LIMITATIONS ON THE USE OF PHENOTHIAZINES AND LOCAL ANAESTHETICS AS INDICATORS OF CALMODULIN FUNCTION IN INTACT CELLS

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1. Introduction

The calcium-binding protein calmodulin is thought to regulate a wide range of cellular and enzymic functions [1,2]. The antipsychotic phenothiazine family of drugs inhibits the actions of calcium–calmodulin complexes [3], and this has led to the widespread use of these drugs as indicators of calmodulin involvement in specific biochemical and cellular functions. Drugs with local anaesthetic activity also inhibit calmodulin function in isolated systems [4,5]. Among the enzymes regulated by calmodulin is myosin light chain kinase, which controls the activity of cytoskeletal actomyosin ATPase. This complex is thought to be involved in many processes related to cell motility, including cap formation on lymphocytes [6,7], and as part of a study of the latter process we have tested some of the reported calmodulin antagonists. To attribute any effects of the drugs to the inhibition of calmodulin, it is necessary to show that they do not have other independent metabolic effects. In particular we have examined their effects on lymphocyte adenine nucleotide levels, since we had shown that there is a quantitative dependence of cap formation on the ATP level [8]. We have also tested the effects of the drugs on lymphocyte lactate output, since changes in adenine nucleotide levels would be expected to affect the glycolytic flux.

The results indicate that each of the drugs tested can reduce lymphocyte ATP levels, greatly reducing the phosphorylation potential, expressed as log [ATP]/[ADP]. This finding may explain many of the effects of the drugs, independently of any inhibition of calmodulin function, and therefore seriously limits their use as indicators of calmodulin function in intact cells.

Abbreviations: FITC-RaMIg, fluorescein-conjugated rabbit anti-mouse IgG; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline

2. Materials and methods

Spleen cells and thymocytes from 4–8-week old BALB/c mice and 6-month old Wistar rats were isolated as in [8] and were suspended at $1-2 \times 10^7$ cells/ml in RPMI 1640 medium (Flow Labs.) buffered at pH 7.3 with 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes; Searle). Cell viabilities, as judged by the exclusion of sodium eosin, were initially >95%, and drug-induced cytolysis was assessed by release of lactate dehydrogenase (LDH) as described below. Aliquots of the cell suspension were mixed with freshly prepared solutions of the inhibitors in medium or DMSO as appropriate, and were then divided into samples for assay of ATP and ADP, lactate output, LDH release or cap formation. Control incubations contained equivalent additions of medium or DMSO, which were ≤1% (v/v).

ATP was assayed using the luciferin–luciferase method as in [8], and ADP was estimated from the increase in ATP in the neutralised lymphocyte extracts after incubation for 30 min at 37°C with 0.5 mM phosphoenolpyruvate and 50 μg pyruvate kinase/ml (Boehringer) [9]. For the determination of lactate output, cell samples were briefly centrifuged (3 s at 14 000 × *g*), and the supernatants (250 μl) were added to assay cocktail (750 μl) to give final conc. 0.4 M glycine, 0.3 M hydrazine and 2 mM NAD^+ (pH 9.2) [10]; the increase in absorption at 340 nm after incubation for 30 min at 37°C with 20 μg lactate dehydrogenase/ml (Boehringer) was measured in a Perkin-Elmer model 557 spectrophotometer, relative

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to samples of medium that had been similarly processed. LDH release was assessed by the rate of decrease in absorbance at 340 nm after the addition of supernatants to assay cocktail (final conc. 1 mM pyruvate, 0.1 mM NADH in medium) at 37°C, and the total cellular LDH activity was determined by the addition of the detergent NP40 (1%, v/v) to cell samples. Cap formation on mouse spleen cells was initiated by the addition of 300 μg/ml of fluorescein-conjugated rabbit anti-mouse IgG (FITC-RaMIg; Miles Labs.). After 15 min at 37°C the cells were fixed by the addition of 2% formaldehyde in phosphate-buffered saline (PBS), and were then washed twice in ice-cold PBS before examination under ultra-violet illumination in a Leitz Diavert Microscope. Between 100–200 stained cells were counted per sample, and capping was scored as the percentage of these cells with caps covering <50% of the cell. The results thus obtained are expressed as a percentage of capping on control samples.

Trifluoperazine dihydrochloride and fluphenazine dihydrochloride were obtained from Smith Kline and French Labs. and E. R. Squibb and Sons, respectively, and all other inhibitors were the generous gift of Dr J. M. Young of the Department of Pharmacology, University of Cambridge.

3. Results

The effects of trifluoperazine, chlorpromazine, dibucaine and lignocaine on the ATP levels of rodent lymphocytes are shown in fig.1 (upper): there is a progressive reduction in ATP as the concentration of

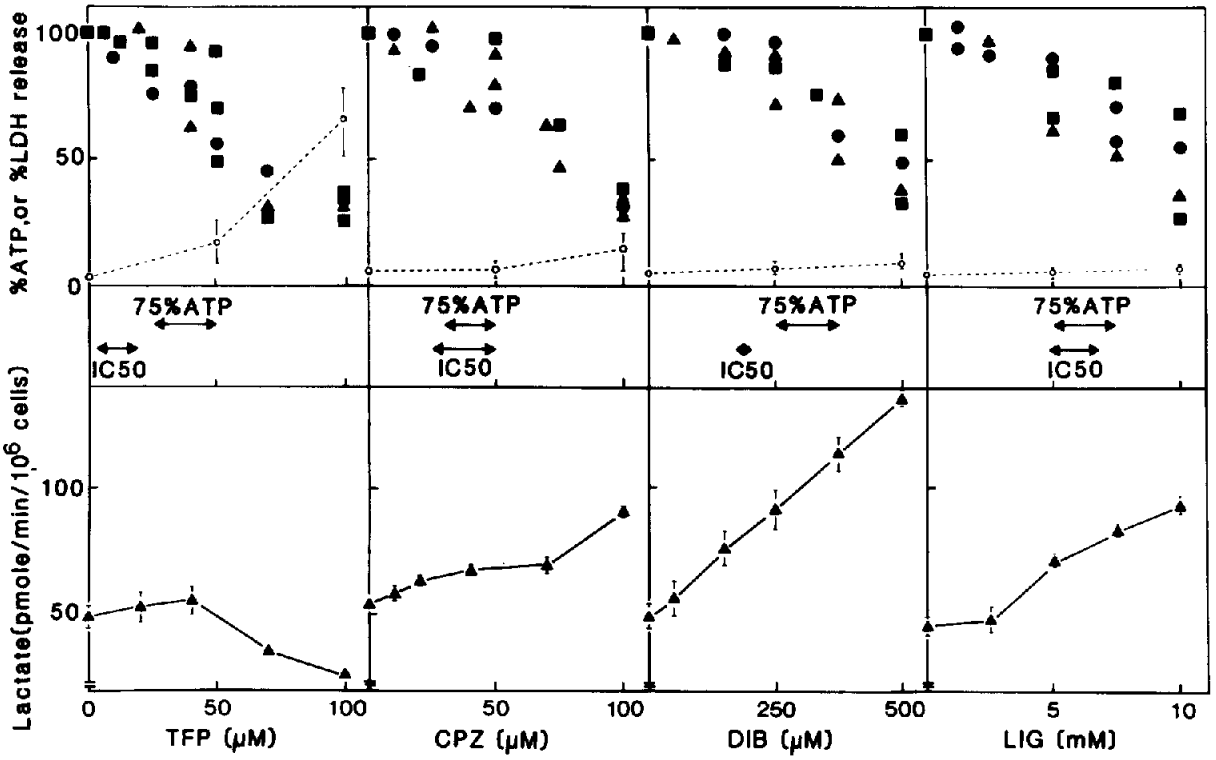

Fig.1. Effects of trifluoperazine, chlorpromazine, dibucaine and lignocaine on lymphocyte ATP levels and lactate output. Rat spleen cells (•), mouse spleen cells (▪) or mouse thymocytes (▲) were incubated at 37°C with the indicated drug concentrations. Upper: ATP (closed symbols) or lytic LDH release (○) were assayed after 15 min as in section 2. ATP results are the mean and range of duplicates from 1–3 expt with each cell type; LDH results are the combined mean and range from these experiments. Middle: The range of drug concentrations reducing the ATP level to 70–80% of control is indicated '75% ATP', and the range of drug concentrations reported to give 50% inhibition of calmodulin-mediated enzymic processes is indicated 'IC_{50}'. References: trifluoperazine [3,4,11,12]; chlorpromazine [3,5,11,13]; dibucaine [4,5]; lignocaine [5]. Lower: Lactate output from thymocytes was assayed after 60 min as in section 2. The results are the mean and range from 2 duplicate expt.

each drug is increased. Also shown is the drug-induced release of LDH from the cells in the same experiments; comparison of the effects of trifluoperazine with those of lignocaine shows that there is no consistent correlation between reduction of ATP and release of LDH. Although LDH release is frequently used as an assay for toxic effects of drugs added to cells, the reduction in ATP is a more sensitive measure of functional impairment. For example, lymphocytes can withstand several hours of ATP depletion by sodium azide and then recover (on removal of azide by washing) to give normal capping [6,8].

When the ATP level is reduced to 70–80% of control, there is a marked decrease in the phosphorylation potential, expressed as log [ATP]/[ADP]. The ratio of ATP to ADP in resting lymphocytes is 7–8, but when the ATP level falls to <75%, the ratio falls to 2, independent of the drug used (phenothiazines, local anaesthetics, or the mitochondrial inhibitor sodium azide (fig.2)). In general, any enzymic process that involves the hydrolysis of ATP will be sensitive to such changes in the phosphorylation potential, irrespective of any calcium (or calmodulin) regulation of the process. Therefore, the ranges of drug concentrations that reduced the ATP level to 70–80% of control were compared with the concentration ranges reported to give 50% inhibition of isolated calmodulin-regulated processes (fig.1, middle; ranges marked '75% ATP' and 'IC_{50}', respectively). On the basis of the relationship between these 2 concentration ranges, the phenothiazines and local anaesthetics can be divided into 2 groups. Trifluoperazine and dibucaine have little or no effect on lymphocyte ATP levels when used at their reported IC_{50} for calmodulin, but give a depletion of ATP at concentrations that are not very much higher. However, chlorpromazine and lignocaine give significant depletion of ATP when they are used at their reported IC_{50} for calmodulin, and have no effect on ATP levels only when used at concentrations which would not (according to previous reports) interact significantly with calmodulin. This also applies (not shown) to fluphenazine, a phenothiazine, and the β-adrenergic antagonist propranolol, which has local anaesthetic and anti-calmodulin activities [4].

As in other aerobic cells, glycolytic flux in lymphocytes is increased when, as a result of the impairment of mitochondrial function, the ATP level is lowered. Fig.1 (lower) shows that trifluoperazine, chlorpromazine, dibucaine and lignocaine each stimulated lymphocyte lactate output, although the magnitude of the effects varied widely and lytic concentrations of trifluoperazine (and of fluphenazine, not shown) caused a sharp decrease in output. Chlorpromazine, lignocaine and non-lytic trifluoperazine concentrations increased the lactate output when they reduced the ATP level over the range where the phosphorylation potential falls; a similar relationship was obtained using sodium azide (not shown). Dibucaine, however, gave an increase in lactate output over a

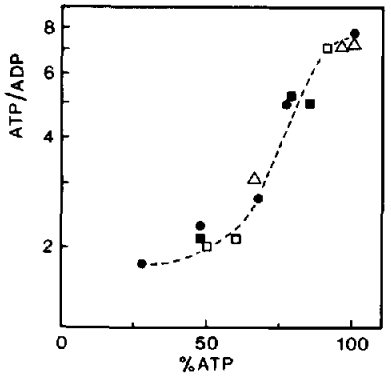

Fig.2. Effects of phenothiazines and local anaesthetics on log [ATP]/[ADP] as a function of ATP level. Mouse spleen cells were incubated for 15 min at 37°C with: trifluoperazine (10–100 μM, •); chlorpromazine (10–100 μM, ▪); fluphenazine (10–100 μM, ▲); dibucaine (150–500 μM, □); or lignocaine (2.5–10 mM, △); and were then assayed for ATP and ADP as in section 2. Also shown (– – –) is the profile obtained using sodium azide (0.075–1.5 mM).

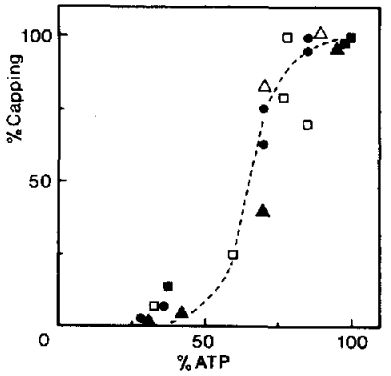

Fig.3. Effects of phenothiazines and local anaesthetics on cap formation as a function of ATP level. Mouse spleen cells were incubated for 15 min at 37°C as in fig.2, and were then assayed for ATP or capping of surface immunoglobulin as in section 2. Also shown (– – –) is the profile obtained using sodium azide (0.075–1.5 mM).

range of concentrations that included those reported to interact with calmodulin, despite the lack of effect of these concentrations on cellular ATP levels. Local anaesthetics are known to affect the activity of membrane proteins [14], and since one of the controlling factors in lymphocyte glycolytic flux is glucose uptake [15], the various effects of the drugs on lactate output might reflect, for example, a combination of effects on the glucose-transport system and cellular energy levels.

In fig.3 we show that there is no inhibition of cap formation by phenothiazines and local anaesthetics at concentrations that do not reduce the ATP level, so that the effects of the drugs on capping may be explained simply in terms of their effects on nucleotide levels [16]. The possible involvement of calmodulin, through, e.g., myosin light chain kinase activation, cannot be assessed in these experiments, since several aspects of cytoskeletal function (e.g., monomer–polymer steady-states [17,18]) may be controlled by nucleotide levels.

4. Discussion

It is clear that under the conditions used here the phenothiazines and local anaesthetics cannot be used to infer the involvement of calmodulin in either mitogenic stimulation (as indicated by lactate output [15]), or cap formation. This conclusion may apply more generally to the use of these drugs as indicators of calmodulin function in intact cells, since the effects demonstrated here on cellular energy metabolism would be expected to interfere with any system for which ATP is either a substrate or a regulator. It may therefore be invalid to ascribe effects of trifluoperazine noted only at high concentrations to the inhibition of calmodulin [19,20], and it may be premature to suggest that local anaesthetics might act on calcium-mediated systems through the antagonism of calmodulin, while other mechanisms apply to calcium-independent systems [4]. Similarly, it would not be valid to assume that any effects of these drugs result from the severance of calcium-dependent linkages between membrane proteins and the cytoskeleton [7,21,22]. This limitation has been noted for the effects of chlorpromazine and other drugs on macrophage functions [23], and has been stressed for the effects of local anaesthetics on capping, where an ATP-capping profile similar to fig.3 was demonstrated [16]. The use of phenothiazines as probes for the involvement of calmodulin in α-adrenergic responses has also been questioned, since trifluoperazine and chlorpromazine act as α-adrenergic antagonists in hepatocytes at similar concentrations to those reported to interact with calmodulin [24,25].

A comparison of our data with those of other workers reveals considerable variability in the concentrations of trifluoperazine reported to inhibit capping. Thus, in [26], little effect of 20 μM trifluoperazine was found, while significant inhibition by 10 μM trifluoperazine was reported in [27], and half-maximal inhibition by 3 μM trifluoperazine was found in [28]. Where suitable metabolic controls have been performed, there is again much variation in the reported effects. Our results (fig.1) are intermediate between those in [29] (increased release of LDH after only 2 min preincubation of neutrophils with 10 μM trifluoperazine) and [28] (no depletion of neutrophil ATP after 20 min incubation with higher trifluoperazine concentrations). At least some part of this variability may be due to different amounts of (phospholipid) material present in the system (as shown for the new, highly hydrophobic calmodulin inhibitor R24571 [30]), and this emphasizes the need for the appropriate metabolic controls to be performed in each system.

Recent studies have correlated the antagonism of calmodulin by the local anaesthetics and chlorpromazine with their octanol–water partition coefficients [5,31,32], which raises the possibility that they may cause general membrane perturbations [31]; the effect on lymphocyte ATP levels probably results from interference with mitochondrial membrane function (compare [33,34]), although at higher concentrations cytolytic effects also contribute. The demonstration that the correlation between calmodulin antagonism and the octanol–water partition coefficient extends to the specifically-designed calmodulin inhibitor W-7 [5], suggests that the use of this drug (and others such as R24571) may be subject to limitations similar to those described here.

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