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INHIBITION OF SECRETION OF PROTEINS AND TRIACYLGLYCEROL FROM ISOLATED RAT HEPATOCYTES MEDIATED BY BENZIMIDAZOLE CARBAMATE ANTIMICROTUBULE AGENTS

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Abstract—The benzimidazole carbamates, nocodazole and parbendazole, inhibited the secretion of newly labelled proteins and very low density lipoprotein triacylglycerol by isolated rat liver cells. At a concentration of 10 μM, nocodazole produced colchicine-like inhibition over a full 6hr time course whilst parbendazole produced a less regular pattern of inhibition. Methyl benzimidazol-2-yl-carbamate at the same concentration had no apparent effect on secretory rates. None of the compounds affected incorporation of the labelled precursors into protein or triacylglycerol, which were recovered from cell suspensions in amounts similar to controls. This post-synthetic block in the export of secretory components together with the previously noted anti-microtubular activity of the benzimidazole carbamate group of compounds is in compliance with the view that microtubules are closely involved with the secretory process.

INTRODUCTION

Increasingly the microtubular apparatus of cells is being implicated in secretory processes of many tissues. Drugs which show a high degree of specific binding to microtubular protein, particularly tubulin, can lead to a decrease in the number of assembled microtubules within the cell. Involvement of the microtubular system in mitosis, organelle orientation, intracellular transport and secretion has been concluded from studies using drugs such as colchicine, vinblastine and vincristine which all show specific binding to tubulin.

Mammalian liver synthesises and subsequently secretes large amounts of plasma proteins and very low density lipoprotein (VLDL). Isolated liver perfused with low concentrations of anti-microtubular drugs secretes significantly decreased amounts of newly synthesised protein [1] and triacylglycerol [2], a marker for VLDL, but continues to synthesise these products throughout at an unchanged rate. Hepatocytes isolated from rat liver by enzymic perfusion and maintained in suspension retain many of the properties and functions of the parent tissue [3, 4] including the synthesis and secretion of both protein and VLDL. As such, hepatocytes present a useful model for studying the role played by microtubules in secretory events [5, 6].

A major class of compounds which show affinity for microtubules is that of the benzimidazole carbamates. Benzimidazole itself has no antimicrotubular activity [7, 8] but with different side group substitutions the potency is considerably increased. It seems likely that it is a common mode of action, the depolymerisation of functional microtubules, which accounts for their efficacy in a range of diverse applications. Methyl benzimidazol-2-yl carbamate (MBC) perturbs fungal mitosis [9] and is the active agent of the fungicide benomyl (methyl-1-[butyl carbamoyl] benzimidazole-2-yl carbamate). Parbendazole (methyl-5-butyl benzimidazol-2-yl carbamate) is one of a number of proven anthelmintics within the group [7]. Nocodazole (methyl-[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate was initially investigated for its anti-cancer properties [10]. Recently is has been reported to be of value for producing synchronous cultures of mammalian cells [11].

Ultrastructural changes brought about by nocodazole administered to non-proliferating primary cultures of isolated rat liver cells have been compared to those brought about by colchicine [12]. Notably, hepatocytes treated with 33 μM-nocodazole were more resistant to spreading, showed a high degree of organelle displacement and contained many more small lipid droplets than did control cultures. Also, far fewer assembled microtubules were visible in the cytoplasm of the nocodazole treated cells than in controls. Whereas nocodazole has been reported to inhibit pancreatic insulin and glucagon secretion [13] no such data are available concerning the release of hepatic secretory products.

In this study, we report suppression of the secretion of [^{3}H]-leucine and [^{3}H]-oleic acid newly incorporated into protein and triacylglycerol, brought about by 10 μM-colchicine, nocodazole and parbendazole. MBC at the same concentration did not have this effect. Neither protein nor triacylglycerol synthesis was decreased by any of the drugs. These results are in agreement with the view that micro-

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tubules play an integral role in hepatic secretion, and that the benzimidazole carbamates are colchicine-like microtubule inhibitors.

MATERIALS AND METHODS

Parbendazole and methylbenzimidazol-2-yl carbamate were a gift from Pfizer (Sandwich, U.K.), Nocodazole was obtained from Aldrich (Gillingham, U.K.), Colchicine and collagenase were from Boehringer (Lewes, U.K.), Eagle's minimum essential medium and foetal bovine serum were obtained from Flow Laboratories (Irvine, Scotland, U.K.) and bovine serum albumin (BSA) was from Armour Pharmaceuticals (Eastbourne, U.K.). Monospecific antibody to rat serum albumin was the kind gift of Professor J. D. Judah. Florisil (100–200 mesh size), firefly lantern extract, L-leucine, oleic acid (Na^+ salt) and antibiotics were all from Sigma (Poole, U.K.), L-[4,5-^{3}H]-leucine (149.5 Ci/mmol), [9,10-^{3}H]-oleic acid (6.4 Ci/mmol), NCS and scintillants were all from the Radiochemical Centre (Amersham, U.K.). All solvents and standard laboratory reagents were of the highest grade available from standard suppliers.

Male Sprague-Dawley rats (200–275 g), inbred at the University of Kent, were used throughout. They had free access to food and water.

Liver cell isolation and incubation. Rats were anaesthetised by the intraperitoneal injection of sodium pentobarbital (60 mg.kg^{-1} body wt) containing heparin (5 mg.kg^{-1} body wt), and hepatocytes isolated essentially by the method of Elliott *et al.* [14]. The perfusion medium used was Krebs-Henseleit buffer minus Ca^{2+} and contained 5.6 mM glucose. Calcium (2.54 mM) was re-added, and the glucose concentration maintained at 10 mM for the cycles of washing. Cells were finally resuspended at approximately 20 mg dry wt/ml in the appropriate incubation medium.

Individual incubations consisting of 0.2 ml of the cell suspension and 1 ml medium were carried out under an atmosphere of O_2/CO_2 (19:1), in 20 ml siliconed scintillation vials. Incubations at 37° were maintained in a gyrotary shaking water bath (Infors type WTR 1), at 130 oscillations per min. All drugs were made up freshly in dimethyl sulphoxide (DMSO) and were warmed to 37° to assist solubility before addition by microsyringe. DMSO was included in all controls at the same final concentration of 0.5 per cent (v/v).

Incubations for studying triacylglycerol synthesis and secretion were carried out in Krebs-Henseleit buffer containing 10 mM-glucose and 2% (w/v) BSA (defatted according to the Method of Chen [15]) containing [^{3}H]-oleate (final 1 mM; 0.4 μCi per vial). For albumin and total protein synthesis and secretion the medium used was Eagle's minimum essential medium supplemented with 10% (v/v) foetal calf serum, 120 μg.ml^{-1} penicillin G, 100 μg.ml^{-1} streptomycin sulphate and 2 μCi [^{3}H]-leucine (final specific radioactivity 4.2 μCi/μmol). Glucose was added to give a final concentration of 10 mM.

Hepatocyte viability was ascertained by measurement of the cellular ATP content [16]. In these experiments the initial value was above 10 nmoles ATP (mg dry weight of cells)$^{-1}$. Incubation for 6 hr in Eagle's medium or 3 hr in Krebs-Henseleit medium resulted in a fall no greater than 10 and 12 per cent respectively; this decrease was unaffected by any of the drugs.

At appropriate times, incubations were terminated by placing the vials in ice. Samples of cell suspension were taken and the medium was obtained after pelleting the cells by a short (1–2 sec; 10,000 rpm) spin in an Eppendorf model 3200 microfuge.

Measurement of triacylglycerol and protein synthesis and secretion. Lipids were extracted from 0.5 ml cell-free medium or 0.4 ml cell suspension by the addition of 20 vol of chloroform:methanol 1:1 (v/v); precipitated protein was removed by centrifugation. The volume of the lower layer was decreased by washing with 0.02 per cent (w/v) $MgCl_2$ before evaporation under N_2 at 50°. Residual lipids were taken up in 0.5 ml 15 per cent (v/v) diethyl ether in petroleum ether, b.p. 60–80°, and applied to 0.5 g columns of Florisil (activated at 105° for 2 hr); triacylglycerol was eluted according to Carrol [17] in 7.5 ml 2 per cent (v/v) methanol in diethyl ether. Samples were transferred to minivials (Hughes and Hughes, Romford, U.K.), solvent was evaporated and the contents were counted for radioactivity in 4ml Cocktail T.

Protein from 0.05 ml samples of suspensions, and 0.10 ml medium alone was precipitated by the addition of an equal volume of ice-cold 10 per cent (w/v) TCA containing 10 mM L-leucine; albumin in 0.05 ml samples of cell-free medium was assayed by specific immuno-precipitation, both as described by Dickson and Pogson [18]. Non-protein [^{3}H]-leucine was removed by washing each pellet three times either with 0.25 ml 5 per cent (w/v) TCA (total protein samples), or with 0.25 ml phosphate-buffered saline (albumin samples); the wash solutions both contained 10 mM L-leucine. In each case the washed pellets were dissolved in 0.03 ml NCS and counted for radioactivity in minivials using 1 ml PCS scintillant.

All scintillation counting was carried out in a Packard liquid scintillation counter (Models 3003 or 3375). All results were corrected by subtraction of counts found at zero time.

Each incubation was carried out in triplicate and experiments were repeated at least once. Where applicable, results are expressed as the mean of three determinations ± S.E.M.

RESULTS

Effect of colchicine on albumin and triacylglycerol secretion

Albumin as a secretory product of the liver is a valid marker of the continuing normal synthetic and secretory activities of isolated liver cells. Of the radiolabelled protein appearing in the medium during 6 hr incubations, a constant 10–12 per cent of the label could be attributed to albumin and this ratio remained unaffected by the presence of colchicine (unpublished observations). In subsequent experiments only total protein radioactivity was measured.

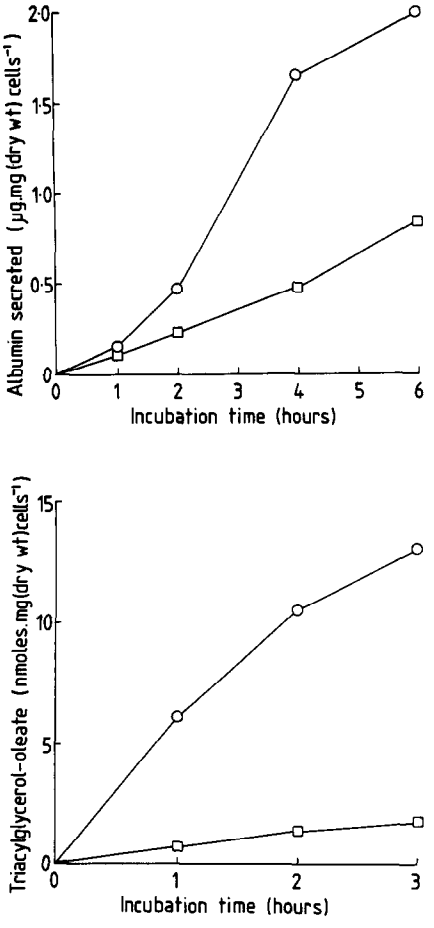

Fig. 1. Effect of colchicine of the secretion of (a) albumin and (b) triacylglycerol from rat hepatocytes. Secretion was determined by the appearance of newly labelled albumin or triacylglycerol in the medium in the absence (○) or presence of 10 μM colchicine (□). In both cases 0.5 per cent DMSO was present. Points represent the mean of triplicate incubations.

From the given leucine content of rat serum albumin [19] and the specific radioactivity of leucine in the medium, the rate of secretion during the 2–4 hr period was 0.65 μg albumin $hr^{-1}.mg^{-1}$ dry wt of hepatocytes [Fig. 1(a)]. This is slightly lower than previously reported rates for isolated cell studies [4, 20]. Triacylglycerol was also released into the medium over a three hour time course [Fig. 1(b)], the measured rates during the second and third hours being 4.4 and 2.5 nmoles triacylglycerol oleate hr^{-1} (mg dry wt of cells)$^{-1}$ respectively.

Colchicine at a concentration of 10 μM brought about rapid and substantial inhibition of both albumin and triacylglycerol secretion. Inhibition of the 2–4 hr rate of albumin secretion and the 1–2 hr rate of triacylglycerol secretion were 84 and 86 per cent respectively. In the cases of both protein and lipid secretion the action of colchicine was sustained and rapidly appreciable, indicating fast uptake of the drug by the hepatocytes.

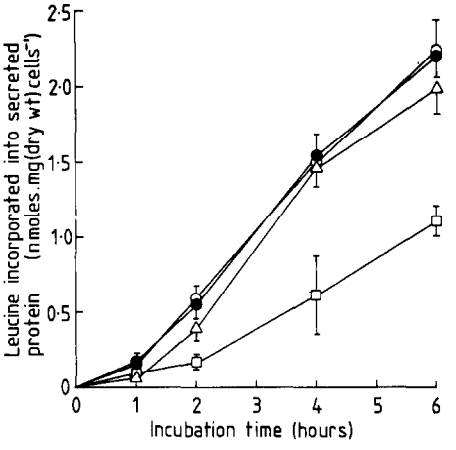

Fig. 2. Secretion of labelled protein by hepatocytes treated with benzimidazole carbamates. The secretion of newly labelled total proteins from hepatocytes incubated in Eagle's minimum essential medium (●) and in the presence of 10 μM MBC (○), nocodazole (□) and parbendazole (△). Points represent the mean of three experiments, the bars S.E.M.

Effects of benzimidazole carbamates on protein secretion and synthesis

Protein secretion over a 6 hr time course (Fig. 2) proved to be stable, the measured rate over the 2–4 hr period being 0.50 ± 0.03 nmoles leucine incorporated hr^{-1} (mg dry weight of cells)$^{-1}$. Incubation with 10 μM-MBC had no effect on secretion, the rate for the same interval being 0.52 ± 0.02 nmoles leucine $hr^{-1}.mg^{-1}$. In contrast, nocodazole at the same concentration elicited a sharp drop in the secretory rate. Initially a high rate of inhibition with respect to the non drug treated cells was observed, 80 per cent during the second hour, but this declined to 60 per cent inhibition during the 2–4 hr interval.

Parbendazole also inhibited secretion within the first hour, but a normal rate of secretion was regained within 2 hr and maintained over the remaining incubation period. Reproducibility of this phenomenon was observed in each experiment conducted.

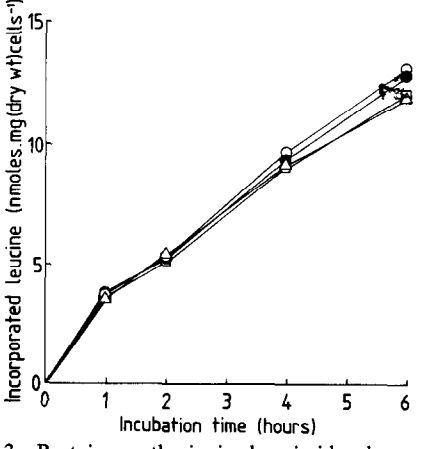

Fig. 3. Protein synthesis in benzimidazole carbamate treated hepatocytes. Incorporation of [^{3}H]leucine into TCA insoluble material from cells and medium. Symbols represent the same as in Fig. 2; the points are the mean of three experiments.

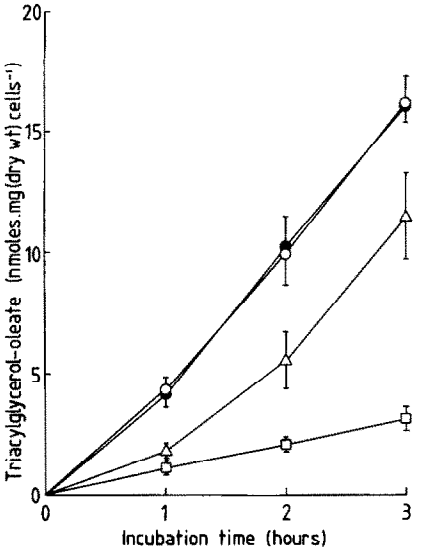

Fig. 4. Triacylglycerol secretion by benzimidazole carbamate treated hepatocytes. Triacylglycerol synthesis and secretion was stimulated by 1 mM oleate included in the Krebs-Henseleit BSA medium. [^{3}H]-oleic acid incorporation into medium triacylglycerol were measured. Hepatocytes were incubated with or without 10 μM drugs, but all included 0.5 per cent DMSO; control (●), MBC (○), nocodazole (□) and parbendazole (△).

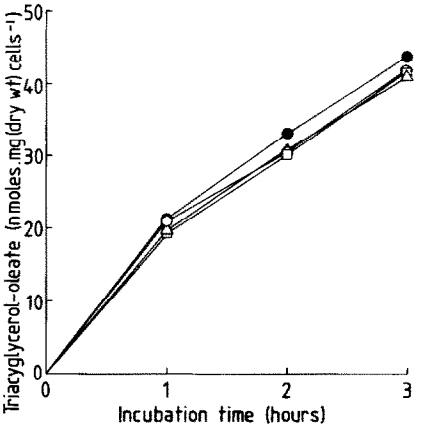

Fig. 5. Synthesis of triacylglycerol by treated and untreated liver cells. Triacylglycerol were isolated as described in the text from cells and medium. The symbols are the same as in Fig. 4. Points represent the mean values of three experiments.

As restriction of protein secretion was apparent during the first hour with both nocodazole and parbendazole it is apparent that the uptake and expression of the drugs are rapid processes.

Incorporation of [^{3}H]leucine into TCA-insoluble material of total cell suspension (Fig. 3), taken as a measure of protein synthesis, remained unaffected by any of the three drugs.

Effects of benzimidazole carbamates on triacylglycerol secretion and synthesis

Rates of appearance of triglyceride in the medium are shown in Fig. 4. Untreated hepatocytes secreted triglyceride at rates of 4.21 ± 0.32, 6.24 ± 0.14 and 5.84 ± 0.25 nmoles triacylglycerol oleate.hr^{-1}.mg^{-1} over the 3 hr respectively. Hepatocytes treated with MBC secreted 4.36 ± 0.23, 5.62 ± 0.50 and 6.17 ± 0.27 nmoles triacylglycerol oleate.hr^{-1}.mg^{-1} dry wt over the same periods. Incubation in the presence of 10 μM-nocodazole decreased medium triacylglycerol levels to 27, 16 and 18 per cent of the control levels during the same periods; with parbendazole the changes were −57 and −38 per cent during the first two hourly periods. Over the third hour, exposure to parbendazole was not associated with a change from the control rate.

The effect of parbendazole showed the same trend as that observed on protein secretion, but was somewhat more pronounced.

Triacylglycerol recovered from whole cell suspensions (Fig. 5) exhibited the same degree of [^{3}H]oleic acid incorporation whether the hepatocytes were incubated with or without any drug.

DISCUSSION

Several authors have recently documented the effects of colchicine upon hepatic secretion of protein and VLDL [2, 5, 21] with which our results are in accord. Inhibition of secretion by 10 μM-colchicine was of the same order for both albumin and triacylglycerol. Electron microscopic evidence [22, 23] indicates that microtubule-directed agents cause small secretory vesicles to accumulate within hepatic tissue, and it is reasonable to assume that they contain the diverted secretory products. In the case of blockage by colchicine, vincristine and vinblastine the 'lost' secretory VLDL and proteins [2, 5, 21] can be recovered by extracting the disrupted washed cells, further evidence that microtubular blocks act at a secretory and not synthetic, level. In these experiments secreted and cellular products were measured together to determine any effects the benzimidazole carbamates may have on synthesis.

Nocodazole and parbendazole at the same concentration as colchicine brought about a similar effect on protein and triacylglycerol secreted, and as neither synthesis of these products nor metabolic integrity (as judged by the levels of cellular ATP (unpublished results)), were affected, the indication is of a block occurring in the secretory pathway. Whilst nocodazole continued to suppress secretion over a 6 hr time course parbendazole only exhibited a transient effect on the secretory rate, more readily appreciable in the case of triacylglycerol secretion. Within 2–3 hr of exposure to the drug hepatocytes were secreting at a more normal rate despite the continuing presence of parbendazole. Clearly this may represent metabolism of the drug by hepatocytes, non-specific binding to vials and plasma membranes, or both. De Brabander *et al.* [12], using radiolabelled nocodazole, observed that, under tissue culture conditions, the drug did come out of solution; however, in their experiment the concentration of the compound was higher (33 μM) and that of DMSO lower (0.1%).

Experiments designed to monitor the assembly of mammalian brain microtubules *in vitro* by light scattering reveal dose-responsive inhibition by various agents. The median inhibitory concentrations of the three benzimidazole derivatives are 3, 5 and >150 μM for parbendazole, nocodazole and MBC respectively [7]. In rank order of effectiveness in blocking protein and triacylglycerol secretion we have nocodazole > parbendazole > MBC. As MBC showed no discernible effects on secretion in this study it is reasonable to assume that its low potency in inhibiting microtubule assembly *in vitro* extends to the cellular level and the microtubules involved in hepatic secretory processes.

Specific binding of benzimidazole carbamates to tubulin has been demonstrated [8, 24] and the competitive nature of binding with colchicine for the same site on the tubulin dimer [24] suggests that the mode of action of the carbamates are those of specific anti-microtubular drugs. These results show that another group of compounds that are chemically unlike colchicine and vinblastine can inhibit hepatic secretion, in a manner similar to that associated with these drugs.

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