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Fast deactivation of guinea-pig isolated ileum to $C5a_{desArg}$: a possible cyclicAMP-dependent mechanism

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1 The fast component of deactivation of guinea-pig isolated ileum to the spasmogenic action of the complement peptide $C5a_{desArg}$ was further differentiated from the slow component which had been previously analysed (Damerau *et al.*, 1985a,b).

2 Fast deactivation differs from the slow component in the following characteristics: (a) it is unspecific in that it is also induced by C3a, another complement peptide, (b) it depends on the spasmogenic effect of the peptides, and (c) it does not occur at 16°C.

3 In contrast to the slow component, in which the deactivation is thought to be caused by blockade of C5a receptors, fast deactivation seems to be due to a transient increase of intracellular cyclicAMP evoked by $C5a_{desArg}$ and C3a; it is prevented by $GDP_{\beta}S$ (5×10^{-4} M) which blocks activation of adenylate cyclase, and prolonged by agents which sustain cyclic AMP elevations, namely 5×10^{-4} M theophylline and 5×10^{-4} M $GTP_{\gamma}S$.

Introduction

In the course of repeated applications, the complement peptides C3a and $C5a_{desArg}$ induce a progressive loss of spasmogenic response in guinea-pig isolated ileum strips. This deactivation/tachyphylaxis has been supposed to be uniform and to be specific for C3 and C5 peptides (for review see Vogt, 1974; Hugli & Müller-Eberhard, 1978), as cross-deactivation has not been observed. However, when we recently analysed various parameters of deactivation of guinea-pig ileum segments by hog $C5a_{desArg}$ it became apparent that it consisted of two components, one fast and one slow. The latter is identical with the well-known specific tachyphylaxis or deactivation (Damerau *et al.*, 1985a). As in leukocytes (Chenoweth & Hugli, 1978), slow deactivation seems to be due mainly to occupation and subsequent blockade and/or loss of the specific receptors for $C5a_{desArg}$. After slow deactivation, the ileum gradually recovers in the course of about 80 min, probably by recycling of the receptors (Damerau *et al.*, 1985a,b).

In contrast to the slow component, there is recovery from fast deactivation within a few minutes. This is reminiscent of similar reactions to other agents such as epidermal growth factor, sympathomimetics and insulin (for review see Lefkowitz *et al.*, 1980). We have studied fast deactivation to $C5a_{desArg}$ in the guinea-pig isolated ileum, its stimulus-specificity, time course and the influence of drugs which affect the intracellular cyclic AMP level.

Methods

Determination of spasmogenic activity

Guinea-pig ileum segments (about 2.5 cm in length, weight of animals 300–400 g) 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 agents studied, oxygenated with a mixture of 95% O_2 and 5% CO_2. After 30 min incubation acetylcholine (ACh) was given 5 to 10 times to achieve constant reactivity. When not otherwise stated (see legends to figures), $C5a_{desArg}$ 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 30 s (contact time of ACh was 15 s) = zero time of the next cycle.

Effects of $C5a_{desArg}$ and histamine are usually given in relation to the effect of a supramaximal ACh concentration, which was taken as 1.0.

Substances

$GDP_{\beta}S$ (guanosine-5'-O-(2-thiodiphosphate) trilithium salt) and $GTP_{\gamma}S$ (guanosine-5'-O-(3-thiotriphosphate) tetralithium salt) were purchased from Boehringer (Mannheim, F.R.G.), theophylline

from Merck (Darmstadt, F.R.G.). The complement peptides C3a and $C5a_{desArg}$ were generated by yeast-activation of hog serum (C3a in the presence of 1 M epsilon aminocaproic acid, according to the method of Vallota & Müller-Eberhard, 1973) and purified as described by Zimmermann *et al.* (1980).

Results

(1) *Fast deactivation is rapidly reversible*

In a previous paper we showed that the progress of deactivation depends on the time interval between subsequent applications of $C5a_{desArg}$ (Damerau *et al.*, 1985a). When the peptide was applied at intervals of 15 s, ileum strips had completely failed to respond, by the third challenge. When given at longer intervals, 135 s and 360 s, deactivation proceeded much more slowly, being complete only after the 7th and 10th application of $C5a_{desArg}$, respectively. These results gave the first hint that, in addition to the slow deactivation, $C5a_{desArg}$ causes a further component of deactivation which is characterized by rapid onset and disappearance within a few minutes. However, as this observation alone did not prove the assumed fast component, we looked for additional characteristics.

(2) *Dependence on temperature of recovery from fast deactivation*

In this series, ileum strips were first stimulated with a moderately supramaximal concentration of $C5a_{desArg}$ (0.1 $\mu g\,ml^{-1}$) for 30 s to achieve substantial deactivation. Thereafter, one group of test organs was kept at 16°C for 20 min. These strips were then quickly warmed up to 34°C again, for the next test; upon subsequent exposure to a smaller dose of $C5a_{desArg}$

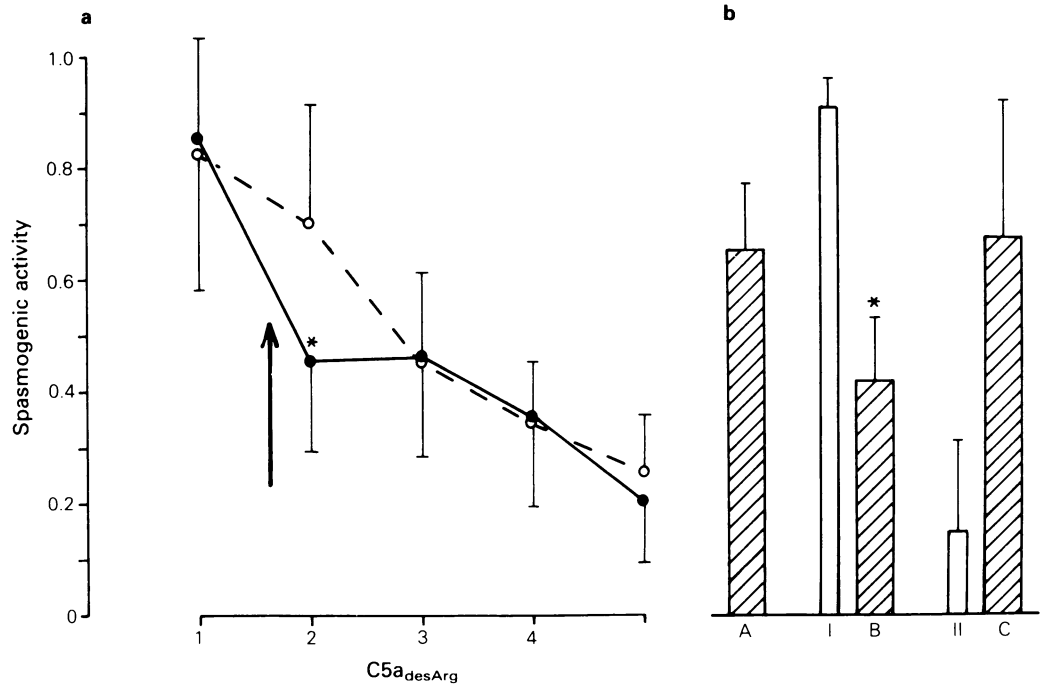

Figure 1 (a) Induction of fast deactivation to $C5a_{desArg}$ by C3a ($n = 9$, mean values with s.d. shown by vertical lines). Broken lines shows responses of ileum segments treated with 0.02 $\mu g\,ml^{-1}$ $C5a_{desArg}$ repeatedly (number of applications, abscissa scale) in the usual test rhythm, i.e. with two intercalated doses of ACh. Solid line: instead of the 2nd dose of ACh, C3a (0.32 $\mu g\,ml^{-1}$, causing maximal response, arrow) was applied 75 s before $C5a_{desArg}$. Ordinate scale: spasmogenic effect of $C5a_{desArg}$ relative to ACH response. *Significantly decreased response ($P < 0.001$). (b) Dependence of fast deactivation on the spasmogenic effect (test procedure the same as in (a); $n = 5$). Hatched columns, responses to $C5a_{desArg}$: (A) without previous application of C3a, (B) given 75 s after a bolus injection of C3a (contact time 15 s, open column (I) = effect of C3a), (C) given 45 s after a continuous infusion of C3a (lasting 30 s, C3a washed out after 40 s, effect shown in (II). *Statistically significant decrease when compared with (A) $P < 0.001$, and compared with (C) $P < 0.05$.

($0.05\ \mu g\ ml^{-1}$) one minute later, they showed marked loss of contractile response due to 'fast' deactivation (0.19 ± 0.04 spasmogenic effect relative to maximal ACh activity; $n = 6$) which was hence not reversed at the low temperature. Recovery then followed during a further interval of 4 min at 34°C, and correspondingly the next dose of $C5a_{desArg}$ gave a larger response. Control organs which were brought intermittently to 34°C for 5 min during the 20 min rest period at 16°C after the first deactivating dose, did not show such a severe reduction of contractile force (0.50 ± 0.08 spasmogenic effect relative to ACh response; $n = 6$); they had apparently recovered during the 5 min. Thus recovery from fast deactivation requires elevated temperatures and is inhibited at a lower temperature.

(3) *Lack of specificity and effect-dependence of fast deactivation*

In contrast to the slow deactivation, which is induced by contact with the specific agonist itself, fast deactivation of guinea-pig ilea to $C5a_{desArg}$ is not as specific. When contracted by another anaphylatoxin, C3a, the ileum strips also gave a reduced response to a subsequent dose of $C5a_{desArg}$ provided it was applied not later than about 3 min after the C3a injection (Figure 1a). On the other hand, the effect is not entirely unspecific: (1) if the organs were stimulated by ACh instead of C3a between two tests with $C5a_{desArg}$, the decline in responses followed the normal, regular progress of slow deactivation only; (2) after a conditioning maximally contracting dose of C3a, histamine became fully active again in 60 s (Figure 2).

Again unlike slow deactivation, the fast process is not induced simply by contact with C3a or $C5a_{desArg}$ but becomes apparent only if they have evoked a contraction. As seen from Figure 1b, C3a failed to cause deactivation to $C5a_{desArg}$ when it was slowly infused into the bath, preventing a marked response. Similarly when $C5a_{desArg}$ was applied as the conditioning agent at 16°C, and hence did not cause a contraction, no fast reversible deactivation was seen during the next test with $C5a_{desArg}$ at 34°C (data not shown).

Since C3a does not cause any slow (specific) deactivation towards $C5a_{desArg}$ it can be used—more effectively than $C5a_{desArg}$ itself—to show the time course of recovery from fast deactivation. As demonstrated in Figure 2, fast deactivation is overcome completely after about 300 s.

(4) *Effect of theophylline and $GTP_\gamma S$*

The lack of specificity indicated that fast deactivation is not caused by blocking of C5a receptors (the latter being probably the main cause of slow deactivation;

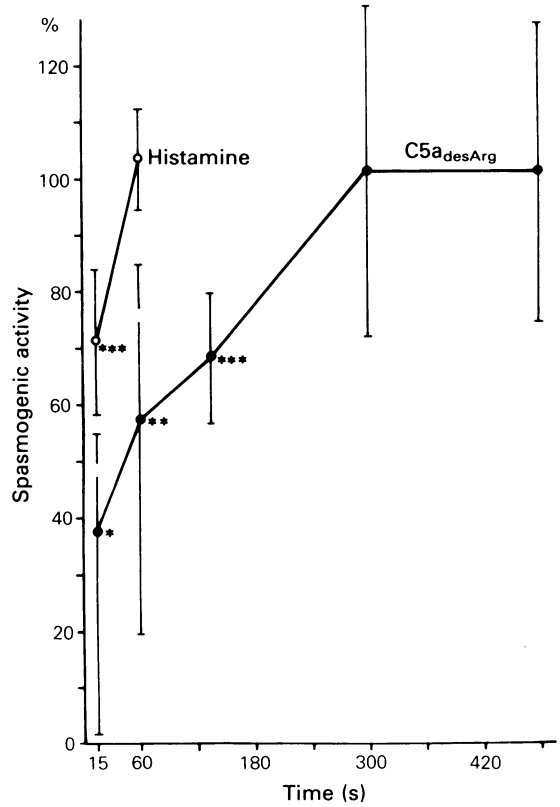

Figure 2 Time course of recovery from fast deactivation to $C5a_{desArg}$ induced by a single dose of C3a ($n = 6$, mean values with s.d. shown by vertical lines). Ordinate scale: spasmogenic effect of $C5a_{desArg}$ (●) and histamine (○), given as % of the response of control segments which were stimulated with ACh (10^{-7} M, contact time 15 s) instead of C3a ($0.32\ \mu g\ ml^{-1}$, contact time 15 s) before application of $C5a_{desArg}$ or histamine. Abscissa scale: time interval (in s) between washing out of C3a or ACh and application of $C5a_{desArg}$ ($0.02\ \mu g\ ml^{-1}$) or histamine (10^{-8} M). When possible, intercalated doses of ACh were given in the normal test rhythm. Asterisks indicate statistically significant inhibition by C3a; $^*P < 0.005$, $^{**}P < 0.005$ (9 experiments), $^{***}P < 0.001$.

Damerau *et al.*, 1985a), as these do not interact with C3a. The dependence on temperature and effect pointed to biochemical processes accompanying cell activation, and it seemed reasonable to investigate the role of cyclic AMP, as its intracellular level is transiently (for about 300 s) increased in leukocytes and mast cells after stimulation. The latter cells are involved in the spasmogenic action of $C5a_{desArg}$ on the guinea-pig ileum (Sorgenfrei *et al.*, 1982). Therefore, the effect of theophylline and $GTP_\gamma S$ was inves-

tigated; both agents induce an increase in cyclic AMP, theophylline by inhibiting phosphodiesterase and $GTP_\gamma S$ by maintaining activation of adenylate cyclase (Birnbaumer *et al.*, 1980).

Neither theophylline (2×10^{-4} M) nor $GTP_\gamma S$ (5×10^{-4} M) affected the first response to $C5a_{desArg}$ but considerably enhanced the progress of deactivation by subsequent applications (shown for theophylline in Figure 3a). This effect could be caused by an enhancement of the fast deactivation and/or by an inhibition of its reversal. The first possibility was excluded by the experiments shown in Figure 3b: immediately after the first response to $C5a_{desArg}$ (in the presence or in the absence of $GTP_\gamma S$) the ileum segments were cooled to 16°C for 10 min to allow sufficient effects of $GTP_\gamma S$ on the respective tissues without reversing fast deactivation. They were then rewarmed to 34°C, thereafter both control and $GTP_\gamma S$-treated organs showed a similarly decreased second response to $C5a_{desArg}$.

About 5 min later the control tissues had markedly recovered to the third challenge with $C5a_{desArg}$, whereas those treated with $GTP_\gamma S$ had not (Figure 3b). These results show that $GTP_\gamma S$ does not enhance fast deactivation but that it inhibits recovery therefrom. The same result were obtained with theophylline (5×10^{-4} M; $n = 6$, data not shown).

(5) *Effect of $GDP_\beta S$*

The sulphur-substituted GDP analogue $GDP_\beta S$ binds to the regulatory unit of the adenylate cyclase complex and inhibits stimulation of the enzyme (Eckstein *et al.*, 1979). One might, therefore, expect

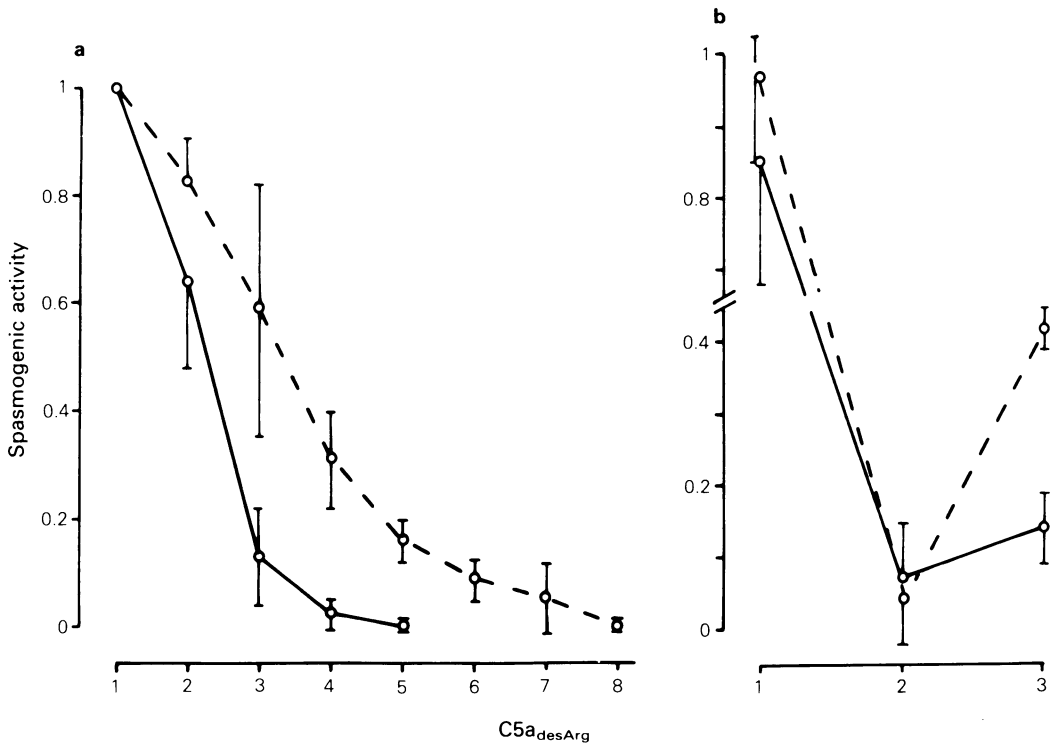

Figure 3 (a) Theophylline (2×10^{-4} M) accelerates the progress of deactivation (solid line) on repeated stimulations with $C5a_{desArg}$ (0.05 μg ml^{-1}; ordinate scale: spasmogenic effect relative to the first application of $C5a_{desArg}$; abscissa scale: number of applications). Control tests in the absence of theophylline (broken line). The time intervals between $C5a_{desArg}$ doses were 240 s. $n = 5$, mean values with s.d. shown by vertical lines. (b) Inhibition of recovery from fast deactivation by 5×10^{-4}M $GTP_\gamma S$ (n = 5–6; abscissa scale: number of applications of $C5a_{desArg}$; ordinate scale: spasmogenic effect relative to ACh response). Ileum strips were first stimulated at 34°C with $C5a_{desArg}$ (0.08 μg ml^{-1}) inducing maximal contraction (first response); they were then immediately washed, incubated at 16°C for 10 min, then rewarmed to 34°C and tested with 2 further doses of $C5a_{desArg}$. Control strips, incubated in drug-free medium (broken line), strips treated with $GTP_\gamma S$ added 3 min before the first test with $C5a_{desArg}$ and kept present throughout the experiment (solid line).

effects opposite to those of theophylline and $GTP_\gamma S$. Indeed, when present during stimulation with $C5a_{desArg}$, 5×10^{-4} M $GDP_\beta S$ suppressed fast deactivation completely (Figure 4; after the first response to $C5a_{desArg}$ the ileum segments were cooled and washed for 10 min at 16°C to remove $GDP_\beta S$ completely from the tissue without allowing recovery from the fast component).

Discussion

It has previously been shown that the well-known tachyphylaxis of smooth muscle organs to stimulation by peptides such as $C5a_{desArg}$ is reversed with time (Friedberg *et al.*, 1964; Bodammer & Vogt, 1970). In a preceding paper we demonstrated that the tachyphylactic phenomenon consisted of two different deactivation processes characterized by special time courses of onset and of recovery (Damerau *et al.*, 1985a). One might argue that this is insufficient for the postulation of two processes, as recovery from a single process of deactivation could proceed asymptotically, beginning fast and then following a slower time course. This argument can be refuted. The two different components can be clearly differentiated. In contrast to the slow component the fast one (1) is not as specific (since it is also induced by C3a), (2) is not simply caused by contact of the target with the agonist but occurs only when the latter has evoked a contraction, and (3) does not occur at 16°C. These characteristics indicate that it is not due to the blockade of C5a receptors which is thought to be the main cause of the slow component (Damerau *et al.*, 1985a). Also it does not just represent an unspecific loss of the contractility of the smooth muscle cells since ACh still produces a normal response. Furthermore, it is unlikely to be caused by the secondary mediator of $C5a_{desArg}$, histamine. High concentrations of endogenous histamine can indeed transiently desensitize smooth muscle cells or inhibit further histamine release from mast cells by interacting with H_2-receptors. The latter mechanism has been proposed to exist in human basophils (Lichtenstein & Gillespie, 1973). However, both mechanisms are unlikely to induce deactivation in our model as the reaction to exogenous histamine was only slightly reduced for about 15 s and had fully recovered by 60 s; the H_2-receptor blocker ranitidine (2×10^{-4} M, $n = 11$, data not shown) which should block the negative feedback inhibition did not decrease the degree of fast deactivation.

The characteristics indicate that post-receptor events are involved in the fast deactivation process and the temperature sensitivity points to the participation of biochemical processes. The experimental evidence suggests that the fast component is a cyclic AMP-dependent reaction stimulated by the complement peptides as it is aggravated and/or prolonged by agents which lead to an increase in intracellular cyclic AMP (theophylline, $GTP_\gamma S$) and it is suppressed by $GDP_\beta S$ which blocks stimulation of adenylate cyclase. Unfortunately, these functional findings could not be supplemented by direct measurements of cyclicAMP levels, since the subserosal mast cells which

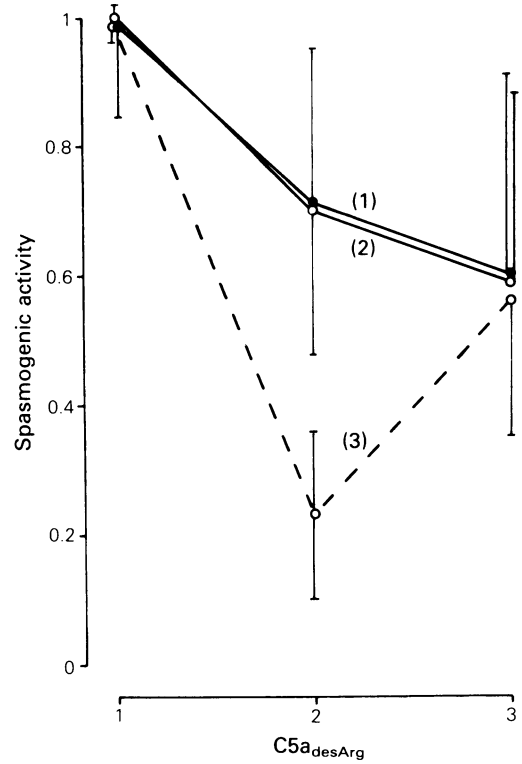

Figure 4 Suppression of fast deactivation by $GDP_\beta S$ ($n = 4$, mean values with s.d. shown by vertical lines). Ordinate scale: spasmogenic effects, relative to ACh response, of 3 successive tests (abscissa scale) with 0.05 μg ml^{-1} $C5a_{desArg}$. Curve (1): 3 min before the first test 5×10^{-4} M $GDP_\beta S$ was added to the bath and maintained through the experiment. After the first test and washing, the organs were kept at 16°C for 10 min and were then warmed up to 34°C. One minute later the 2nd test was performed and after 210 s at 34°C the third one. Curve (3): same procedure as in 1 but $GDP_\beta S$ omitted. Marked reduction of contractile response due to fast deactivation with fast recovery; no comparable deactivation in curve 1. Curve (2): same test procedure as in curve (3), but the bath temperature after the first test was kept at 34°C for 3 min, so that deactivation was reversed.

are major targets for the action of $C5a_{desArg}$ amount to less than 1% of the total cell mass of the ileum.

The rapid recovery from the fast component (for recovery, half-times of 40–60 s can be extrapolated from the curve in Figure 2) indicates that cyclic AMP levels of the target cells of $C5a_{desArg}$ are increased transiently, their decline correlating with the fading of deactivation. Similar changes in intracellular cyclic AMP have been observed in mast cells and granulocytes (Jackowski & Sha'afi, 1979; Simchowitz *et al.*, 1980; Smolen *et al.*, 1980; Ishizaka *et al.*, 1983) and in other cells, and with different agents these changes were shown to induce a transient hyporesponsiveness as in our model (for review see Lefkowitz *et al.*, 1980). As inhibition of adenylate cyclase by $GDP_{\beta}S$ abolished the fast component but did not affect the spasmogenic action of $C5a_{desArg}$ proper, cyclic AMP is most probably not required (as a second messenger) for signal transfer and activation of the target cells after challenge with $C5a_{desArg}$. Similar conclusions have been drawn about the role of cyclic AMP in granulocyte activation (Smolen *et al.*, 1980; Smolen & Weissmann, 1981).

Finally, fast deactivation in guinea-pig ileum appears to be a further example of cyclic AMP-dependent 'heterologous desensitization' (according to Lefkowitz *et al.*, 1980), because it is also induced by another complement peptide, C3a, which acts via specific receptors different from those for $C5a_{desArg}$. However, the heterologous character of the fast component is limited: ACh and histamine do not induce it. The most plausible explanation for this restriction is that the complement peptides act primarily on different targets from those affected by ACh and histamine.

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