7. J. D. Brooker, G. Srivastava, B. K. May and W. H. Elliot, *Enzyme* **28**, 109 (1982).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **93**, 265 (1951).
9. D. Mauzerall and S. Granick, *J. biol. Chem.* **219**, 435 (1956).
10. V. A. Percy and B. C. Shanley, *J. Neurochem.* **33**, 1267 (1979).
11. F. DeMatteis and D. E. Ray, *J. Neurochem.* **39**, 551 (1982).
12. M. Doss, R. Von Tiepermann and J. Schneider, *Int. J. Biochem.* **12**, 823 (1980).
13. D. Shemin, *Phil. Trans. R. Soc. (B)* **273**, 109 (1976).
14. J. R. Paterniti, J. J. Simone and D. S. Beattie, *Archs Biochem. Biophys.* **189**, 86 (1978).
15. F. DeMatteis, P. Zetterlund and L. Wetterberg, *J. Biochem, Tokyo* **196**, 811 (1981).
16. H. L. Bonkowsky and W. Schady, *Semin. Liver Dis.* **2**, 108 (1982).

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Differential effects of chymotrypsin on magnesium, sodium, and guanine nucleotide regulation of α_2-adrenoreceptors of human platelets

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The membrane-bound receptor–adenylate cyclase complex consists of at least three macromolecules, namely the receptors [R] located at the external surface of the plasma membrane, the catalytic unit of the enzyme [C] anchored in the inner surface of the plasma membrane, and the regulatory proteins [G] acting as couplers between the receptors and the catalytic unit of the enzyme [1]. The receptor–adenylate cyclase complex in human platelets where activation of α_2-adrenorcceptors leads to inhibition of adenylate cyclase is regulated by divalent [Mg^{2+}] and monovalent [Na^+] cations and by guanine nucleotide [GTP] [2]. We recently reported that pretreatment of human platelet membranes with trypsin eliminated the Mg^{2+} and GTP but not the Na^+ effect [3] and suggested that two distinct proteins are involved in Na^+ and GTP binding. In the present study, we investigated the effects of chymotrypsin on Mg^{2+}, GTP, and Na^+ regulation of the α_2-adrenoreceptors.

Materials and methods

The following chemicals and enzymes were purchased: chymotrypsin (bovine pancreas), trypsin inhibitor (soybean), 5′-guanylyllimidodiphosphate (Sigma Chemical Co.) and [*O-methyl*-^{3}H]yohimbine (90 Ci/mmol; Amersham).

Platelet membranes were prepared from platelet-rich plasma by differential centrifugation as described by Periyasamy and Somani [4].

Binding of [^{3}H]yohimbine to platelet membranes was carried out as previously described [4]. An 8nM concentration of [^{3}H]yohimbine was used in all experiments to measure total binding except in saturation binding experiments where concentrations of [^{3}H]yohimbine ranging from 0.5 to 20 nM were used. Nonspecific binding was measured in the presence of 10 μM unlabeled yohimbine, and specific binding was calculated by subtracting nonspecific from total binding.

Platelet membranes were treated with chymotrypsin as follows. Membranes were incubated with or without chymotrypsin [0.5 mg of enzyme · (mg membrane protein) · ml^{-1}] in a buffer containing 25 mM Tris–HCl + 0.5 mM EDTA (pH 7.5) at 35° for 3 min. Proteolytic activity of chymotrypsin was terminated (3 min later) by adding 2 parts of soybean trypsin inhibitor to 1 part of chymotrypsin by weight. The chymotrypsin-treated membranes were washed twice by repeated centrifugation at 40,000 *g* for 30 min. An aliquot of the washed membrane was used for binding assay.

Results

Effect of chymotrypsin on [^{3}H]*yohimbine binding.* Specific binding data generated from the saturation binding studies were transformed into a Scatchard plot, and the data were analyzed with computer-assisted linear regression analysis. As shown in Fig. 1, chymotrypsin reduced the number of binding sites from 302 ± 25 to 125 ± 20 fmol/mg protein without significantly affecting the affinity [K_d] of the receptors for [^{3}H]yohimbine. It also did not modify the affinity of the receptors for other antagonists such as phentolamine since the IC_{50} of phentolamine (82.0 ± 8.0 nM) was similar in control and treated membranes. However, the affinity of the receptors for the agonist was reduced, the IC_{50} for *l*-epinephrine being 950 ± 100 and 2300 ± 130 nM in control and treated membranes respectively.

Effect of chymotrypsin on the regulation of α_2-adrenoreceptors–agonist interactions by Mg^{2+}, GTP, and Na^+. Figure 2A shows that the epinephrine-displacement curve was shifted to the right by 2-fold in treated membranes as compared to control. Mg^{2+} increased the affinity of the receptors to epinephrine as shown by a shift in the displacement curve to the left; however, the ability of Mg^{2+} to increase the affinity of the receptors for epinephrine was consistently 2-fold less in treated membranes as compared to control, indicating that part of the Mg^{2+} effect was destroyed by chymotrypsin. GPP[NH]p reduced the affinity of the receptors for the agonist in control membranes but this effect of GPP[NH]p was lost in chymotrypsin-treated membranes (Fig. 2A). These results suggest that chymotrypsin inactivated the GTP binding component of the receptor–adenylate cyclase complex.

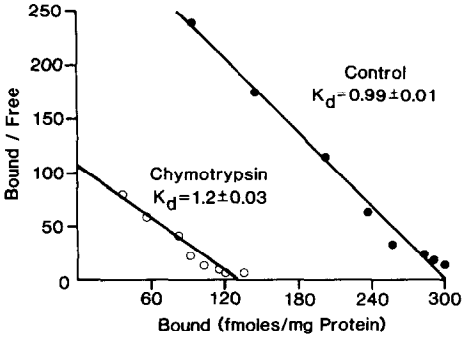

Fig. 1. Equilibrium binding data of specific [^{3}H]yohimbine binding to human platelet membranes pretreated with or without chymotrypsin. The data were converted into a Scatchard plot and were fitted by straight lines using linear regression analysis [μ = 0.97]. The data are the mean of three experiments conducted in duplicate.

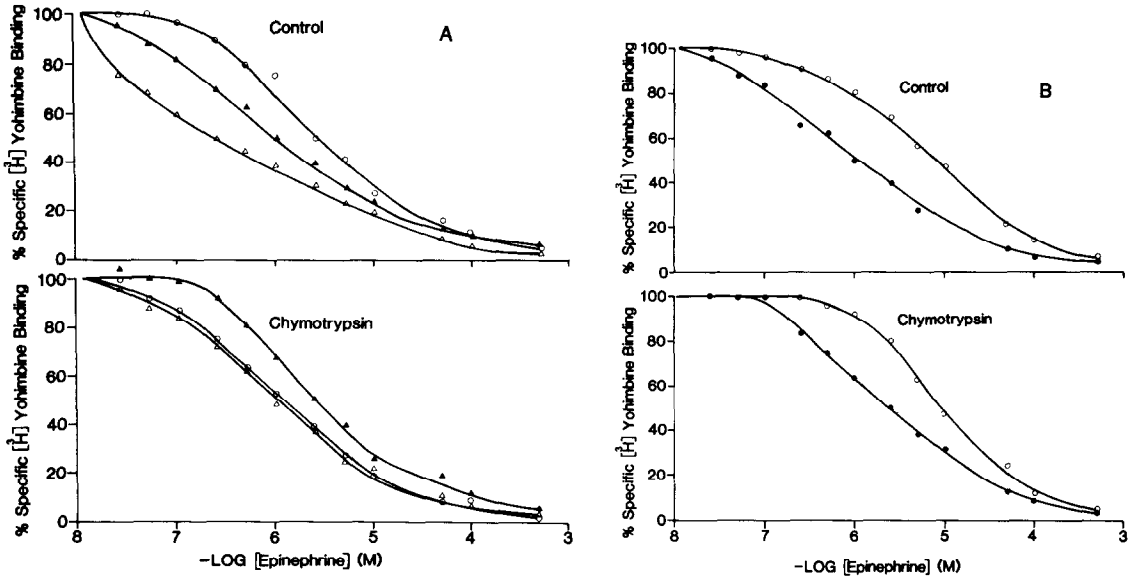

Fig. 2. (A) Specific [^{3}H]yohimbine binding to human platelet membranes pretreated with or without chymotrypsin as a function of (1) epinephrine alone (▲); (2) epinephrine + 5 mM Mg^{2+} (△); and (3) epinephrine + 5 mM Mg^{2+} + 0.1 mM GPP[NH]p (○). (B) Specific [^{3}H]yohimbine binding to human platelet membranes as a function of epinephrine alone (●) and epinephrine + 100 mM NaCl (○). Specific [^{3}H]yohimbine binding was determined as described in the text, and the specific binding in the absence of epinephrine was taken as 100%. The data are the mean of four experiments conducted in duplicate.

As shown in Fig. 2B, in the presence of 100 mM NaCl, the displacement curve of epinephrine was shifted to the right in control as well as chymotrypsin-treated membranes, and the IC_{50} value for epinephrine in the presence of Na^+ was found to be similar in both preparations. These findings suggest that the Na^+-regulatory component of the receptor–adenylate cyclase complex was not disrupted by pretreatment with chymotrypsin.

Discussion

Pretreatment of human platelet membranes with chymotrypsin did not alter the affinity of the receptors for the antagonist but reduced the number as well as the affinity of the receptors for the agonist. These findings suggest that chymotrypsin did not alter the affinity of the receptors *per se*. Further, chymotrypsin treatment produced conversion of the high-affinity state of the receptors to the low-affinity state, reduction in the affinity of the receptors for the agonist, and abolition of the GTP effect (Fig. 2A); all of these changes can be explained on the basis of a single action, i.e. inactivation of GTP binding protein by chymotrypsin. A similar effect has been observed in several other receptor systems in the presence of *N*-ethylmaleimide [5–7]. The present results are slightly different from those of Ferry *et al.* [8] who reported that treatment of human platelet membranes by chymotrypsin did not alter the total number of binding sites but abolished the GTP effect on epinephrine-induced inhibition of [^{3}H]yohimbine binding. However, these investigators did not study the effect of either Mg^{2+} or Na^+ on epinephrine-induced inhibition of [^{3}H]yohimbine binding.

Mg^{2+} increased the affinity of the receptors for the agonist in the untreated membranes, and this effect of Mg^{2+} is mediated through binding to GTP proteins [9] and also through facilitating the physical interactions between the receptors and the GTP binding proteins [10]. On the other hand, in the treated membranes, Mg^{2+} only partially increased the affinity of the receptors for agonist while the effect of GPP[NH]p was lost completely. These data suggest that, in addition to binding to GTP protein, Mg^{2+} also binds to some other component not susceptible to proteolysis by chymotrypsin. Similar results were obtained for cardiac muscarinic receptors when cardiac membranes were treated with *N*-ethylmaleimide or pertussis toxin [11]. Although the second binding site of Mg^{2+} may be on the receptor itself, this is unlikely since, under similar experimental conditions, pretreatment of platelet membranes with trypsin completely abolished the Mg^{2+} effect [3].

Although earlier studies suggested that the Na^+ regulates the receptors by binding to GTP protein [12, 13], recently we and others have shown that Na^+ and GTP mediate their effects through two distinct proteins [3, 14]. The present data with chymotrypsin, which completely eliminated the GTP effect but not the Na^+ effect, provide further support that two different proteins are involved in the regulatory effects of Na^+ and GTP.

In summary, the effects of chymotrypsin were investigated on the regulatory effects of Mg^{2+}, GTP, and Na^+ on α_2-adrenoreceptors of human platelets. The results suggest that GTP and Na^+ regulate the receptor–adenylate cyclase complex by binding to two distinct proteins, and the regulatory effects of Mg^{2+} occur by interacting with the GTP binding protein and also a second site which was not susceptible to chymotrypsin.

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REFERENCES

1. L. T. Williams, D. Mullikin and R. J. Lefkowitz, *J. biol Chem.* **253**, 2984 (1978).
2. K. H. Jakobs, K. Aktories, M. Minuth and G. Schultz, *Adv. Cyclic Nucleotide Res.* **19**, 137 (1985).
3. S. M. Periyasamy and P. Somani, *Fedn Proc.* **45**, 563 (1986).
4. S. M. Periyasamy and P. Somani, *Eur. J. Pharmac.* **116**, 17 (1985).
5. S. J. Korn, M. W. Martin and K. Harden, *J. Pharmac. exp. Ther.* **224**, 118 (1983).
6. J. W. Wei and P. V. Sulakhe, *Naunyn-Schmiedeberg's Archs Pharmac.* **314**, 51 (1980).
7. N. E. Larsen, D. Mullikin-Kilpatrick and A. Blume, *Molec. Pharmac.* **20**, 255 (1981).
8. N. Ferry, S. Adnot, A. Borsodi, M. L. Lacombe, G. Guellan and J. Hanoune, *Biochem. biophys. Res. Commun.* **108**, 708 (1982).
9. S. Y. Cech and M. E. Maguire, *Molec. Pharmac.* **22**, 267 (1982).
10. S. Y. Cech, W. C. Broaddus and M. E. Maguire, *Molec. cell. Biochem.* **33**, 67 (1980).
11. K. K. McMahon and M. M. Hosey, *Molec. Pharmac.* **28**, 400 (1985).
12. K. Aktories, G. Schultz and K. H. Jakobs, *Biochim. biophys. Acta* **676**, 59 (1981).
13. J. J. Mooney, W. C. Horne, R. I. Handin, J. J. Schildkraut and R. W. Alexander, *Molec. Pharmac.* **21**, 600 (1982).
14. L. E. Limbird and J. L. Speck, *J. Cyclic Nucleotide Res.* **9**, 191 (1983).

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Liver regeneration and hepatic microsomal changes in rats administered cyclosporin A

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Cyclosporin A (CsA), a cyclic immunosuppressant, is widely used in the transplantation of organs, including the liver. Generally, hepatotoxicity is less involved than nephrotoxicity (which can be ameliorated to some extent by dosage reduction and substitution therapy). Hepatic changes, notably hyperbilirubinemia, have been reported in human organ recipients on CsA [1–4], in dogs with orthotopic liver transplants [5] and in rats over periods of 21–49 days [6, 7] or with isolated rat liver perfusion [8]. It has been suggested that liver dysfunction in human organ recipients may stem from intercurrent infection [9]. The metabolites of CsA in humans, and in species such as the dog and the rat, retain the basic cyclic oligopeptide structure, the most prominent biotransformations being hydroxylation alone and in conjunction with N-demethylation [10]. Microsomal mixed-function oxidase system elements in rats administered 50 mg of CsA per kg, daily for 14 days, were followed by Cunningham *et al.* [11]; cytochrome P-450 decreased slightly, whereas aminopyrine *N*-demethylase (APdM) was little affected. These variables were elevated markedly in animals treated also with Aroclor 1254, an enzyme inducer. For 7 weeks, at an oral dose of CsA of 25 mg/kg daily, cytochrome P-450 was unchanged; APdM decreased during the first 4 weeks, rose during remission, and fell during the relapse period in relation to nephrotoxic effects. The levels of NADPH–cytochrome *c* reductase activity paralleled APdM activity from week 3 onward [12]. In another report by Cunningham *et al.* [13], enzyme induction in the presense of CsA ensued with continued i.p. injection of phenobarbital just as with Aroclor 1254, but not with 3-methylcholanthrene. Augustine and Zemaitis [14] corroborated in the rat, several of the above findings concerning cytochrome P-450 and demonstrated a type I binding spectrum for CsA. Depression of aniline hydroxylase was greater than that of APdM as observed by Cunningham *et al.* [11], and NADPH–cytochrome *c* reductase was most sensitive to the action of CsA at oral doses of 25 or 50 mg/kg daily for 9 days, whereas protein and cytochrome b_5 levels were not influenced appreciably at either level. CsA has also been shown to inhibit cytochrome P-450 and the enzymes, APdM and benzo[*a*]pyrene hydroxylase (BPH), in male Swiss mice [15]. With heightened hepatic microsomal drug metabolism or enzyme induction, concomitant nephrotoxicity of CsA appears to diminish as a result of a lower drug level in blood. Effects of enzyme induction are also apparent with phenytoin [4, 16] but not with inhibitors such as ketoconazole [17–19] and melphalan [18].

The current report describes findings on CsA in relation to the extent of liver regeneration in partially hepatectomized rats and hepatic microsomal variables as such and after enzyme induction by phenobarbital. The data are compared with those for intact animals. The intention of this study was to delineate liver regenerative changes that may be applicable to human post-transplantation grafts. According to a recent communication [20], CsA does not affect hepatic thymidine kinase or ornithine decarboxylase in Fischer male rats over a period of 48 hr after partial heptectomy and, in fact, pretreatment with CsA leads to potentiation of their levels.

Materials and methods

CsA, furnished by Sandoz, Inc., East Hanover, NJ, was dissolved in olive oil heated to 60° to yield a solution of 15.0 mg/ml. Phenobarbital was dissolved in low alkaline pH medium (80 mg/ml). Male rats of the Charles River (COBS) strain, averaging 300 g in weight, were partially hepatectomized in the mid-morning under ether anesthesia; two-thirds of the organ was removed [21] and dried to constant weight at 100°. All animals were housed in individual cages and given Purina rat meal and water *ad lib.* CsA (25 $mg \cdot kg^{-1} \cdot day^{-1}$) was administered by gavage in a volume of 0.5 ml for the first 7 days; controls received the vehicle. At least four animals of each group were injected with phenobarbital i.p. (80 $mg \cdot kg^{-1} \cdot day^{-1}$) on days 7–9, inclusive. All rats were killed by exsanguination on day 10, at which time the entire livers were removed, drained and weighed; tared amounts were deep-frozen for microsomal studies and the remaining organ was dried as stated above. Very small sections were reserved for microscopic examination. The amount of tissue regeneration (liver increment, g) was calculated from the dry weights at surgery (L_s) and necropsy (L_n) according to: $L_n - (0.46)L_s$ [22]. Other males from the same shipment were treated similarly except that they were not sham-operated; wet and dry liver weights as g/100 g body weight were measured for each rat. The precipitation method of Schenkman and Cinti [23] was used to isolate liver microsomes. Procedures for analyses of protein and nucleic acid contents of homogenates and subfractions as well as of cytochrome P-450