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INHIBITION BY TRIFLUOPERAZINE AND DIGITONIN OF SEVERAL FORMS OF PINOCYTOSIS

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Abstract—The effect of the calmodulin antagonist trifluoperazine on fluid phase, adsorptive and receptor-mediated pinocytosis in cultured human fibroblasts and mouse peritoneal macrophages was studied. Uptake in each case was reversibly inhibited by about 20% without toxic effects. Low concentrations of the detergent digitonin had a similar effect. Thus the three uptake mechanisms have common features, and we suggest that in both fibroblasts and macrophages they may reflect uptake within vesicles participating in receptor-mediated endocytosis. These effects of trifluoperazine may operate directly on membranes without the participation of calmodulin.

Pinocytosis, the internalization of molecules by cells by means of membrane invagination and subsequent intracellular vesicle formation, can be usefully subdivided into three types on the basis of the degree of membrane association of the internalized molecules. These are fluid phase, adsorptive and receptor-mediated pinocytosis, according to whether molecules enter the cells respectively in the fluid phase, or partly bound to heterogeneous sites on the membrane, or bound mainly to specific receptors [1]. In previous work [2] we have shown that the temperature dependence of all three processes in mouse peritoneal macrophages is very similar, which would largely exclude the earlier suggestion that pinocytosis can also be divided into two mechanistic forms (macro- and micropinocytosis) on the basis of vesicle size and energy dependence [3]. Our work indicated that the three types of pinocytosis mentioned above have common mechanistic components. In all three types, pinocytosis rate falls with declining temperature, and ceases at about 15° [2]. Previous literature has also suggested that another common feature of pinocytosis is its dependence on extracellular calcium [3], and thus in the present work we have attempted to study the possible role of calmodulin, a protein which transduces calcium signals (reviewed in [4]) within many cells, by using the antagonist of its function, trifluoperazine. Since trifluoperazine has some direct effects on membranes [4], we have compared its effects with those of the membrane-active detergent digitonin.

MATERIALS AND METHODS

Most materials were as described before [2]. Trifluoperazine (Stellazine) was a kind gift of Smith, Kline & French Ltd. (Welwyn, Herts., U.K.).

The preparation of mouse peritoneal macrophages and the maintenance of the mouse macrophage-like cell line P388D$_1$ were as described previously [5]. Cultures of normal and hexosaminidase-deficient (Sandhoff's disease) human fibroblasts were maintained in EMEM† containing 10% (v/v) heat-inactivated foetal calf serum plus antibiotics. All cultures were maintained at 37° and gassed with 5% (v/v) CO_2 in air.

Beta-hexosaminidase (*N*-acetyl-beta-D-glucosaminidase, EC 3.2.1.30) was assayed at pH 4.5 using 2 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-beta-D-glucopyranoside [6]. One unit of activity was the amount of enzyme which catalyzed the release of 1 nmole of 4-methylumbelliferone per hr. Protein was measured by the Lowry method [7].

Endocytosis was quantitated by adding radioactively labelled molecules or lysomal enzymes directly to the culture medium of both fibroblasts and macrophages, and measuring their subsequent association with the cells. Tracers were used at the following concentrations, which were shown in preliminary experiments to be within the range in which uptake was linearly dependent on tracer concentration: [^{3}H]sucrose, 4 μg/ml; [^{125}I]dHSA, 5 μg/ml; hexosaminidase secreted spontaneously in 24 hr [5] by P388D$_1$ cells, an activity of 100 units/ml of culture medium, as previously [5]. At various times after presentation of tracers, the media were harvested and the cells washed four times with PBS. In the case of uptake of sucrose, 0.01% (w/v) non-radioactive sucrose was also included in the PBS for washing [2]. Cells were then lysed by addition of 0.1% (v/v) Triton X-100 in PBS for measurement of radioactivity or enzyme. In the case of dHSA, some intracellular degradation occurred during the experiments; thus total uptake was measured as the sum of degradation products [molecules soluble in 5% (w/v) TCA, from both cell and media fractions] and intact macromolecules (precipitable by 5% TCA) in the cell fractions (as described in detail previously [2]). Degradation was expressed as degradation products as % of total uptake [2]. Uptake was expressed as the Endocytic Index: the number of microlitres of fluid whose contained substrate has

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† Abbreviations: EMEM, Eagle's Minimal Essential Medium; [^{125}I]dHSA, ^{125}I-labelled denatured human serum albumin; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TFP, trifluoperazine.

been internalized at the specified time, per mg cell protein. In the case of hexosaminidase from P388D$_1$ cells, 2 mM mannose-6-phosphate could completely prevent uptake of activity by Sandhoff's fibroblasts; thus confirming that uptake was specifically mediated by the receptor for glycoprotein mannose-6-phosphate ligands (as before[5]). Uptake of hexosaminidase was expressed as total units of enzyme activity internalized per culture during the incubation (22 hr).

Cell lysis was determined in the various experimental conditions by prelabelling either proteins (in the case of macrophages which do not divide *in vitro* and thus incorporate little thymidine) or nucleic acids (in the case of fibroblasts) and measuring the subsequent release of *intact* labelled macromolecules into the medium. Macrophages were labelled for 24 hr with [^{14}C]leucine (in leucine-free medium); fibroblasts for 24 hr with [^{3}H]thymidine in medium containing 5 μg/ml cold thymidine. Each cell type was then washed extensively with PBS containing the appropriate cold precursor. Cold precursors (leucine: 10 mM; thymidine 5 μg/ml) were also present during the subsequent experimental periods, after which the release of total radioactivity (from thymidine-labelled cells) or radioactivity precipitable by 5% TCA (in the case of leucine-labelled cells) was measured, and expressed as a percentage of the total radioactivity in the system (summed cell fractions plus summed medium fractions), to give % lysis.

Samples were counted either in Triton X-100/toluene (3:7, v/v) scintillant (beta isotopes), or directly by a gamma-counter (gamma isotopes). For beta counting, quench correction was by an automated channels ratio method based on an external standard, and was performed by the Packard 460CD instrument.

Data presented are means ± S.D. (on the figures only when S.D. exceeds the span of the point symbol) from experiments with triplicate replicates, and are representative of several similar experiments.

RESULTS AND DISCUSSION

In general, previous workers have used TFP concentrations between 10 and 100 μM [4], so in preliminary experiments we assessed the effect of a range of concentrations, and found that in the case of macrophages toxicity precluded the use of concentrations above 15 μM, and the effect on sucrose intake of 15 and 10 μM TFP was very similar. Toxicity was judged by cell rounding and detachment (as seen by light microscopy), and by measurements of cell lysis (as described in Materials and Methods). Thus we chose to conduct all subsequent experiments at the relatively low concentration of 10 μM. At this concentration, TFP could reduce by about 20% the rate of uptake of tracers representing all three classes of pinocytosis, in experiments with both macrophages and fibroblasts. Figure 1 shows the kinetics of this effect for fluid phase pinocytosis (of sucrose) by both cell types. Inhibition was established within 2 hr, and remained effective throughout the experiments, up to 24 hr. The inhibition of uptake of dHSA by macrophages was similar in all respects: thus after 24 hr of uptake control cells had internalized the

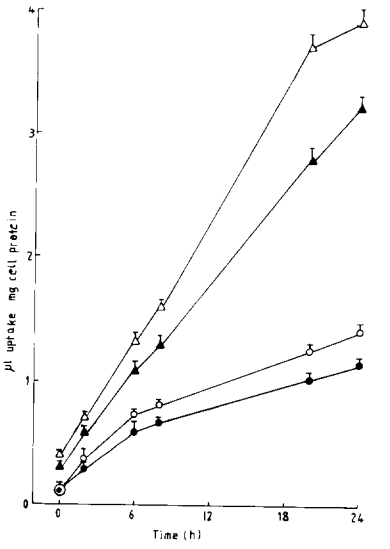

Fig. 1. The effect of TFP on uptake of [^{3}H]sucrose by macrophages and fibroblasts. Cells endocytosed sucrose for 24 hr in control conditions (△, macrophages; ○, fibroblasts) or in the presence of 10 μM TFP (▲, macrophages; ●, fibroblasts). The degree of inhibition estimated at the various times was from 18 to 23% for macrophages, and from 15 to 19% for fibroblasts.

equivalent of 45 ± 0.9 μl of fluid/mg protein, while the corresponding figure for cells exposed to 10 μM TFP was 34 ± 1, representing an inhibition of approximately 25% (Fig. 2). The degradation of dHSA which followed its endocytosis was unaffected by TFP (Fig. 2). To assess the effect of TFP on receptor-mediated endocytosis, we have utilized the uptake of lysosomal enzymes bearing mannose-6-phosphate ligands by their receptors on fibroblasts [8]. We measured the internalization of hexosaminidase by Sandhoff fibroblasts, which are deficient in the enzyme, and thus in which accumulation of exogenous enzyme can be accurately measured [5]. The uptake was, as previously described, largely

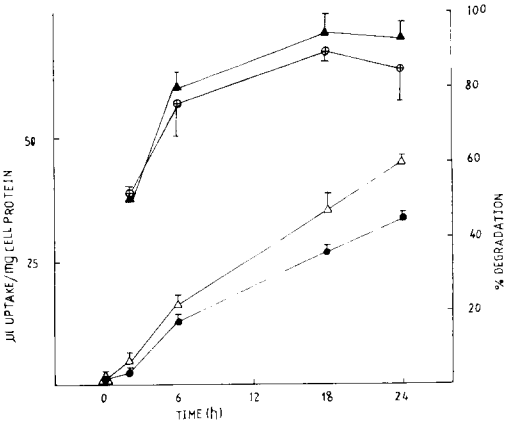

Fig. 2. The effect of TFP on the uptake (△, ●) and degradation (▲, ⊕) of [^{125}I]-human serum albumin by macrophages. Cells were exposed to HSA with (●, ⊕) or without (△, ▲) TFP for various periods, and the uptake and degradation measured. TFP reduced uptake by between 19 and 25%.

inhibited by 2 mM mannose-6-phosphate, and thus was by means of the mannose-6-phosphate-glycoprotein receptor. As shown in Table 1, such internalization was also inhibited by TFP by 20%.

In order to assess the nature of this consistent inhibition of all three types of endocytosis by TFP, we first established that cell death was not responsible. Using the techniques in Materials and Methods, we found that 10 μM TFP caused no more cell lysis than occurred under control conditions. This lysis was: for Sandhoff fibroblasts, less than 10% at 22 hr (cf. Table 1); and for macrophages, less than 10% at 24 hr (cf. Fig. 1). Furthermore, these estimates of cell lysis are overestimates since they do not allow, on the one hand, for degradation of nucleic acids, and, on the other, for secretion of proteins. Thus the effects of TFP could not be attributed to cell death.

Since TFP has been reported to have several 'detergent-like' effects [4] operating on cellular membranes in addition to those operating on calmodulin, we next compared its effects with those of a relevant low concentration (10 μg/ml) of the detergent digitonin, as previously described by Baker *et al.* [9]. Table 1 shows that digitonin causes similar inhibition of receptor-mediated uptake by fibroblasts to that due to TFP. It had parallel consequences on endocytosis of sucrose by macrophages: while control cells internalize in 24 hr 2.6 ± 0.1 μl/mg cell protein, cells exposed to 10 μg/ml digitonin internalized only 2.2 ± 0.05, revealing a statistically significant reduction of 16%. Again experiments on cell lysis showed that the effects of digitonin were not due to enhanced cell death. In other work in our laboratory (Bodmer, unpublished) we have found that 10 μM TFP and 10 μg/ml digitonin virtually abolish binding of zymosan particles to the cell surface of macrophages, which indicates that they perturb the plasma membrane, and hence that such an effect could be responsible for the changes in endocytosis we have described. Thus the inhibitory effect of TFP could not clearly be ascribed to an action on calmodulin, although this is not formally excluded.

We finally assessed the reversibility of the effects of both TFP and digitonin. For this purpose, macrophages were incubated either under control conditions or in the presence of 10 μM TFP or 10 μg/ml digitonin, for 6 hr. This duration of treatment was chosen because, as shown in Fig. 1, it was sufficient to establish maximal inhibition. Cells were then washed four times with PBS, and sucrose was added for an uptake period of 18 hr. As shown in Fig. 3, the rates of uptake for all three groups were indistinguishable, and were equal to those in a control group which was endocytosing sucrose throughout the 24 hr and which was not washed at 6 hr. Positive controls for the effects of TFP and digitonin when present continuously were included and gave results as before. This experiment clearly established that the inhibitory effects of both TFP and digitonin were rapidly reversible, again indicating that toxic effects are unlikely to be involved.

There are previous indications that calmodulin participates in receptor-mediated uptake by coated vesicles [10]. However, the present data neither further support nor undermine this view, since the site(s) of action of neither TFP nor digitonin is clear. Perhaps the most interesting aspect of the present data is the striking similarity in the degree of inhibition achieved in all three uptake systems at the chosen concentration of both agents. Thus, the present evidence on TFP and digitonin implies that fluid, adsorptive and receptor-mediated uptake share crucial features, in agreement with our previous work on temperature dependence of endocytosis in macrophages [2].

Table 1. Effect of TFP and digitonin on the uptake of hexosaminidase by Sandhoff fibroblasts

Condition	Uptake of enzyme (units/culture)	% lysis of cells
Control	10.0 ± 1.0	9.0 ± 1.0
TFP, 10 μM	7.0 ± 0.2	8.5 ± 2.0
Digitonin, 10 μg/ml	7.2 ± 0.1	10.0 ± 1.0

Uptake of enzyme was measured after 22 hr of presentation (150 nmole/hr of enzyme, in a total medium volume of 1.5 ml). Controls were run in parallel to allow for the slight hexosaminidase activity in the fibroblasts (incubations of cells without added enzyme); results presented are nett uptake after subtraction of such endogenous activity. Uptake was comparable in magnitude to that observed in our previous work [5], and was essentially abolished by the presence of 2 mM mannose-6-phosphate in all experimental conditions.

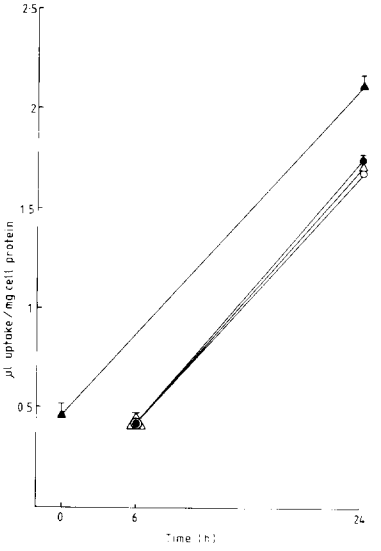

Fig. 3. The reversibility of the effects of TFP and digitonin on uptake of [^{3}H]sucrose by macrophages. Cells were incubated with medium alone (△), or medium containing 10 μM TFP (●) or 10 μg/ml digitonin (○). After 6 hr these groups were washed four times with PBS, and then received for a subsequent 18-hr period medium containing radioactive sucrose. A fourth group of cells (▲) were incubated normally with radioactive sucrose throughout the 24 hr of the experiment. The cultures from each experimental group were harvested at the indicated times. The rates of uptake for all four groups were indistinguishable.

The evidence of very similar inhibition by digitonin and TFP of uptake by all three pinocytic routes might indicate that three distinct sets of membrane internalization sites are equally inhibited by both agents. However, Pearse and Bretscher [11] have argued on a quantitative basis that all the fluid intake of fibroblasts could be explained by coated-vesicle internalization (which is responsible for receptor-mediated intake of lysosomal enzymes and other molecules [8]). This would explain rather more economically the equal inhibition of uptake of all three tracers by both agents. Thus, it is possible that the present data imply a similar situation in macrophages, in which receptor-internalizing vesicles may again be responsible for the majority of intake, whether of fluid, of adsorbed or of receptor-bound molecules.

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