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Pharmacological characterization of the slow component of deactivation of guinea-pig isolated ileum to the spasmogenic action of $C5a_{desArg}$

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1 The slow component of deactivation of guinea-pig isolated ileum to $C5a_{desArg}$ was studied to analyse the mechanism of loss and subsequent recovery of sensitivity.

2 Neither cycloheximide (10^{-3} M) nor colchicine (5×10^{-5} M), vinblastine, lumicolchicine, or cytochalasin B (each 2×10^{-5} M) affected significantly the spasmogenic effect of $C5a_{desArg}$ or the course of deactivation produced by repeated applications; chloroquine (2×10^{-4} M) inhibited the spasmogenic effect unspecifically without interfering with deactivation.

3 Recovery from slow deactivation was totally blocked by chloroquine and considerably diminished by colchicine and vinblastine, but was not affected by the other agents.

4 It is proposed that recovery involves lysosomal processing of C5a receptors (occupied by the peptide) but does not require biosynthesis of new receptors.

Introduction

Recently we have demonstrated that deactivation of guinea-pig isolated ileum to the spasmogenic action of $C5a_{desArg}$ consists of fast and slow components (Damerau *et al.*, 1985a). In this paper we analyse the slow deactivation and recovery therefrom using agents which affect lysosomal function (chloroquine), protein synthesis (cycloheximide) and cytoskeleton structures (colchicine, vinblastine, cytochalasin B). The slow deactivation is most probably due to occupation and blockade of specific receptors by $C5a_{desArg}$. It has been shown that it is an exponential process with a half-time of about 60 s and that the extent of deactivation is proportional to the sum of contact times with $C5a_{desArg}$ (Damerau *et al.*, 1985a). Deactivation does not depend on the spasmogenic effect of the peptide nor on exhaustion of histamine, as it develops under conditions which suppress contraction, for instance during incubation in Ca^{2+}-free medium or at 16°C. Therefore, post-receptor events such as signal transfer and activation of target cells (mast cells, muscle cells) are apparently not involved.

After total deactivation, the test organs gradually regain their sensitivity to the peptide. Recovery may start after a few minutes and reach a maximum after about 80 min. It is highly temperature-dependent (not occurring at 16°C). In its time course and temperature sensitivity it is reminiscent of receptor recycling which has been shown to occur with receptors for chemotactic *N*-formylated oligopeptides, transferrin and other peptide ligands (Sullivan & Zigmond, 1980; Zigmond *et al.*, 1982; Ciechanover *et al.*, 1983).

The present findings support the assumption that slow deactivation is due to progressive blockade of the receptor by $C5a_{desArg}$, and that recovery probably involves internalization and recycling of receptors.

Methods

Determination of spasmogenic activity

Segments of guinea-pig ileum about 2.5 cm long were mounted in a 6.3 ml organ bath and connected to a strain gauge isometric recording system (basal tension 1 g). The medium was Tyrode solution at 34°C, alone or containing one of the drugs studied, oxygenated with a mixture of 95% O_2 plus 5% CO_2. After 30 min incubation acetylcholine (ACh) was applied 5–10 times to achieve constant reactivity. Then $C5a_{desArg}$ alternating with two ACh applications was tested, using the following time schedule: change of bath fluid at zero time; injection of spasmogenic substance after 60 s; change of bath fluid after the time periods indicated in the legends to the Figures (contact time of ACh was 15 s) = zero time of the next cycle.

Effects of $C5a_{desArg}$ are given in relation to the effect of supramaximal ACh concentrations (taken as 1.0).

Substances

Cycloheximide, cytochalasin B and vinblastine sulphate were purchased from Sigma (Munich, Germany), colchicine from Serva (Heidelberg, Germany). Lumicolchicine was formed by treatment of colchicine with u.v. light, the conversion was determined photometrically by shift of the characteristic absorption maxima.

The complement peptide $C5a_{desArg}$ was generated by yeast-activation of hog serum and purified as described by Zimmermann *et al.* (1980).

Results

(1) Effect of chloroquine on contractility, slow deactivation and recovery

At a concentration of 2×10^{-4} M, chloroquine totally suppressed contractile responses to all agents tested: 0.03 μg ml^{-1} $C5a_{desArg}$, 10^{-7} M ACh and 10^{-7} M histamine (data not shown). This effect was, however, completely reversible by repeatedly washing the tissues over a period of 30 min. Chloroquine-treated ileum segments then showed a response to $C5a_{desArg}$ of 0.27 ± 0.15 relative to maximal ACh effects, non-treated controls a mean response of 0.29 ± 0.13 (n = 5).

To test the effects of chloroquine on deactivation, two sets of ileum segments were treated repeatedly with $C5a_{desArg}$, one in the absence, the other in the presence of 2×10^{-4} M chloroquine (Figure 1). The

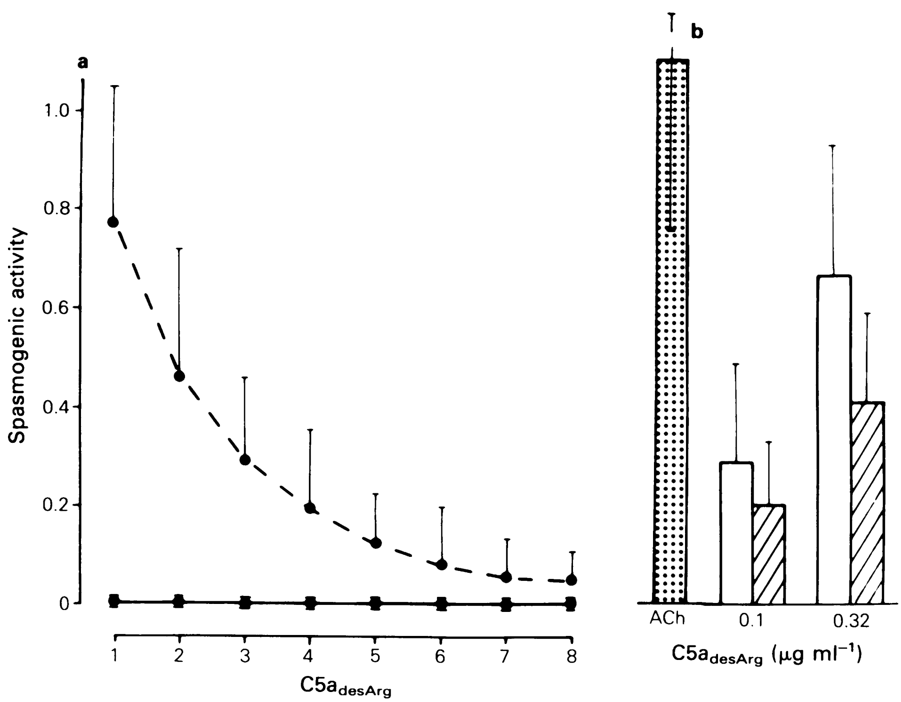

Figure 1 Influence of 2×10^{-4} M chloroquine on the spasmogenic effect of (a) and on the extent of deactivation (b) induced by repeated applications of 0.05 μg ml^{-1} $C5a_{desArg}$ (number of applications on abscissa scale; contact times 60 s). n = 5; mean values with s.d. shown by vertical lines. Ordinate scale: spasmogenic effect of $C5a_{desArg}$ relative to ACh response in the absence (broken line) and presence of chloroquine (solid line). After the initial test series (a) all ileum segments were incubated in fresh Tyrode solution (renewed every 5 min to wash out the drug) at 16°C for 45 min. The organs were then warmed up to 34°C again and after 5 min (with 3 intercalated ACh applications; ACh response of chloroquine-treated strips relative to contractions of controls shown by dotted column) they were tested with 0.1 μg ml^{-1} and 0.32 μg ml^{-1} $C5a_{desArg}$ (b) (normal test rhythm, contact times 30 s). The responses are given by the columns in (b) (hatched columns = chloroquine-pretreated organs; open columns = controls).

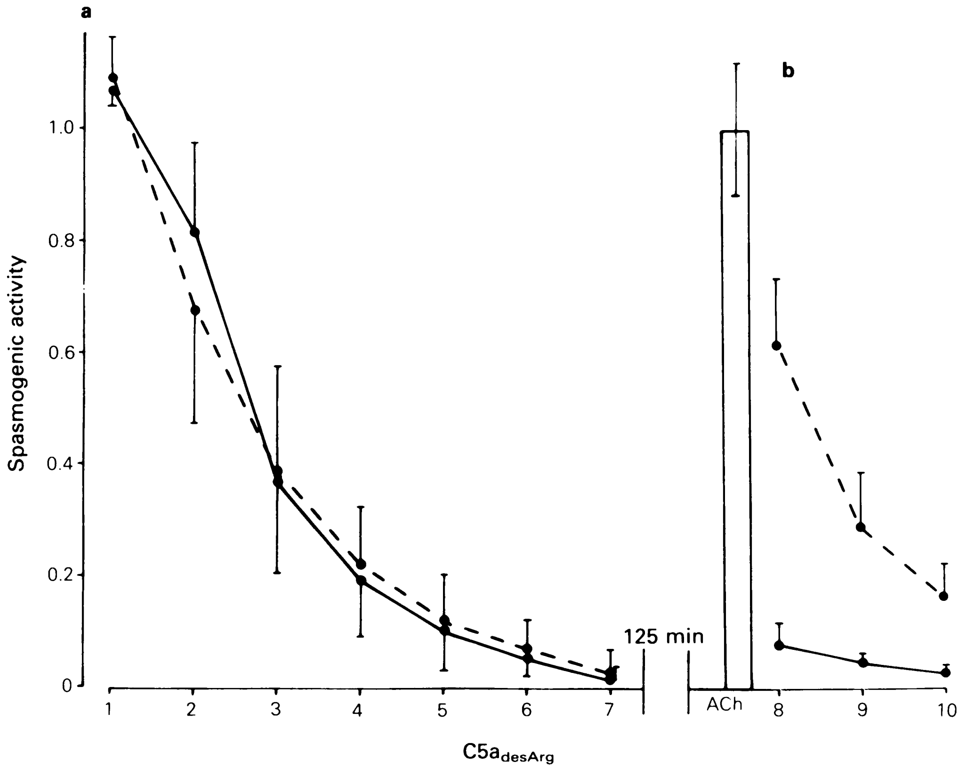

Figure 2 Inhibition by 2×10^{-4} M chloroquine of recovery from slow deactivation. $n = 5$; mean values with s.d. shown by vertical lines. Ordinate scale: spasmogenic effect of $C5a_{desArg}$ relative to ACh response; abscissa scale: number of applications. (a) Course of deactivation induced by 7 subsequent applications of 0.1 $\mu g\, ml^{-1}$ $C5a_{desArg}$. Curves in (a) show the results from pairs of identically treated test tissues, which were afterwards incubated either for 80 min in Tyrode solution alone followed by 10 min in the presence of chloroquine (controls; broken line) or for 90 min in the presence of chloroquine (solid line). All pieces were then incubated in fresh Tyrode solution which was renewed every 5 min at 16°C (to block further recovery) for 30 min. (b) 5 min after warming up to 34°C (and after 3 applications of ACh; ACh response of strips treated with chloroquine for 90 min, relative to contraction of controls, shown by the column) the ileum segments were tested three times with 0.1 $\mu g\, ml^{-1}$ $C5a_{desArg}$.

control tissues gradually lost their sensitivity to the peptide. The organs in chloroquine-containing medium were not able to respond from the beginning. After 8 tests with $C5a_{desArg}$ both sets of tissues were brought to 16°C and washed extensively at this temperature (to remove chloroquine without allowing recovery from deactivation). Then the ilea were rewarmed to 34°C and tested again with $C5a_{desArg}$. The chloroquine-treated ileum segments had lost as much, or even slightly more sensitivity, as the controls (compare hatched and open columns, Figure 1). Hence, although they had never contracted to $C5a_{desArg}$ they became deactivated in the presence of chloroquine.

A clear effect of chloroquine was seen on recovery, as shown by the following test series. Pairs of ileum segments were first deactivated by repeated applications of $C5a_{desArg}$ (0.1 $\mu g\, ml^{-1}$); the almost identical deactivation curves shown in Figure 2a demonstrate the high reproducibility of the tests. After subsequent suspension in Tyrode solution alone, for 90 min at 34°C the test tissues had regained most of their sensitivity to $C5a_{desArg}$ (broken line, Figure 2b). In contrast, those ileum segments which during the 90 min rest period had been incubated with chloroquine (2×10^{-4} M) were only minimally contracted by $C5a_{desArg}$ thereafter (solid line, Figure 2b) but responded normally to ACh (column). Hence, chloroquine inhibits recovery from slow deactivation. The possibly even greater deactivation of chloroquine-treated ileum segments seen in Figure 1 may be due to this effect. In contrast, in 6 experiments 2×10^{-6} M chloroquine did not affect recovery from the fast component of deactivation (data not shown).

(2) Effect of cycloheximide

From the previously published results (Damerau *et al.*, 1985a) we concluded that slow deactivation is probably caused by $C5a_{desArg}$ binding to its specific binding sites/receptors and blocking them. It was hypothesized that recovery could occur by the appearance of newly available receptors on the surface of the target cells either after biosynthesis of new or by recruitment of preformed silent or recycled receptors. Experiments with the protein synthesis inhibitor, cycloheximide, now indicate that recovery does not depend on biosynthesis of new receptors (data not shown). At a concentration of 10^{-3} M cycloheximide had no effect on the spasmogenic effect of $C5a_{desArg}$ (0.05 $\mu g\ ml^{-1}$) or on the course of deactivation upon repeated peptide applications or on recovery (tested after 90 min incubation at 34°C).

(3) Effect of colchicine, vinblastine, lumicolchicine and cytochalasin B

Colchicine inhibits formation of microtubules and, mainly via this mechanism, influences several cell functions such as mitosis, exocytosis, motility and shape changes.

At a concentration of 5×0^{-5} M colchicine reduced the spasmogenic effect of $C5a_{desArg}$ (0.06 $\mu g\ ml^{-1}$) by about 18% (Figure 3a). After washing (20 min, 34°C) control and colchicine-treated organs responded similarly to the next test with $C5a_{desArg}$ given in a three fold higher concentration, whereas deactivation of the colchicine-treated pieces by the following applications was markedly accelerated (Figure 3b). A shift of the deactivation curve similar to that shown in Figure 3a persisted, when the ilea had been pretreated for 60 min at 34°C with 2×10^{-5} M colchicine and were washed subsequently before starting the test with $C5a_{desArg}$ (Figure 4a).

When deactivated ileum segments were incubated with colchicine and then washed, recovery was considerably retarded. These organs responded much less to $C5a_{desArg}$ given 90 min after deactivation than control segments which had been incubated during the recovery period without the drug (Figure 4b). Even when the result of the first test with $C5a_{desArg}$ was corrected for the inhibitory effect of colchicine on its spasmogenic activity, the decrease in the extent

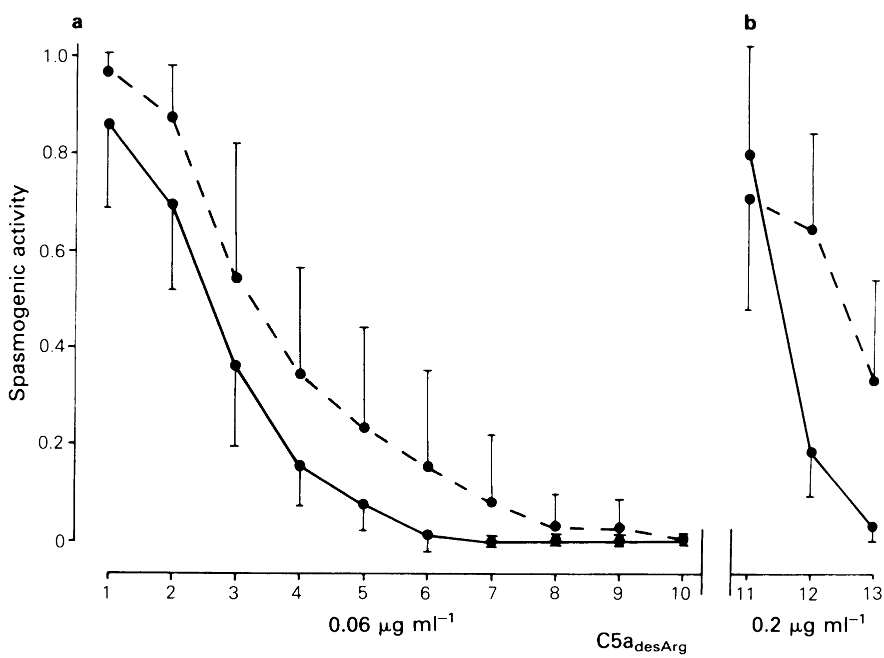

Figure 3 (a) Effect of 5×10^{-5} M colchicine on deactivation induced by 10 subsequent applications of 0.06 $\mu g\ ml^{-1}$ $C5a_{desArg}$ (solid line). Results of control pieces tested in colchicine-free medium (broken line). (b) After 20 min washing at 34°C in Tyrode solution alone the ileum pieces were tested 3 times with 0.2 $\mu g\ ml^{-1}$ $C5a_{desArg}$. Ordinate scale: spasmogenic effect of $C5a_{desArg}$ relative to ACh response; abscissa scale: number of applications (contact time 30 s). $n = 5$; mean values with s.d. shown by vertical lines.

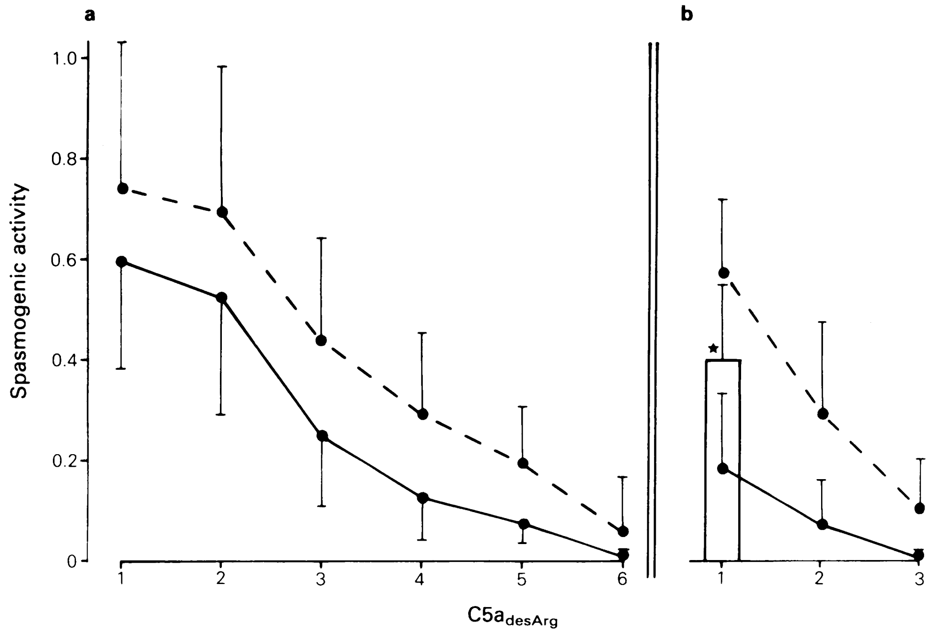

Figure 4 (a) Effect of pretreatment of ileum segments with 5×10^{-5} M colchicine (for 60 min at 34°C, solid line) on the course of deactivation induced by repeated applications of $C5a_{desArg}$ (0.03 $\mu g\ ml^{-1}$, contact times 30 s, number of applications = abscissa scale; tests performed in colchicine-free medium). Results of control pieces which had been preincubated in the absence of colchicine (broken line). Ordinate scale: spasmogenic effect of $C5a_{desArg}$ relative to ACh response. $n = 6$; mean values with s.d. shown by vertical lines. (b) Effect of 5×10^{-5} M colchicine on recovery from slow deactivation. Ileum segments from the same animals as in (a) had been completely deactivated in the absence of colchicine by 9 subsequent applications of 0.04 $\mu g\ ml^{-1}$ $C5a_{desArg}$ (normal test rhythm, contact times 30 s; data not shown). They were then incubated for 60 min at 34°C in the absence (broken line) or presence of colchicine (solid line) and thereafter tested in colchicine-free medium with the same concentration of $C5a_{desArg}$ (number of applications = abscissa). Column: response of colchicine-pretreated tissues to the 1st application of $C5a_{desArg}$ corrected for inhibitory effect of the drug shown in (a).

of recovery is statistically significant ($P < 0.025$; column in Figure 4b).

Vinblastine, another inhibitor of microtubule formation, also reduced recovery considerably, usually by about 30% (Table 1). In contrast, lumicolchicine, which is a u.v.-treated, inactive derivative of colchicine, did not affect it (Table 1). Therefore, inhibition of recovery by colchicine and vinblastine seems to be due to their anti-microtubule properties.

Cytochalasin B (2×10^{-5} M), which disturbs the integrity of microfilaments, did not change the course of deactivation (results not shown) nor did it affect recovery (Table 1). Hence, microfilaments seem not to be involved in deactivation and recovery.

Discussion

Of the drugs used to analyse further the slow component of deactivation, chloroquine, colchicine and vinblastine had definite effects. One of the drugs, chloroquine, unspecifically blocked spasmogenic responses to $C5a_{desArg}$ and other motor stimulants. This effect depended, however, on the presence of the drug and could be fully reversed by intensive washing at low temperature, e.g. under conditions which did not allow recovery from deactivation during the washing time. Therefore, the effect of chloroquine on deactivation and recovery could be studied after this manipulation. Chloroquine at 2×10^{-4} M did not significantly modify the extent of deactivation, but it totally blocked recovery from slow deactivation. Both findings became manifest after chloroquine had been washed out. Chloroquine is known to accumulate in lysosomes (de Duve *et al.*, 1974) and to inhibit intralysosomal proteolysis (Wibo & Poole, 1974; Mego & Chung, 1979). It thereby blocks degradation of the ligand/dissociation of ligand receptor complexes (King *et al.*, 1980; Ciechanover *et al.*, 1983). If chloroquine is acting in a similar manner in the

Table 1 Influence of vinblastine, lumicolchicine and cytochalasin B (CB) on recovery from slow deactivation

Substances present in the medium	*Relative spasmogenic activity*	*Spasmogenic effect as % of controls*	n
None	0.40 ± 0.12		
Vinblastine (2×10^{-5} M)	0.27 ± 0.13	69 ± 29	5
None	0.67 ± 0.20		
Lumicolchicine (2×10^{-5} M)	0.65 ± 0.26	99 ± 40	5
None	0.54 ± 0.25		
Cytochalasin B (2×10^{-5} M)	0.50 ± 0.37	97 ± 59	6

Procedure: after complete deactivation by 6–10 subsequent $C5a_{desArg}$ applications (0.05 or 0.06 $\mu g\ ml^{-1}$; number of applications constant in each test series) the ileum segments were incubated either for 80 min at 34°C in fresh Tyrode solution (controls) or for 60 min in the presence of the test compound and thereafter for 20 min in fresh Tyrode solution. In the experiment with CB the corresponding times were: 70 min incubation in the presence of CB or its solvent, 0.1% DMSO (controls), followed by 20 min in fresh Tyrode solution. Then the organs were tested for recovery with $C5a_{desArg}$ in the same concentrations used before. Effects are given as relative spasmogenic activity (2nd column), and related to the results of the respective control experiments (3rd column).

guinea-pig ileum, it is possible that the complexes of $C5a_{desArg}$ and its receptors are also internalized and are processed in the lysosomes of the target cells as has been demonstrated to occur in macrophages and leukocytes (Chenoweth *et al.*, 1982; Chenoweth & Goodman, 1983).

As chloroquine is not a specific inhibitor for lysosomal activities other actions have to be considered, too. The first step of the action of $C5a_{desArg}$, namely ligand binding seems not to be affected by chloroquine, because deactivation was not prevented. The next step, endocytosis of $C5a_{desArg}$-receptor complexes, might be inhibited by the drug; such an effect has been shown in receptor-mediated pinocytosis of glycosylated lysosomal enzymes in fibroblasts (Gonzalez-Noriega *et al.*, 1980) but it was not detected in the uptake of epidermal growth factor or of transferrin (King *et al.*, 1980; Ciechanover *et al.*, 1983). The results in Figure 2, however, make inhibition by chloroquine of uptake of $C5a_{desArg}$-receptor complexes improbable: in these experiments, the ileum segments were gradually deactivated during a time period (about 25 min) sufficiently long to allow completion of receptor-mediated uptake, before chloroquine was added (from several other cell models it is known that such uptake is finished after a few minutes; for review see Szego & Pietras, 1984) and still, recovery was blocked.

Unspecific inhibition of the smooth muscle cells' responsiveness persisting even after washing can be refuted since the inhibitory effect of chloroquine on the spasmogenic action of ACh, histamine and $C5a_{desArg}$ could be washed out completely. This is in accordance with the findings of Famaey *et al.* (1977). Furthermore, other lysosome-independent actions of chloroquine and other antimalarials such as changes in membrane properties, prostaglandin biosynthesis or replacement of Ca^{2+} from phospholipid bilayers (Lüllman *et al.*, 1980; Dise *et al.*, 1982; Tauber & Simons, 1983) are unlikely to be causally involved in the effects on the ileum, as extralysosomal effects are essentially removed by washing whereas chloroquine is trapped in the lysosomes and can act there for longer periods of time (Manku & Horrobin, 1976; Famaey *et al.*, 1977; Ohkuma & Poole, 1978; Gonzalez-Noriega *et al.*, 1980).

As recovery from C5a deactivation is not affected by cycloheximide, it is not due to biosynthesis of new C5a receptors but rather to re-expression of existing receptors. Clearly there are differences in receptor recycling in different models. Cycloheximide has no influence on recycling of receptors for *N*-formylated oligopeptides in rabbit neutrophils (Sullivan & Zigmond, 1980) but inhibits re-expression of C5a receptors in human neutrophils by 60% (Chenoweth & Goodman, 1983).

Microfilaments seem not to participate in slow deactivation because cytochalasin B, which blocks actin filament elongation (Flanagan & Lin, 1980), did not modulate the effect of $C5a_{desArg}$, the course of deactivation or recovery.

Both colchicine and vinblastine markedly reduced recovery. The effect of both agents is probably specifically mediated by interactions with microtubules, since lumicolchicine which lacks antimicrotubule activity was without effect. The exact mechanism of their effect on recovery is not yet known. It may be that colchicine and vinblastine inhibit early steps of receptor processing, as intracellular degradation of C5a in neutrophils is inhibited by vinblastine (Chenoweth & Goodman, 1983). Furthermore, it is possible that they disturb receptor re-expression, as has been suggested for the effect of colchicine on the processing of insulin receptors (Whittaker *et al.*, 1981).

Colchicine (at 5×10^{-5} M) suppressed recovery and reduced the spasmogenic activity of $C5a_{desArg}$ but did not change the rate of deactivation. The reduction of spasmogenic activity is possibly due to inhibition of histamine release which has been demonstrated previously in rat peritoneal mast cells by Grant *et al.* (1977) and by Hook & Siraganian (1977). An unspecific decrease of contractility is unlikely, as colchicine did not affect the spasmogenic effect of ACh and histamine.

In conclusion, it appears that slow deactivation is not affected by intracellular reactions involving lysosomes and the cytoskeleton, but is a consequence of ligand binding and subsequent blockade of the receptors. Recovery after slow deactivation seems to involve re-expression of regenerated receptors, probably after intralysosomal processing of receptor-ligand complexes.

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