The effect of low-density lipoproteins on the synthesis of cyclic nucleotides induced by prostacyclin in isolated platelets

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Isolated platelets are strongly sensitized by the presence of low-density lipoproteins (LDL) so that they aggregate with very low concentrations of other agonists or exhibit spontaneous aggregation. Prostacyclin (PGI₂) is a potent inhibitor of aggregation, but its action was reversed by LDL. This effect of LDL was accompanied by a decrease in the synthesis of cyclic AMP induced by PGI₂, but its efficacy depended on the relative concentrations of LDL and PGI₂. PGI₂ also enhanced the synthesis of cyclic GMP, but this was completely reversed by the presence of LDL. LDL did not remove inhibitory prostaglandins, e.g. E₁, from their receptor sites. The lipoproteins also decreased cyclic AMP synthesis induced by forskolin, which has its effect on the GTP-sensitive protein or the catalytic unit of the adenylate cyclase enzyme complex. It is proposed that LDL may act on the enzyme catalytic unit via an inhibitory GTP-sensitive protein or by a separate mechanism which indirectly impedes the production of cyclic AMP.

Plasma lipoproteins, particularly the low-density fraction, have been shown to enhance the sensitivity of platelet-rich plasma to some agonists (Farbyszewski *et al.*, 1969; Hassall *et al.*, 1983a). A similar effect of LDL was observed in platelets isolated by gel filtration when LDL were present in the medium (Koller *et al.*, 1979). When platelets are isolated in the presence of PGI₂ (Vargas *et al.*, 1982), they retain a greater sensitivity to all agonists after the PGI₂ has degraded. Platelets isolated by this technique were 1000-fold more sensitive to adrenaline and other agonists after brief incubations with LDL, which also induced spontaneous aggregation at high concentrations (Hassall *et al.*, 1983b). It was also observed that LDL reversed the inhibition of platelet activity by PGI₂, a very potent platelet inhibitor. PGI₂ is known to inhibit platelet activity by elevating the intracellular concentrations of cyclic AMP (Tate-son *et al.*, 1977; Gorman *et al.*, 1977). Receptors exist on the surface of the platelet for both LDL (Koller *et al.*, 1982) and PGI₂ (Siegl *et al.*, 1979); the latter are considered to activate the membrane-bound adenylate cyclase enzyme complex. It was

the purpose of the present study to investigate the interrelationships between lipoprotein and PGI₂ and their effects on the synthesis of cyclic nucleotides in platelets.

Materials and methods

Materials

PGE₁, PGD₂, cyclic AMP, N⁶,O^{2'}-dibutyryl-adenosine 3',5'-cyclic monophosphate (dibutyryl cyclic AMP), N²,O^{2'}-dibutyrylguanosine 3',5'-cyclic monophosphate (dibutyryl cyclic GMP) and 3-isobutyl-1-methylxanthine were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Forskolin (7 β -acetoxy-8,13-epoxy-1 α ,6 β ,9 α -tri-hydroxylabd-14-en-11-one) was obtained from Calbiochem-Behring, San Diego, CA, U.S.A., and [5,6(n)-³H]PGE₁ from Amersham International, Amersham, Bucks, U.K.

Isolation of platelets and lipoproteins

Platelets were isolated in the presence of PGI₂ as described previously (Hassall *et al.*, 1983b). In some cases a similar procedure was adopted, i.e. centrifugation and chromatography on Sepharose 2B (Pharmacia, Uppsala, Sweden), except that dibutyryl cyclic AMP (final concn. 1 mM) was substituted for PGI₂ as an inhibitor during isolation.

Abbreviations used: LDL, low-density lipoproteins; PGI₂, prostacyclin; PGE₁, prostaglandin E₁; PGD₂, prostaglandin D₂.

The activity of the platelets recovered after 2 h. Platelets were also isolated without an inhibitor, but at pH 6.5 to decrease activation, as described by Lagarde *et al.* (1980). The pH was restored to 7.4 in Tyrode's buffer after isolation, but in general these platelets were less responsive to agonists, particularly to adrenaline, than were platelets isolated by the other procedures.

Platelet-aggregation tests and the isolation of LDL in Tyrode's buffer, pH 7.4, have been described previously (Hassall *et al.*, 1983b).

Incubations of platelets and lipoproteins

Platelet suspensions (4×10^{11} /l) were incubated with an equal volume of Tyrode's buffer, pH 7.4, containing 0–4 g of LDL protein/l at 20°C for 0–10 min. The incubation medium contained 0.25 mM-3-isobutyl-1-methylxanthine as a phosphodiesterase inhibitor and 0–300 nM-PGI₂, which was added either 1 min before or 5 min after LDL. In some incubations 20 μ M-forskolin was added in place of PGI₂. Each incubation was stopped by addition of HClO₄ (final concn. 0.5 M) to denature and precipitate the protein and to release intracellular cyclic nucleotides. The supernatant was neutralized with K₃PO₄, and samples were used for the assay of cyclic AMP and cyclic GMP (5 μ l and 50 μ l respectively).

Assay of cyclic AMP and cyclic GMP

Measurements of cyclic nucleotide concentrations were performed by radioimmunoassay techniques devised by Steiner *et al.* (1972) and modified by Harper & Brooker (1975). Antisera raised to the albumin-succinyltyrosine methyl esters of the nucleotides were kindly supplied by Dr. M. Schumacher, Department of Experimental and Clinical Endocrinology, University of Hamburg, Germany.

Cross-reactivity tests were carried out for other adenine and guanine nucleotides. The assay for cyclic AMP was unaffected by cyclic GMP, ATP, ADP or AMP over a wide range of concentrations; only adenosine showed some cross-reactivity at high concentrations (above 0.1 mM). The cyclic GMP assay was unaffected by high concentrations of cyclic AMP or other adenine nucleotides. Only guanosine or GMP showed appreciable cross-reactivity, but only at high concentrations (10 μ M in both cases). The influence of LDL on the radioimmunoassays was also assessed because of the turbidity produced after precipitation of protein by HClO₄; LDL did not interfere with the assay of cyclic AMP at any of the concentrations tested. At concentrations above 2 g of LDL protein/l (the maximum used in the experiments), some interference in cyclic GMP assays occurred.

Binding studies with prostaglandin E₁

Isolated platelets (0.5 ml; 4×10^{11} /l) were incubated at 25°C for 30 min with a range of concentrations of [³H]PGE₁ (sp. radioactivity 54 Ci/mmol). After these incubations, 0.5 ml of Tyrode's buffer, pH 7.4, or Tyrode's buffer containing LDL (final concn. 2 g of LDL protein/l) was added, and the platelets were incubated for a further 5–10 min.

At the end of the incubation, 3 ml of ice-cold platelet wash buffer, pH 7.6 (Gaintner *et al.*, 1962), was added to each tube and the contents were rapidly filtered through a Whatman GF/C glass-microfibre filter under reduced pressure (Schafer *et al.*, 1979). Each assay tube and filter was then washed successively with 4 $\times$ 5 ml of ice-cold platelet wash buffer, pH 7.6. Filters were then resuspended in 5 ml of Scinttran cocktail T liquid-scintillation mixture (BDH, Poole, Dorset, U.K.) and counted for radioactivity in a Packard Tri-Carb 3255 scintillation counter with an overall efficiency of 65%. The amount of bound prostaglandin E₁ was calculated from the known specific radioactivity and M_r . Separate tubes containing PGE₁-labelled platelets with either buffer alone or LDL in buffer were included in each experiment to determine 'non-specific binding' by the addition of 10 μ M non-radioactive PGE₁. These values were subtracted from those for total binding to yield 'specific binding'.

Results

Effects of LDL on prostaglandin-induced inhibition of platelet aggregation

PGI₂, PGE₁ and PGD₂ inhibited the aggregation of isolated platelets induced by adrenaline (5 μ M), but a lower dose of PGI₂ was required than for the other prostaglandins (Fig. 1). The presence of LDL in the medium brought about a shift to the right in the dose-response curve for inhibition of aggregation by PGI₂ (Fig. 1a). This effect of LDL was greatest at a concentration of 2 g of protein/l, which caused a maximal sensitization of isolated platelets to other agonists, including adrenaline (Hassall *et al.*, 1983b). This means that LDL reverses the inhibition of aggregation by PGI₂, except at high concentrations of the prostaglandin. Similarly, LDL reversed the inhibition by PGE₁ and PGD₂, but again not at higher prostaglandin concentrations (Figs. 1b and 1c).

Cyclic AMP synthesis in the presence of PGI₂ and LDL

Since PGI₂ stimulates adenylate cyclase activity, it was first necessary to show that the basal concentration of cyclic AMP in platelets prepared in the presence of PGI₂ is similar to that in platelets not exposed to PGI₂. In the absence of

Fig. 1. Effect of LDL on the inhibition of platelet aggregation induced by PGI_2 , PGE_1 and PGD_2

Platelets ($4 \times 10^{11}/\text{l}$) were isolated in the presence of PGI_2 (see the Materials and methods section) and responded to adrenaline and other agonists after 2 h. PGI_2 was added to 0.5 ml samples of isolated platelets for 1 min at concentrations of 0–150 nM, and then LDL at final concentrations of (●) 0, (△) 0.5, (▲) 1.0 and (○) 2.0 g of LDL protein/l was added to each incubation at 20°C (a). After 5 min, aggregation tests were performed with $5 \mu\text{M}$ -adrenaline and the percentage inhibition of the maximum response to adrenaline was calculated. The same procedure was used to observe the effects of LDL on inhibition by PGE_1 (b) and PGD_2 (c), but with a concentration range of 0–5 μM for these prostaglandins.

phosphodiesterase inhibitor, the basal cyclic AMP concentration after a 4 h period in PGI_2 -isolated platelets was $4.8 \pm 0.5 \text{ pmol}/10^8$ cells ($\pm \text{S.E.M.}$; $n = 4$), compared with $2.5 \pm 0.5 \text{ pmol}/10^8$ cells in platelets from the same donors prepared by the techniques of Lagarde *et al.* (1980), in which platelet activity during isolation is inhibited by buffers of low pH. In isolated platelets synthesis of cyclic AMP was very slow, rising only slightly above basal values even in the presence of a

Fig. 2. Production of cyclic AMP induced by PGI_2 in isolated platelets in the presence of LDL

Isolated platelets were incubated with PGI_2 (30 nM) for up to 10 min, in the absence (●) of LDL or with LDL present (2 g LDL of protein/l), which were added either 5 min before (■) or 1 min after (□) the PGI_2 , and the synthesis of cyclic AMP was measured by radioimmunoassay. Cyclic AMP production in untreated platelets (○) and those incubated with LDL alone (▲) was also measured. Each result is the mean $\pm \text{S.E.M.}$ for three experiments, or for two experiments where no S.E.M. is indicated, and is expressed as pmol synthesized/ 10^8 platelets.

phosphodiesterase inhibitor (Fig. 2). This rate was accelerated greatly by the presence of 30 nM- PGI_2 (Fig. 2), although the responsiveness varied between individual donors. In the presence of LDL, whether added before or after the PGI_2 , cyclic AMP production was decreased, but was still higher than the basal activity. LDL appeared to decrease the rate slightly below the basal activity that exists in the absence of PGI_2 .

The effect of PGI_2 on cyclic AMP synthesis was apparent over a wide range of concentrations of this prostaglandin. However, the amount of cyclic AMP produced was greater after addition of 300 nM- PGI_2 than at the lower (30 nM) concentration (Fig. 3a). LDL (2 g of protein/l) lowered the rate of cyclic AMP synthesis at all concentrations of PGI_2 , but at a lipoprotein concentration of 1 g of LDL protein/l LDL was effective only at low PGI_2 concentrations (30 nM).

Since these platelets had been exposed to PGI_2 during the isolation procedure, other platelets were prepared by a similar method in which 1 mM-dibutyl cyclic AMP was used as an inhibitor during isolation. The platelets recovered their activity as the dibutyl cyclic AMP was degraded. Nevertheless 2 h after isolation the platelets had a high basal cyclic AMP concentration of 23 pmol/ 10^8 platelets; some of it may have been

Fig. 3. Effect of LDL on production of cyclic AMP induced by various concentrations of PGI_2 in isolated platelets prepared by different methods

Isolated platelets were prepared (a) in the presence of PGI_2 or (b) in the presence of dibutyryl cyclic AMP. The platelets were then incubated with PGI_2 at a range of concentrations in the presence of LDL (■, 2g, □, 1g, or △, 0.5g, of LDL protein/l) or in the absence of LDL (●). Cyclic AMP production was measured by radioimmunoassay after 5 min from addition of PGI_2 and expressed as pmol synthesized/ 10^8 platelets. The results are means $\pm$ S.E.M. for three or more experiments, or for two experiments where no S.E.M. is given.

extracellular. There was no cross-reactivity of dibutyryl cyclic AMP in the cyclic AMP assay. Again, the addition of PGI_2 increased the cyclic AMP concentrations to a similar extent to those in platelets isolated in the presence of PGI_2 , indicating that desensitization of the PGI_2 receptor had not occurred at the first exposure to the prostaglandin (Fig. 3b). Furthermore, high concentrations (2mg of LDL protein/l) of LDL decreased the cyclic AMP synthesis, over a wide range of PGI_2 concentrations, but not to basal values. The effectiveness of the LDL was decreased at lower concentrations of lipoprotein, again only influencing cyclic AMP production at lower PGI_2 concentrations.

In separate experiments (results not shown) the aggregation of isolated platelets was inhibited by dibutyryl cyclic AMP at concentrations as low as 0.1mM. In the presence of LDL (2g of LDL protein/l) this inhibition was reversed, but not if the dibutyryl cyclic AMP concentration was above 0.4mM.

Cyclic GMP synthesis in the presence of PGI_2 and LDL

In some of the experiments, the concentrations of cyclic GMP were also assayed. The basal concentrations of cyclic GMP were considerably lower than those for cyclic AMP. Addition of PGI_2 (30nM) brought about an increase in cyclic GMP

synthesis so that the final concentrations were between 2- and 4-fold above basal, depending on the sample of platelets used (Fig. 4a). This effect appeared to be maximal at 15nM- PGI_2 (Fig. 4b). The addition of LDL (2g of LDL protein/l) before or after PGI_2 decreased the cyclic GMP production to below basal values within 5min (Fig. 4a), irrespective of the concentration of PGI_2 used (Fig. 4b). In the absence of PGI_2 , LDL decreased the basal concentration of cyclic GMP by almost 50% after 10min (Fig. 4b).

In other experiments (results not shown) the addition of dibutyryl cyclic GMP to platelets had a slightly inhibitory effect on aggregation by ADP (5 μ M), eliminated aggregation induced by 5 μ M-adrenaline and had no effect on aggregation by thrombin (100 units/l). However, this occurred only at concentrations of 2mM-dibutyryl cyclic GMP or above, and was completely reversed by the presence of LDL (2g of LDL protein/l).

Effects of LDL on forskolin-stimulated cyclic AMP synthesis

The adenylate cyclase enzyme system is regulated by membrane-bound receptors (e.g. receptors for PGI_2) by stimulation of a guanine nucleotide regulatory protein (G_s), which in turn activates the catalytic unit of the enzyme (Rodbell, 1980). Forskolin, a diterpene, stimulates the production of cyclic AMP by its interactions with G_s and with

Fig. 4. Production of cyclic GMP induced by PGI₂ in isolated platelets both before and after addition of LDL. Isolated platelets prepared in the presence of PGI₂ (see the Materials and methods section) were incubated with PGI₂ (30 nM) for up to 10 min (a) or with a range of concentrations of PGI₂ for 5 min (b). This was done either in the absence of LDL (●) or with LDL (2 g of LDL protein/l) added after PGI₂ (□). In some cases LDL was added before PGI₂ (■) or in the absence of PGI₂ (△). The production of cyclic GMP (pmol synthesized/10⁸ platelets) in these incubations was compared with those of controls in which no PGI₂ or LDL was added (○). The results are means $\pm$ S.E.M. for three experiments or two experiments where no S.E.M. is shown.

the catalytic unit, but not by stimulation of the surface receptors (Wong & Martin, 1983).

Forskolin inhibited the isolated platelet preparations in their aggregation responses to adrenaline, ADP, thrombin and collagen at concentrations as low as 1 μ M (results not shown). It was added to the platelets in ethanol, but in sufficiently low concentrations of this solvent to avoid inhibition of aggregation. At concentrations of 5 μ M-forskolin and above, LDL did not reverse the inhibition of aggregation, but at 1 μ M-forskolin some response to thrombin (100 units/l) and ADP (2 μ M) re-appeared; the adrenaline response remained suppressed.

Production of cyclic AMP and cyclic GMP was measured in the presence of 20 μ M-forskolin, and also after the addition of LDL. Control incubations with ethanol (17 mM) showed that the solvent had no effect on cyclic nucleotide production (Fig. 5). Forskolin greatly increased cyclic AMP production, but had no effect on that of cyclic GMP. This gave further evidence of the absence of cross-reactivity of cyclic AMP in cyclic GMP assays. The addition of LDL decreased the effect of forskolin on cyclic AMP production, but not completely, so that the concentrations of cyclic AMP were well above basal concentrations. This may account for the observation that aggregation was still suppressed with this dose of forskolin.

These results suggest that the LDL may influence cyclic AMP synthesis by a mechanism which does not involve the membrane receptors for agonists which stimulate adenylate cyclase. However, it was possible that LDL simply stripped away these agonists from their receptors, thereby decreasing their effects.

Influence of LDL on PGE₁ binding to isolated platelets

Isolated platelets were incubated with [³H]PGE₁ (see the Materials and methods section), and the amount of bound prostaglandin label was determined after washing on a filtration apparatus. The degree of specific and non-specific binding was evaluated by chasing the label with an excess of unlabelled PGE₁. The experiments were performed in the absence and presence of LDL (2 g of LDL protein/l), which was introduced into the incubation after the PGE₁ was loaded on the platelets and the lipoproteins were allowed to incubate for a further 5 min. The results in Fig. 6 showed that LDL had no effect on the specific and non-specific binding of PGE₁.

Discussion

LDL do not significantly decrease the basal concentration of cyclic AMP (Hassall *et al.*, 1983b)

Fig. 5. Production of cyclic AMP and cyclic GMP by forskolin in isolated platelets in the presence of LDL. Isolated platelets were incubated with forskolin (20 μ M) in the absence (●) or presence (□) of LDL (2 g of LDL protein/l) for periods up to 10 min. The production of cyclic AMP (a) and cyclic GMP (b) was measured by radioimmunoassay in these incubations and compared with those in controls without forskolin and LDL (○) or in other controls containing 17 mM-ethanol (▲), since forskolin was introduced from a solution in ethanol. The results represent the mean values from two experiments.

in isolated platelets in the absence of phosphodiesterase inhibitors. The activity of adenylate cyclase in isolated platelets was also not significantly changed by LDL, which suggests that their ability to activate these cells must occur by another mechanism. Platelets that have been sensitized to other agonists by enrichment in cholesterol from liposomes also fail to show any change in adenylate cyclase activity (Insel *et al.*, 1978). However, it is clear that LDL can reverse the inhibition of aggregation by PGI₂ and decrease the high concentrations of cyclic AMP produced by this prostaglandin (Hassall *et al.*, 1983b). Furthermore, in the present study the activation of adenylate cyclase by PGI₂ was decreased by LDL, depending on the relative concentrations of the prostaglandin and the lipoproteins. At high doses of PGI₂, LDL (2 g of LDL protein/l) does not reverse the inhibition by PGI₂, but cyclic AMP production is inhibited by 50% or more. At lower concentrations of PGI₂, both high and low LDL concentrations reverse the inhibition caused by PGI₂ and bring about a decrease in cyclic AMP production. In patients with familial hypercholesterolaemia (elevated LDL) it was observed that the platelets had a lower sensitivity to inhibition by PGI₂, although this did not appear to relate to the activity of adenylate cyclase (Colli *et al.*, 1983). Platelets isolated in the presence of dibutyryl cyclic AMP

aggregate very satisfactorily, even though the total basal cyclic AMP contents are well above those for PGI₂-isolated or low-pH-isolated platelets. In the presence of LDL, aggregation may occur at relatively high concentrations of cyclic AMP, but there may be limits to this, and greater stimulation of adenylate cyclase by PGI₂ may overcome the activating effects of LDL.

The action of LDL on cyclic AMP production may be considered in the light of the current proposals for the regulation of adenylate cyclase. As indicated above, the enzyme is activated by surface receptors for various agonists via the G_s protein. Forskolin stimulates the G_s protein, which in turn activates the catalytic unit of the enzyme (Rodbell, 1980; Insel *et al.*, 1982). The enhanced synthesis of cyclic AMP by forskolin was decreased by LDL. This suggested that LDL influenced the catalytic unit of adenylate cyclase through a route which did not involve the receptors for the prostaglandins or other agonists for the enzyme. The LDL receptor may inhibit the G_s protein or the catalytic unit directly or through another intracellular mediator yet to be recognized. Another possibility is that LDL stimulated the inhibitory GTP-sensitive protein (G_i), the existence of which has been proposed by Jakobs *et al.* (1981) to give a more refined level of control over adenylate cyclase activity. This stimulation

Fig. 6. Effect of LDL on the specific binding of [3 H]PGE₁ to its receptor

Isolated platelets were incubated at 25°C for 30 min with [3 H]prostaglandin E₁ (54 Ci/mmol) in Tyrode's buffer. More buffer or buffer containing LDL (final concn. 2 g of LDL protein/l) was added to the platelets and incubated for a further 10 min. The platelets were then filtered and washed (see the Materials and methods section) and the total amount of bound radioactivity remaining in the presence (●) and absence (○) of LDL was determined. In some incubations, 10 μ M unlabelled PGE₁ was included in the second part of the incubation so that the amount of non-specific binding could be determined: □, +buffer; ■, +LDL. By subtracting this from the total binding, the amount of specific binding could be calculated: △, +buffer; ▲, +LDL. The results are the means $\pm$ S.E.M. for three experiments.

may be direct or through another intracellular regulator, which may be the same as that which activates platelets in the presence of LDL alone.

An increase in intracellular Ca²⁺ concentration is known to activate platelet aggregation (Gerrard *et al.*, 1981), and this is opposed by a rise in cytosolic cyclic AMP (Feinstein *et al.*, 1983). No information is available on the effects of LDL on Ca²⁺ mobilization or on that of univalent ions, which also inhibit adenylate cyclase in platelets (Steer & Wood, 1981).

It has been suggested by others that cyclic GMP concentrations rise during platelet aggregation (Haslam, 1975), but this may be a consequence of aggregation rather than the nucleotide playing a regulatory role (Haslam *et al.*, 1978). In the present study cyclic GMP synthesis was increased by PGI₂ and decreased by LDL in cells which had been stimulated by PGI₂. LDL decrease the basal

production of this nucleotide, which was in any case very low. High concentrations of dibutyryl cyclic GMP had a weak inhibitory effect on the aggregation of isolated platelets. It therefore seems unlikely that variations in the concentration of cyclic GMP have an important regulatory role in platelet aggregation or that it is the main activator in the effects of LDL.

The effects of LDL on platelet activation require the binding of the lipoproteins to the receptor, since modification of LDL by cyclohexanedione abolished their effects (Hassall *et al.*, 1983b). Little of the bound LDL is internalized (Koller *et al.*, 1982; Masurov *et al.*, 1982), a process which commonly occurs in other cell types. Scatchard analysis of lipoprotein binding to isolated platelets indicated the presence of 1470 high-affinity LDL receptors per platelet, which saturate at a concentration of less than 0.05 g of LDL protein/l (Koller *et al.*, 1982). This is well below the concentration required to activate platelets or to reverse the stimulation of adenylate cyclase by PGI₂ as shown above. These receptor numbers are greater than those for prostaglandin high-affinity receptors (Siegl *et al.*, 1979; Schafer *et al.*, 1979), namely PGE₁ 26, PGI₂ 93 and PGD₂ 760 per platelet. However, there are more numerous low-affinity sites for some prostaglandins, e.g. PGI₂ 2700. Most cell types are exposed to low concentrations of LDL in extracellular fluid, but blood platelets will normally experience high concentrations in the range used in these experiments. LDL may have effects through low-affinity sites by a secondary action on other membrane constituents, which in turn influence the activity of adenylate cyclase or enhance the sensitivity of the platelets to other agonists. LDL reversed the inhibition of aggregation by PGE₁, PGD₂ and PGI₂. PGI₂ and PGE₁ are thought to share the same receptor (Schafer *et al.*, 1979), but the effects of LDL are indiscriminate, since PGD₂ inhibition is also reversed. The studies with PGE₁ binding suggest that LDL does not interfere with the binding of prostaglandins to their receptors. LDL must therefore have other effects on intracellular events, which may account for their ability to reverse inhibition by various agents.

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