolayer in the presence of the test compound.

3. Results and discussion

We first wanted to reproduce by our methods the effects of the phenothiazines on the motility of Molt-4 cells, as they had been described [12]. Molt-4 cells are irregularly shaped, with most cells showing at least one major surface protrusion. In the presence of TFP or CP, the extension factor dropped, indicating that the cells became round, devoid of pseudopodia. The effect was obvious at 4.5 μM, and maximal at 18 μM (Fig. 1A). The structurally unrelated calmodulin inhibitor W7 also caused complete rounding of the cells at 18 μM, but not at 4.5 μM. PIM had only a marginal effect, even at 18 μM. Haloperidol up to 9 μM had no effect on the morphology of the cells at all, at 18 μM there was a minimal rounding. The solvent DMSO did not affect cell morphology at concentrations up to 0.1% (which is at least twofold higher than the concentrations in the drug treated cultures). Migration of Molt-4 cells through polycarbonate filters was inhibited concomittantly: at 9 μM, TFP and CP were strongly inhibitory, W-7 was somewhat weaker, and PIM and haloperidol had no effect (Fig. 1B). DMSO up to

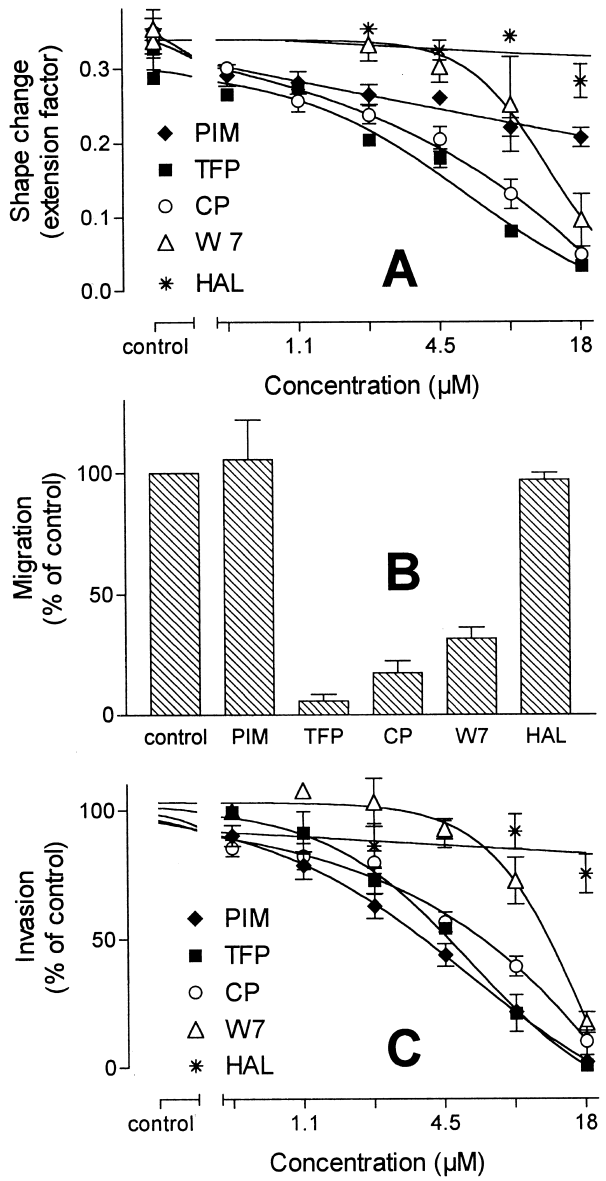

Fig. 1. Effect of calmodulin inhibitors and haloperidol (HAL) on the motile and invasive behaviour of Molt-4 cells. (A) Inhibition of shape changes. Molt-4 cells were treated for 1 hr at the concentrations indicated, and then the contours of 50 cells in each condition were analyzed (Mean and standard error of 2 independent experiments). (B) Inhibition of migration through polycarbonate filters. Molt-4 cells were treated for 1 hr with 9 μM and then inoculated on filters in the presence of the drugs for another 4 hr. (Mean and standard error of 4 independent experiments). (C) Inhibition of monolayer invasion. Molt-4 cells were treated for 1 hr at the concentrations indicated, and then inoculated on confluent fibroblast monolayers in the presence of the drugs for another 3 hr. (Mean and standard error of 3 independent experiments).

0.1% had no effect either. We also confirmed that the phenothiazine-induced rounding of the cells is related to an effect on the polymerization of actin into microfilaments: TFP caused a significant decrease of F-actin content in the Molt-4 cells ($P < 0.05$ at 9 μM, $P < 0.005$ at 18 μM), whereas PIM caused no significant changes. So far, our observations are in full agreement with the findings and conclusions of Matthews et al. [12]: phenothiazines like TFP and CP, but not PIM, cause rounding of T-cells, but this effect is unrelated to their activity as calmodulin inhibitors. In fact, of the compounds used in this study PIM is the most potent calmodulin inhibitor (IC_{50} = 7 μM, compared to 17, 28, 40, and 65 μM for TFP, W-7, CP and haloperidol, respectively [7]).

In the fibroblast monolayer invasion assay, it takes several hours before an appreciable number of T-cells have moved underneath the monolayer [13,14]. With the Molt-4 cell line we found that after 3 hr of confrontation about 25% of the inoculated cells were lying underneath the fibroblasts. Since the above described effects on T-cell motility became obvious approximately 0.5 hr after addition of the drugs and disappeared within a few hours after removal, we preincubated the Molt-4 cells for 1 hr, and then inoculated them on the monolayers for 3 hr in the continuous presence of the test compounds. As we expected, the dose response curves for TFP, CP and W-7 in the monolayer invasion assay closely matched the curves for inhibition of shape change of the Molt-4 cells (Fig. 1c): full inhibition at 18 μM with all three compounds, and 50% inhibition at 4.5 μM with TFP and CP but not with W-7. Haloperidol up to 9 μM had no effect on invasion, 18 μM was only slightly inhibitory. The solvent DMSO 0.1% was inactive. These observations are concordant with our earlier findings with various cell lines and compounds that interfere with actin polymerisation and shape change in T-cells: invasion critically depends on actin polymerization and the extension and retraction of pseudopodia by the lymphoid cells [15,16].

An unexpected result was that PIM, which was initially included in this study as an inactive control compound, inhibited monolayer invasion at least as efficiently as TFP and CP. The inhibition was significant even at 2.25 μM. Thus, PIM interferes with invasion of T-cells across cellular monolayers, without affecting lymphoid cell shape, migration through filters, or F-actin content. Moreover, invasion was inhibited at concentrations of PIM that had no effects on the morphology of the monolayer either, and staining of F-actin in the fibroblasts did not reveal changes in stress fiber number or distribution (not shown).

To assess whether PIM exerted this novel invasion inhibiting effect on the lymphoid cells or on the monolayer cells, separate cultures of these cells were preincubated overnight with 4.5 μM of PIM, and then the invasion assay was performed in the absence of drug. Preincubation of the monolayers with 4.5 μM of PIM caused significant inhibition of invasion (Fig. 2) whereas preincubation of the lymphoid cells had no effect. Preincubation of the monolayers with 4.5 μM of TFP was less effective than with PIM, and the effect of CP was still weaker (Fig. 2). Since the IC_{50} for calmodulin inhibition by these three compounds follows the same order, it is likely that this effect on the monolayer cells was caused by inhibition of calmodulin.

Calmodulin regulates the activity of many kinases, and

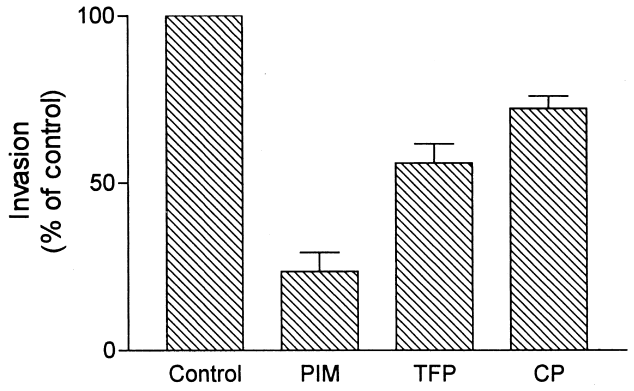

Fig. 2. Inhibition of invasion after pretreatment of the fibroblast monolayers with phenothiazines. The monolayers were treated overnight, and then changed with medium without drugs before the Molt-4 cells were inoculated for a 3-hr invasion assay (Mean and standard error of 4 independent experiments).

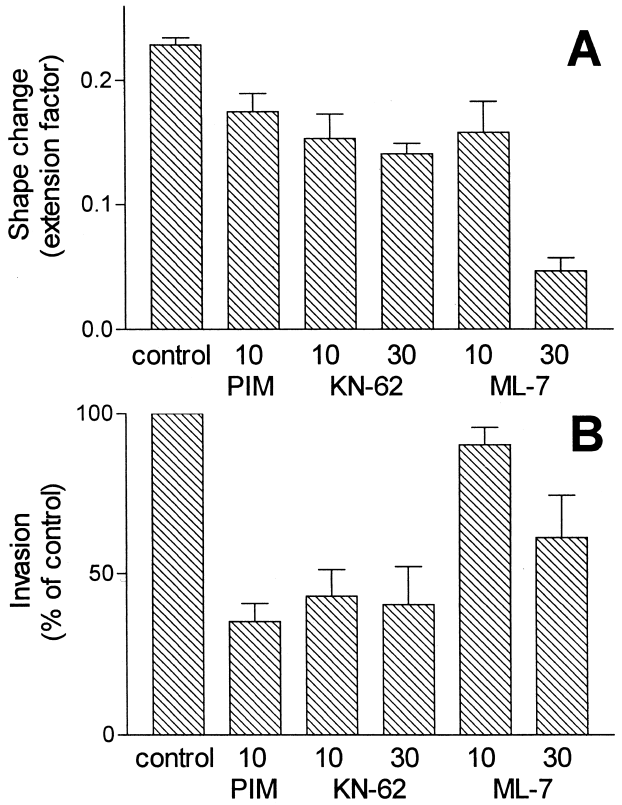

Fig. 3. Effect of PIM, KN-62, and ML-7 on the motile and invasive behaviour of Molt-4 cells. (A) Inhibition of shape change. Molt-4 cells were treated for 1 hr at the concentrations indicated (10 or 30 μM), and then the contours of 50 cells in each condition were analyzed (Mean and standard error of 3 independent experiments). (B) Inhibition of invasion after pretreatment of the fibroblast monolayers. The monolayers were treated overnight, and then changed with medium without drugs before the Molt-4 cells were inoculated for a 3-hr invasion assay (Mean and standard error of 3 independent experiments).

we wondered whether one of these could be associated with the effect of PIM on invasion of T-cells across fibroblast monolayers, downstream of calmodulin. MLCK seemed to be a plausible candidate: it is a major calmodulin-regulated kinase, and invasion of neutrophils across endothelial cell monolayers had been shown to depend on induction of MLCK activity in the monolayer cells [19]. Therefore we assessed the effect of the MLCK inhibitor ML-7 on motility and invasion of the Molt-4 cells in our model. For comparison, we also tested KN-62, which is a specific inhibitor of another major calmodulin-regulated kinase CamKII. CamKII is abundant in the brain and is involved in spatial learning. Very recently, it was found to play a role in T-cell memory as well [20], but there were no indications that CamKII might control mechanical properties of lymphocytes or connective tissue cells. Surprisingly, the effects of KN-62 but not ML-7 were similar to the effects of PIM (Fig. 3). KN-62 inhibited invasion when the monolayer was pretreated with concentrations that did not affect the shape change of the lymphoma cells. In contrast, the effects of ML-7 were more reminiscent of TFP: ML-7 inhibited monolayer invasion only at concentrations which also impaired shape change of the lymphoma cells.

In conclusion, invasion of lymphocytes across cellular monolayers is inhibited both by PIM and by phenothiazines like TFP and CP, but via distinct mechanisms: TFP and CP inhibit lymphocyte motility via a calmodulin independent pathway, whereas PIM impairs the monolayer's tolerance for invasion, most likely via a calmodulin and CamKII dependent pathway.

The present findings have implications and raise questions that will need further investigation. The different, complementary mechanisms by which PIM and TFP inhibit lymphocyte invasion across cellular monolayers in culture may point out opportunities for the containment of tissue infiltration *in vivo* by metastasising lymphoma cells, or by inflammatory T-cells. Thus, inhibition of T-cell migration and invasion may at least in part be responsible for the suppression of delayed type hypersensitivity reactions by phenothiazines [2,4]. Because the *in vitro* effects described here were seen in the micromolar range, *in vivo* experiments to investigate anti-invasive actions of PIM and TFP will be hampered by the neuroleptic or cardiotoxic effects [10]. A further unraveling of the pathways involved in the *in vitro* effects however may allow for the identification of more potent and more specific inhibitors. In the first place, it would be interesting to find out how PIM affects the properties of monolayer cells, downstream of CamKII.

On the other hand, the calmodulin independent effect of TFP on lymphocyte motility ([12] and our results) warrants against use of TFP as a specific calmodulin inhibitor. In our experiments, the effects TFP rather resembled those of MLCK inhibitors. Interestingly, TFP was shown recently to bind directly to a regulatory myosin from scallop, and to lock it in an inactive conformation [21]. Although the latter effect was seen only at high concentrations (from 50 μM onwards) it is possible that TFP directly disturbs proper myosin function in living cells at lower concentrations as

well. Perhaps PIM may be a better choice when a specific calmodulin inhibitor is required for cell culture experiments.

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