Evidence that calmodulin may be involved in phytohaemagglutinin-stimulated lymphocyte division

Sheila MAC NEIL*, S. W. WALKER**, B. L. BROWN**, and S. TOMLINSON*

*Department of Medicine, Clinical Sciences Centre, Northern General Hospital, Herries Road, Sheffield S5 7AU, U.K.; and **Department of Human Metabolism and Clinical Biochemistry, Sheffield University Medical School, Beech Hill Road, Sheffield S10 2RX, U.K.

Received 13 September 1982

Phytohaemagglutinin-stimulated and non-stimulated incorporation of [3H]thymidine into human peripheral blood lymphocytes is inhibited by the calcium antagonist PY 108-068 and by the calmodulin antagonists trifluoperazine and 6-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W7). It is argued that calmodulin may be involved in both non-stimulated [3H]thymidine uptake in lymphocytes and also in the lymphocyte response to phytohaemagglutinin.

Phytohaemagglutinin (PHA) is mitogenic for lymphocytes obtained from peripheral venous blood (Nowell, 1960; MacKinney et al., 1962). The mitogenic response depends upon the presence of extracellular Ca2+ (Kay, 1971; Whitney & Sutherland, 1972), and is accompanied by uptake of radiolabelled 45Ca2+ (Whitney & Sutherland, 1973). Further, Ca2+ ionophores such as A23187 will also, even in the absence of mitogen, stimulate lymphocyte division (Luckasen et al., 1974).

A wide range of intracellular Ca2+-dependent processes occur through the binding of Ca2+ to calmodulin, the ubiquitous calcium-dependent regulatory protein, which is thereby activated (Klee et al., 1980). Therefore, we have examined the effects of a drug reported to be a potent calcium antagonist, PY 108-068 (Hof et al., 1981), and of two calmodulin antagonists, trifluoperazine (Weiss et al., 1980) and 6-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W7) (Hidaka et al., 1978) on PHA-stimulated incorporation of [3H]thymidine into human peripheral-blood lymphocytes.

Materials and Methods

Lymphocyte separation medium was from Flow Laboratories (Irvine); the Hanks' balanced salt solution, RPMI-1640 culture medium, foetal calf serum, penicillin, and streptomycin were obtained from Gibco-Europe (Paisley); PHA was from Wellcome Research Laboratories (Beckenham); [3H]thymidine and [3H]cyclic AMP were obtained from Amersham International. Bovine heart 3':5'-cyclic-nucleotide

©1982 The Biochemical Society
phosphodiesterase (activator-deficient, Sigma P0520) was obtained from
the Sigma Chemical Company, Poole, Dorset, U.K. Purified pig brain
calmodulin was obtained from the Boehringer Corporation (London)
Ltd. Trifluoperazine (TFP) and trifluoperazine sulphoxide were gifts
from Smith, Kline and French Laboratories (Welwyn Garden City,
Herts); PY 108-068 was a gift from Sandoz Products Ltd. Dr. H.
Hidaka kindly provided N-(6-aminohexyl)-5-chloro-1-naphthalene
sulphonamide (W7).

Lymphocytes were prepared from human peripheral venous blood
using standard Ficoll-Hypaque density-gradient centrifugation. The
cells were washed twice in Hanks' balanced salt solution and resus-
pended in RPMI-1640 medium containing 10% foetal calf serum,
heat-inactivated (56°C for 45 min), supplemented with 100 units/ml
penicillin/streptomycin. PHA, when present, was added at a final
concentration of 1 μg/ml, and was present throughout the 72-h
incubation. The drugs were made up freshly in normal saline, except
for PY 108-068 and TFP sulphoxide, which were first solubilized in
DMSO, then diluted in normal saline. All drugs were present for 72 h,
except in some experiments with TFP (see 'Results'). Incubations
were carried out in triplicate with 220-μl vols. of 10⁶ cells/ml in
microtitre plates (Sterilin) incubated at 37°C in 5% CO₂. At 68 h, 4
μCi of [³H]thymidine was added to each well and the incubation
continued for a further 4 h. At 72 h, the samples were harvested on
glass-fibre filters (Dynatech) using a Dynatech multimesh automatic
sampler. The radioactivity was measured in a Packard liquid-
scintillation counter, and results expressed as means ± S.E.M. of
triplicate determinations.

The calmodulin-inhibitory potency of the drugs was determined
using a calmodulin-deficient bovine heart phosphodiesterase, measuring
phosphodiesterase activity by the method of Thompson et al. (1979).
After optimization of assay conditions for the activation of this
enzyme by calmodulin, the final reaction mixture contained, in 100 μl
vol., 40 mM TRIS/HCl pH 7.0 at 37°C, 4 mM 2-mercaptoethanol, 5
mM MgCl₂, cyclic [³H]AMP (2 x 10⁵ c.p.m./tube), 100 μM cyclic
AMP, 25 μM CaCl₂, 2 munits of enzyme/tube, and calmodulin.
Half-maximal activation was produced by 7.6 ng of calmodulin/tube
(7.6 ± 1.0; 9 ± S.E.M. [n = 6]). In determining the inhibitory potency
of the drugs, 20 ng of calmodulin/tube was used in all assays. This
produced approximately 80% of activation of the enzyme (maximal
activation was 4-fold over non-activated levels for this enzyme). In
the studies with the calcium antagonist, incubations were carried out
at 5, 25, and 125 μM calcium. All incubations were for 15 min at
37°C. The concentration of drug required to produce half-maximal
inhibition of the response to calmodulin (IC₅₀) was calculated from
a minimum of 2, but usually 3, expts. with each drug.

Results and Discussion

The [³H]thymidine incorporation in non-stimulated lymphocytes at a
density of 0.2-1 x 10⁶ cells/ml was 6-9 x 10³ c.p.m. The uptake of
label was increased 16-fold (16.5 ± 5-fold; 9 ± S.E.M. [n = 5]) by a
maximally stimulating concentration of PHA (1 μg/ml).
CALMODULIN-DEPENDENT LYMPHOCYTE DIVISION

3H-Thymidine Incorporation (% of control level)

Fig. 1. Effect of a calcium antagonist (PY 108-068) and three calmodulin antagonists (TFP, TFP sulphoxide, and W7) on PHA-stimulated [3H]thymidine incorporation into peripheral-blood lymphocytes. Lymphocytes were cultured with PHA plus PY 108-068 (■), TFP (●), TFP sulphoxide (○), or W7 (▲) for 72 h as described in 'Materials and Methods'. Results are means ± S.E.M. of triplicate determinations from single representative experiments in which the [3H]thymidine incorporation in the absence of drug is expressed as 100% in order to combine data for different drugs.

Figs. 1 and 2 show that PY 108-068, TFP, W7, and, to a much lesser extent, TFP sulphoxide, produce a dose-dependent inhibition of [3H]thymidine incorporation into PHA-stimulated (Fig. 1) and non-stimulated (Fig. 2) lymphocytes. Trifluoperazine sulphoxide (TFP sulphoxide), which has much less inhibitory action than TFP against calmodulin (Weiss et al., 1980) is significantly less potent than TFP in this system. The results shown in Figs. 1 and 2 are of individual experiments, representative of results obtained on at least two occasions with each drug. With W7 there is an indication that more drug may be required to inhibit the PHA-stimulated [3H]thymidine uptake than for inhibition of the non-stimulated [3H]thymidine uptake, but further work is required to investigate this possibility. Also Figs. 1 and 2 show some stimulation of [3H]thymidine incorporation at drug doses approximately 10-fold less than the IC50 values shown in Table 1. The reason for this is not known.
Fig. 2. Effect of calcium antagonist (PY 108-068) and three calmodulin antagonists (TFP, TFP sulphoxide, and W7) on non-stimulated [3H]thymidine incorporation into peripheral-blood lymphocytes. Lymphocytes were cultured for 72 h, without added mitogen, as described in 'Materials and Methods', with PY 108-068 (■), TFP (●), TFP sulphoxide (○), or W7 (▲). Results are means ± S.E.M. of triplicate determinations from single representative experiments in which the [3H]thymidine incorporation in the absence of drug is expressed as 100% in order to combine data for different drugs.

The concentration of drug required for half-maximal inhibition (IC$_{50}$) of [3H]thymidine incorporation, determined from a minimum of 2 expts. with each drug, was compared to the concentration of drug required to produce half-maximal inhibition of the calmodulin activation of beef-heart phosphodiesterase (Table I). The IC$_{50}$ for the action of these drugs in both systems is similar.

Although PY 108-068 was found to inhibit the calmodulin-activated phosphodiesterase as shown in Table I, this inhibition could be reduced by increasing the amount of calcium present (IC$_{50}$ of 4.6 μM with 5 μM Ca$^{2+}$ present, 5.4 μM with 25 μM Ca$^{2+}$ present, and 18 μM with 125 μM Ca$^{2+}$ present) but not by increasing the amount of calmodulin present (even up to 2 μg per incubation). By contrast, the inhibition
Table 1. Drug potencies for inhibition of \([^3H] \)thymidine incorporation in lymphocytes and for inhibition of calmodulin activation of beef heart phosphodiesterase

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitory potency</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>([IC_{50} (\mu M), \bar{x} \ (range) \ (n)])</td>
<td>[^3H]thymidine incorporation in lymphocytes</td>
<td>Calmodulin activation of phosphodiesterase</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>3.0</td>
<td>3.2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>(1.0-6.0)(4)</td>
<td>(1.4-8.0)(4)</td>
<td>(1.1-5.4)(3)</td>
</tr>
<tr>
<td>Trifluoperazine sulphoxide</td>
<td>&gt;100</td>
<td>*25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(&gt;100,&gt;100)(2)</td>
<td>(22.0-28.0)(2)</td>
<td>(&gt;100,&gt;100)(2)</td>
</tr>
<tr>
<td>W7</td>
<td>5.9</td>
<td>31.6</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>(1.8-10.0)(2)</td>
<td>(17.0-38.0)(3)</td>
<td>(24.0-34.0)(2)</td>
</tr>
<tr>
<td>PY 108-068</td>
<td>3.2</td>
<td>3.3</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>(1.8-5.0)(3)</td>
<td>(1.8-4.8)(3)</td>
<td>(4.1-6.3)(2)</td>
</tr>
</tbody>
</table>

*Higher concentrations of this drug (up to 100 \(\mu M\)) failed to produce complete inhibition of \([^3H]\)thymidine uptake.

produced by TFP and W7 was unaffected by increasing \(\text{Ca}^{2+}\) concentration. These results suggest that PY 108-068 exerts its inhibitory effect in the phosphodiesterase assay by an action on \(\text{Ca}^{2+}\) rather than calmodulin, which is consistent with its reported action as a \(\text{Ca}^{2+}\) antagonist (Hof et al., 1981).

The time course of action of TFP was studied. Fig. 3 shows that 25 \(\mu M\) TFP achieved its maximal inhibition of PHA-stimulated \([^3H]\)thymidine incorporation (97% inhibition) when added at time 0 or at 24 h. When added at 48 h after the start of incubation with PHA, however, the inhibition was much less (23% inhibition). Inhibition remained high even if TFP (and PHA) were removed and the cells extensively washed at 24 h. If PHA alone was restored at this time, incorporation of label was nearly 90% of that seen normally in a 72-h PHA incubation. Hence, the majority of lymphocytes remained viable after exposure to this concentration of TFP.

Calmodulin has been isolated from lymphocytes, where it activates a plasma membrane (\(\text{Ca}^{2+}/\text{Mg}^{2+}\))ATP\(\alpha\)se (Lichtman et al., 1981). TFP has been reported to inhibit ligand-induced receptor capping in T lymphocytes (Bourguignon & Balazovich, 1980) and to inhibit the recruitment of clathrin coats to ligand-receptor clusters on the membrane of B lymphocytes (Salisbury et al., 1980). Salisbury et al. (1981) found that capping in the human lymphoblastoid cell line Wil2 was mostly unaffected by TFP or removal of external \(\text{Ca}^{2+}\). However, using indirect immunofluorescence, the same workers showed that, concurrent with capping of cell-surface receptors for concanavalin A, calmodulin, initially diffusely present throughout the cell, became concentrated in the cytoplasm beneath the cap. This calmodulin redistribution was sensitive to TFP and dependent on external \(\text{Ca}^{2+}\). There is, thus, accumulating evidence that calmodulin is involved at one or more stages of receptor-mediated endocytosis. Hence,
lymphocyte-responsiveness to antigen or mitogens such as PHA might be expected to be impaired by calmodulin antagonists such as TFP. We report here that this, indeed, appears to be the case since two potent calmodulin antagonists, TFP and W7, markedly inhibit \([^3H]\)thymidine incorporation into peripheral-blood lymphocytes following exposure to PHA.

**Acknowledgements**

We are indebted to the Medical Research Council and the Wellcome Trust for financial support. S. Walker holds a Wellcome Clinical Research Fellowship. S. Tomlinson is a Wellcome Trust Senior Lecturer. We are grateful to Mrs. Beck Allan and Dr. George Gray for assistance with the lymphocyte methodology and Dr. John Wright and Carol A. Penning for helpful discussion of the work.
References