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Positive inotropic and chronotropic effects of trypsin and some other proteolytic enzymes in the guinea-pig heart

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1 In atrial preparations of the young guinea-pig (body weight 150–250 g), five proteolytic enzymes (trypsin, chymotrypsin, bacterial-Al-proteinase (nagarse), bromelain and kallikrein) produced concentration-dependent positive inotropic and chronotropic effects, while they exerted only minimal effects on the papillary muscle preparations.

2 To characterize the effects, further experiments were conducted in atrial preparations using trypsin. There was a strong tendency for tachyphylaxis: a second exposure to the same concentration of trypsin resulted in considerably smaller positive inotropic and chronotropic effects. The positive inotropic and chronotropic effects of this substance were not affected by propranolol (5×10^{-7} M). However, an accumulation of cyclic AMP was observed and the positive inotropic and chronotropic effects were potentiated by aminophylline (10^{-4} M) in association with an augmentation of the accumulation of cyclic AMP. In preparations partially depolarized with high K^+ (22 mM) medium (contractions ceased under this condition) trypsin 100 μg ml^{-1} reinstated the contraction. Treatment of the preparation with aprotinin (200 u ml^{-1}) resulted in a strong inhibition of the positive inotropic and chronotropic effects.

3 Islet activating protein (IAP), a specific inhibitor of the 'inhibition specific' guanine nucleotide binding regulatory protein of the adenylate cyclase system, did not produce significant inhibition of the positive inotropic and chronotropic effects of trypsin, whereas it produced a complete inhibition of the negative inotropic and chronotropic effects of carbachol.

4 These results suggest that the positive inotropic and chronotropic effects of proteolytic enzymes are intimately connected with the proteolytic activities through which adenylate cyclase is activated to produce an accumulation of cyclic AMP within the myocardium. The destruction of the 'inhibition specific' guanine nucleotide regulatory protein of the adenylate cyclase was not substantiated as a mechanism of activation of the adenylate cyclase.

Introduction

More than 15 years ago we described the dose-related positive inotropic effects produced by several proteolytic enzymes (trypsin, chymotrypsin, bromelain, bacterial-Al proteinase (nagarse) and kallikrein) in Straub preparations of the frog heart (Imai *et al.*, 1970). It was concluded that these effects were closely related to proteolytic activities, for the effect of trypsin was inhibited by soybean trypsin inhibitor and no inotropic effect was observed with N-ethylmaleimide-treated bromelain. However, the mechanism of the positive inotropic effect was not clear at that time. Several years later, it was found that proteolytic enzymes could stimulate adenylate cyclase and enhance adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production in various tissue cell preparations (Guiraud-Simplot & Colobert, 1977; Lacombe *et al.*, 1977; Wallach *et al.*, 1978; Knopp *et al.*, 1983). As for the adenylate cyclase of the heart, Cros *et al.* (1980) demonstrated recently, with trypsin, a stimulatory effect at low concentrations (0.5–2.5 μg ml^{-1}) and an inhibitory effect at higher concentrations (above 2.5 μg ml^{-1}). Using guinea-pig atrial preparations, Cros *et al.* (1981) also described the positive inotropic and chronotropic effects produced by this substance. According to them, the effects were only observed with a high concentration of 10^{-6} M (24 μgml^{-1}) and no dose-effect relationship was established. The increases in force and rate were only 26.5 and 18.0% of the initial level.

Stimulated by these findings, we have undertaken to

re-examine the inotropic and chronotropic effects of several proteolytic enzymes, using guinea-pig atrial and ventricular preparations to clarify: (1) whether the positive inotropic effects can be induced in the ventricular muscle preparation; (2) whether the positive inotropic and chronotropic effects can be produced at the low concentrations reported to stimulate adenylate cyclase activity; (3) whether concentration-effect relationships can be established, and (4) whether the positive inotropic effect can be related to the initiation of the contraction attributable to the slow inward calcium current. It is well established that guanosine 5'-triphosphate (GTP) plays an essential role in the activation and inhibition of adenylate cyclase. This dual regulation of adenylate cyclase activity by guanine nucleotides is mediated by stimulatory (Ns) and inhibitory (Ni) guanine nucleotide-binding regulatory proteins (Ross & Gilman, 1980; Gilman, 1984; Katada *et al.*, 1984). Trypsin was shown by Yamamura *et al.* (1977) to abolish specifically the GTP inhibitory response of the fat cell membrane. Therefore, the possible involvement of the inhibitory guanine nucleotide binding protein was also tested.

Methods

Preliminary experiments conducted with 100 $\mu g\,ml^{-1}$ trypsin in left atrial preparations, from Hartley guinea-pigs weighing between 150–630 g, demonstrated that marked positive inotropic effects (46.6 ± 4.01% of the maximal response produced by isoprenaline (10^{-7} M), which corresponded to approximately 260% of the initial level, $n = 5$) could be elicited only in young guinea-pigs weighing between 150–200 g; the effects in older guinea-pigs weighing between 480–630 g were much smaller (13.4 ± 3.85% of the maximal response, $n = 6$, $P < 0.01$). In view of these findings the experiments were carried out using young Hartley guinea-pigs weighing between 150–250 g. In some experiments Wistar rats were used instead of guinea-pigs.

Under light ether anaesthesia hearts were rapidly removed and the right and left atria, and papillary muscle of the right ventricle were dissected in ice-cold bathing solution. The preparations were suspended individually in 8 ml organ baths to record isometric contractions. The bathing solution was a Krebs-Henseleit solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11 and was continuously bubbled with 95% O_2 + 5% CO_2 to maintain the PO_2 values above 550 mmHg. Initial tensions of 0.3–0.5 g and 0.1–0.2 g were applied to the atrial and papillary muscle preparations, respectively. After 30 min the optimal resting tension was determined and maintained thereafter.

The right atrium was allowed to beat spontaneously, while the left atrium and papillary muscle were stimulated by square wave pulses of 1 ms duration at a frequency of 1 Hz, and with voltages of about 50% above threshold (usually less than 2 V), supplied by a square-wave pulse stimulator (Nihon Kohden MSE-3) via a pair of the silver-plate electrodes between which the preparations were placed. The isometric contraction was measured by a force-displacement transducer (Toyo Baldwin T7-30-240) connected to a carrier-amplifier (Nihon Kohden RP-5), and the atrial rate was counted by a cardiotachometer (Nihon Kohden RT-5). All measurements were recorded on a thermostylus recorder (Watanabe Sokki Linear Corder Mark V). An equilibration period of 60 min was allowed before starting experiments. The experiments were also performed using the partially depolarized left atrial preparation according to the method described previously (Nabata, 1977). Briefly, the contractions were recorded in normal Krebs-Henseleit solution as described above. After 1 h, the bathing solution was changed to high K^+ (22 mM) Krebs-Henseleit solution and the stimulation parameters changed to 0.4 Hz and 4 ms duration. To maintain iso-osmotic conditions, Na^+ concentration was reduced to 96 mM. Under these conditions contractions ceased.

Myocardial cyclic AMP content was determined using a radioimmunoassay kit (Yamasa Shoyu, Choshi) and expressed as pmol mg^{-1} protein. After dissolving the tissue in 0.5 N NaOH, protein was determined by the Lowry method.

Proteolytic enzymes used in the present study were trypsin, chymotrypsin, bromelain, kallikrein (Sigma Chemicals) and nagarse (Nagase Biochemicals). Drugs used were: isoprenaline (Nikken Kagaku), acetylcholine (Sankyo), propranolol (ICI Pharma.), aminophylline (Eisai), aprotinin (Bayer) and islet activating protein (Kaken Seiyaku). The positive inotropic and chronotropic effect of a proteolytic enzyme is expressed as % of the maximal response evoked by isoprenaline (10^{-7} M) in each preparation.

Data are expressed as mean ± s.e. and statistical significances were estimated by use of Student's unpaired t test.

Results

Effects of the proteolytic enzymes in guinea-pig atrial and ventricular preparations

In the preliminary experiments conducted with 100 $\mu g\,ml^{-1}$ trypsin a marked tendency for tachyphylaxis was found for its positive inotropic effects; a second exposure to the same concentration resulted in a much smaller response, as shown in Figure 1, and cumulative applications of the enzyme at increasing

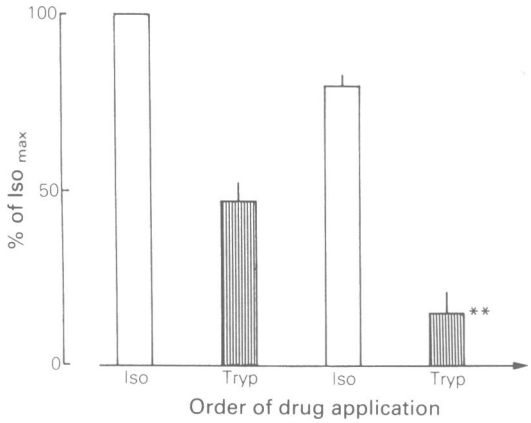

Figure 1 Comparison of the positive inotropic effect, on the left atrial preparation, of a second application of the same concentration of trypsin (Tryp; 100 μg ml^{-1}) and isoprenaline (Iso; 10^{-7} M) with those of the first application. Iso_{max}: the maximal response evoked by isoprenaline (10^{-7} M). Each column indicates mean ($n = 6$) and vertical lines show s.e. **Significantly different from the responses induced by the first application ($P < 0.01$).

concentrations within a narrow range (threefold step) resulted in a rapid diminution of the positive effects. Hence, the positive inotropic and chronotropic effects were assessed only once in each preparation. When tested in this way, all the proteolytic enzymes studied produced concentration-dependent positive inotropic and chronotropic effects in left and right atrial preparations, while the effects of these enzymes on the papillary muscle preparations were minimal (Figures 2 and 3). The maximal responses to the enzymes, except chymotrypsin, obtained in the atria were about 50% of the maximal response produced by isoprenaline. Chymotrypsin produced a maximal response which was about half of those produced by the other enzymes (Figure 2). The magnitude of the inotropic and chronotropic responses were almost the same with all the enzymes except bromelain, which was found to be a preferential positive chronotropic agent (Figure 3). The effects of the proteolytic enzymes started within 30 s after application, were maximal within a few minutes and gradually decreased thereafter.

Effects of propranolol, aminophylline and protease inhibitors on the positive inotropic and chronotropic effects of trypsin

Because of the qualitative similarity of the positive inotropic and chronotropic effects of proteolytic enzymes observed in the atrial muscle preparations, the following experiments were carried out using trypsin. Pretreatment of the preparation with

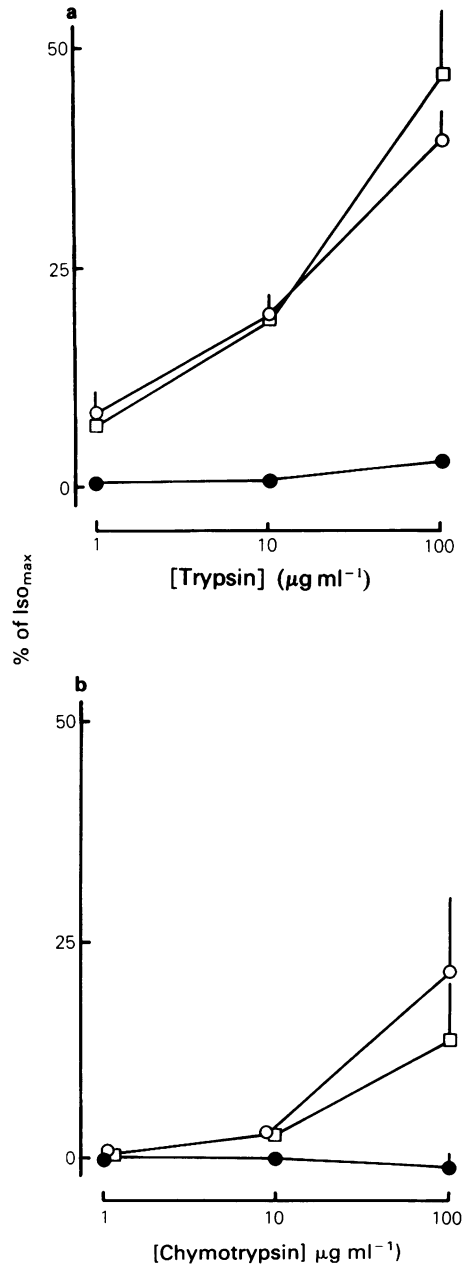

Figure 2 Inotropic and chronotropic effects of (a) trypsin and (b) chymotrypsin on the guinea-pig atrial and ventricular preparations. (□) spontaneous beating rate of the right atria; (○) contractile tension of the left atria; (●) contractile tension of the ventricular preparations. Iso_{max}: the maximal response evoked by isoprenaline (10^{-7} M). Each value is mean ($n = 5$ (a) or 4 (b)) and vertical lines indicate s.e.

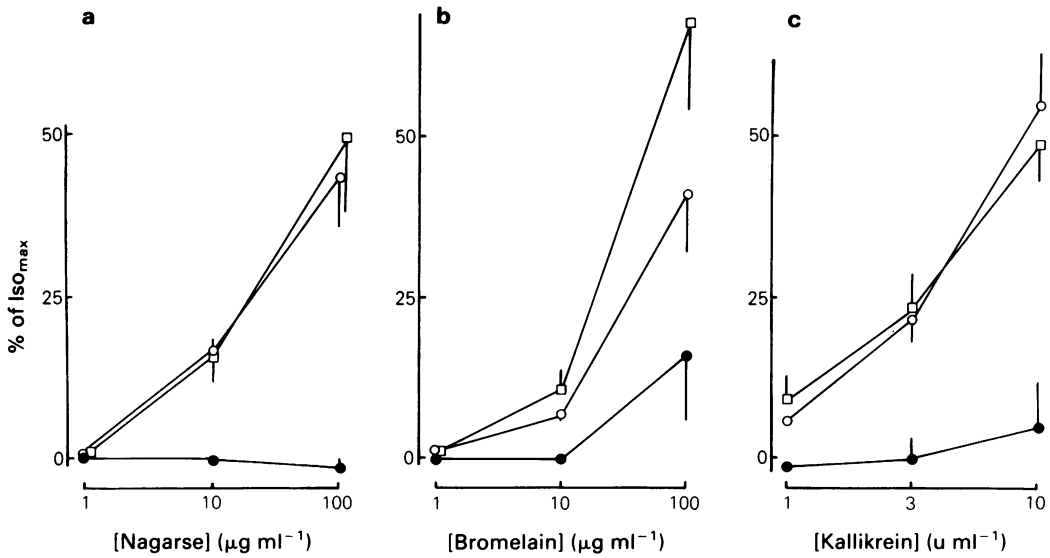

Figure 3 Inotropic and chronotropic effects of (a) nagarse, (b) bromelain and (c) kallikrein on the guinea-pig atrial and ventricular preparations. (□) spontaneous beating rate of the right atria; (○) contractile tension of the left atria; (●) contractile tension of the ventricular preparations. Iso_{max}: the maximal response evoked by isoprenaline (10^{-7} M). Each value is mean ($n = 4$ (a and b) or 5 (c) and vertical lines indicate s.e.

propranolol (5×10^{-7} M) for 30 min scarcely affected the positive inotropic and chronotropic effects of trypsin (Figure 4), while pretreatment with aminophylline (10^{-4} M) for 30 min significantly potentiated the effects of this compound (Figure 4). Therefore, experiments were conducted in the papillary muscle preparations in order to see whether the positive inotropic effect could be induced even in this preparation in the presence of aminophylline. However, a definite positive inotropic effect was not observed. Pretreatment of the preparation with aprotinin (200 u ml^{-1}), a non-specific protease inhibitor, resulted in a complete inhibition of the effects of trypsin (Figure 4).

Effects of trypsin and trypsin + aminophylline on the myocardial cyclic AMP content

At a time when the positive inotropic effects of trypsin attained peak levels, the preparation was frozen with a Wollenberger clamp precooled in liquid nitrogen. Frozen tissues were pulverized in a stainless-steel percussion mortar and homogenized in 0.1 N HCl with a Polytron homogenizer (Kinematica PT10/35) (at maximum speed, 20 s × 2) and heated for 3 min at 100°C to extract the myocardial tissue cyclic AMP. As shown in Figure 5, cyclic AMP content in the trypsin-treated atria was increased. Pretreatment of the preparation with 10^{-4} M aminophylline augmented this increase in cyclic AMP (Figure 5).

Effects of trypsin on partially depolarized atrial preparation

To investigate the underlying mechanism of the positive inotropic effect, the effects of trypsin on the partially depolarized left atrial preparation were also examined. In high K^+ (22 mM) Krebs-Henseleit solution, contractions disappeared within 5 min. Trypsin (10 μg ml^{-1}) reinstated the contractions (data not shown).

Effects of islet activating protein on the positive inotropic and chronotropic effects of trypsin in the rat atria

In order to examine the effects of proteolytic enzymes on the 'inhibition specific' guanine nucleotide regulatory unit (Ni) of the adenylate cyclase system, the effects of islet activating protein (IAP) on the positive inotropic and chronotropic effects of trypsin were studied. IAP, a substance produced by the gram-negative bacillus *Bordetella pertussis*, catalyses the transfer of the ADP-ribose moiety of NAD to the active subunit of guanine nucleotide-binding regulatory protein involved in the inhibition of the adenylate cyclase activity and eliminates the Ni function (Ui, 1984). IAP was administered intravenously to the animal 60–80 h before conducting the experiments, to allow for the slow penetration of the agent into the tissues (Endoh *et al.*, 1985). Neverthe-

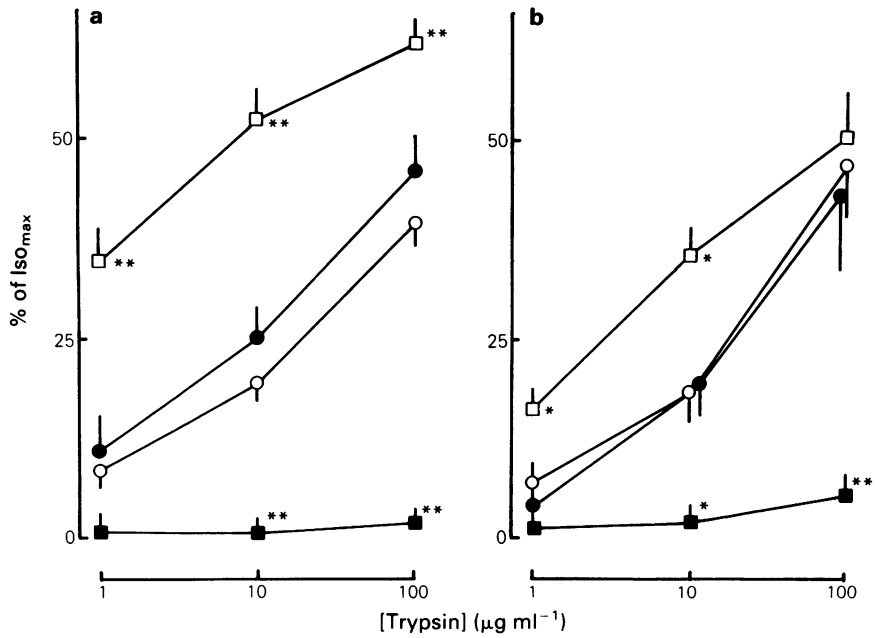

Figure 4 Effects of propranolol, aminophylline and aprotinin on the (a) positive inotropic (contractile force) and (b) chronotropic (heart rate) effects of trypsin in guinea-pig atrial preparations. Shown are responses to (○) trypsin ($n = 5$), (●) trypsin + propranolol (5×10^{-7} M) ($n = 5$), (□) trypsin + aminophylline (10^{-4} M) ($n = 5$), (■) trypsin + aprotinin (200 u ml^{-1}) ($n = 6$). Each value is mean of n results and vertical lines indicate s.e. $*P < 0.05$, $**P < 0.01$, significantly different from the values obtained in the absence of aminiphylline or aprotinin. Iso$_{max}$: the maximal response evoked by isoprenaline (10^{-7} M). Propranolol or aminophylline was added to the bathing solution to make a final concentration of 5×10^{-7} M or 10^{-4} M, respectively, and after 30 min trypsin was added. Aprotinin was added to the bathing solution to make a final concentration of 200 u ml^{-1} and after 10 min trypsin was added.

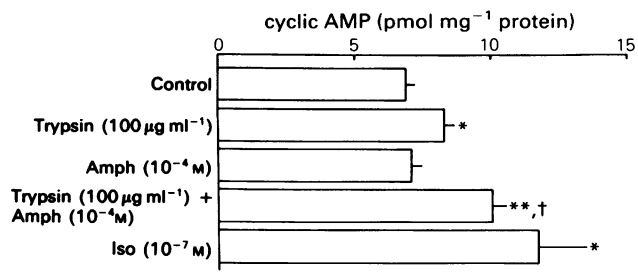

Figure 5 Effects of trypsin and trypsin + aminophylline (Amph) and isoprenaline (Iso) on the cyclic AMP content of atrial preparations of the guinea-pig. $*P < 0.05$; $**P < 0.01$, significantly different from control. $\dagger P < 0.05$, significantly different from effects of trypsin alone.

less, the negative inotropic and chronotropic effects of carbachol (10^{-6} M) on the guinea-pig atria were not affected, even with doses of IAP as high as 100 $\mu g\ kg^{-1}$, which produced a complete inhibition of the effects of carbachol in the rat atria. Hence, experiments were performed in the rat atria. Male Wistar rats weighing 220–295 g were used and IAP (50 $\mu g\ kg^{-1}$) was administered as described above. Negative inotropic and chronotropic effects of carbachol were completely abolished, while no significant reduction of the positive inotropic and chronotropic effects of trypsin was observed (Figure 6).

Discussion

In the present study it was found that proteolytic enzymes could produce positive inotropic and chronotropic effects in the guinea-pig atria at concentrations lower than those used by Cros *et al.* (1981) (1 $\mu g\ ml^{-1}$ in the case of trypsin). Furthermore, the effects observed were greater, attaining a level approximately 260% of the initial tension. As reported by Cros *et al.* (1981) with higher concentrations, the positive inotropic effect of trypsin was associated with an accumulation of cyclic AMP and could be augmented by a phosphodiesterase inhibitor. Further, we succeeded in inducing contractions in partially depolarized left atrial preparations. These findings suggest that the observed positive inotropic and chronotropic effects may be ascribed to the accumulation of cyclic AMP, due to activation of the adenylate cyclase system, and resultant activation of the slow calcium channels. Cros *et al.* (1981) demonstrated that the positive inotropic and chronotropic effects of trypsin were not abolished after treatment of the preparation with phentolamine, promethazine, cimetidine or propranolol. In the present study propranolol had no significant effect. According to Cros *et al.* (1981), reserpine-treatment was also without effect. Thus, the release of catecholamines or histamine and the activation of the respective receptors may be excluded as a cause of the activation of the adenylate cyclase. Rather, the activation may be ascribed to the proteolytic effects, for all the five proteolytic enzymes used in the present study (trypsin, chymotrypsin, bromelain, nagarse and kallikrein) produced qualitatively similar positive inotropic and chronotropic effects. Furthermore, the positive inotropic effects of trypsin were abolished after treatment of the preparations with a natural protease inhibitor, aprotinin.

How then can the proteolysis lead to the activation of the adenylate cyclase system? What component of the adenylate cyclase system is the target for proteolysis? Previous workers (for references see Cros *et al.*, 1981) have suggested the following mechanisms

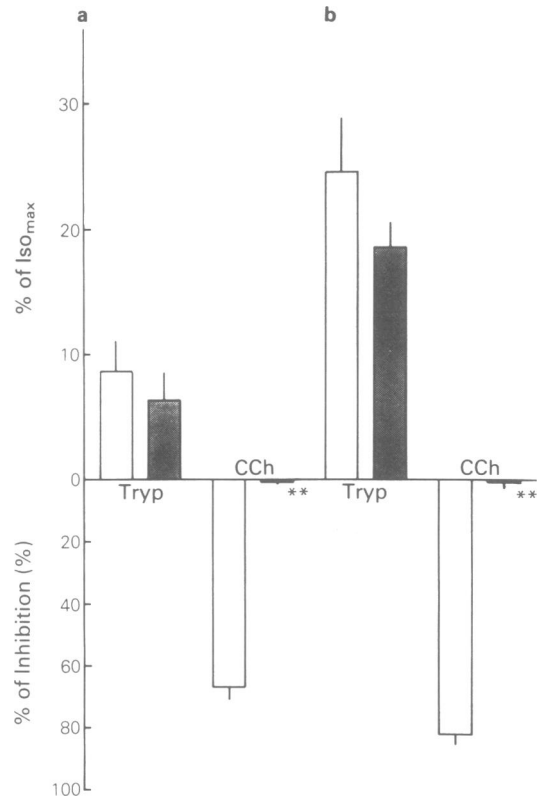

Figure 6 Effects of islet activating protein (IAP) on the positive chronotropic and inotropic effects of trypsin in (a) right and (b) left atria. For comparison effects of IAP on the negative chronotropic and inotropic effects of carbachol are shown. The positive inotropic and chronotropic effects of trypsin (100 $\mu g\ ml^{-1}$) (Tryp) are expressed as % of the maximum positive inotropic and chronotropic effects induced by isoprenaline (10^{-7} M) (Iso). The negative chronotropic and inotropic effects of carbachol (10^{-6} M) (CCh) are expressed as % inhibition of the initial tension. Open columns represent effects in absence of IAP and solid columns those in presence of IAP. **Significantly different from the values obtained without IAP treatment ($P < 0.01$).

of activation of adenylate cyclase by proteolytic enzymes: (1) a non-specific limited proteolysis of receptors or of the catalytic subunit; (2) the destruction of a regulatory protein such as GTP-dependent regulatory protein; (3) a more specific modification of the catalytic unit or a protein closely associated with this unit; and (4) the activation of membrane proteases regulating adenylate cyclase. In a study on the activation of adenylate cyclase of the human platelet by trypsin, Stiles & Lefkowitz (1982) concluded that between the two GTP-dependent regulatory pathways, i.e. the pathways of stimulation and inhibition,

the latter was much more sensitive to trypsin and that the trypsin-sensitive site is localized at the interface of an 'inhibition-specific' guanine nucleotide regulatory unit and the catalytic moiety of adenylate cyclase. Furthermore, three interesting papers have appeared very recently that seem to support the suggestion made by Stiles & Lefkowitz. Hashimoto *et al.* (1985) found, with rat liver, that the release of protein kinase C in the activated form occurs through limited proteolysis of the plasma membrane by trypsin. Jakobs *et al.* (1985) found, with the human platelet, an impairment of the hormone-sensitive inhibitory pathway, dependent on the inhibitory guanine nucleotide binding regulatory protein (Ni), by 12-O-tetradecanoylphorbor 13-acetate (TPA), an activator of protein kinase C. In their experiments, the adenylate cyclase inhibition not involving the Ni protein and the operation of the stimulatory pathway remained unchanged. Katada *et al.* (1985) found the phosphorylation of Ni protein by TPA in association with the impairment of the inhibitory pathway. Prompted by these findings we studied the effects of islet activating protein on the positive inotropic effect of trypsin. IAP has been shown to ADP-ribosylate specifically the active subunit of the guanine nucleotide-binding regulatory protein (Ni) involved in the inhibition of adenylate cyclase and produce a complete loss of Ni function (Ui, 1984). Contrary to expectations, a significant inhibition of the positive inotropic and chronotropic effects of trypsin was not observed, although the negative inotropic and chronotropic effects of carbachol were completely abolished.

The reason why a definite positive inotropic effect was not found in ventricular preparations and in atrial preparations derived from large guinea-pigs is not clear at present. Although differences between atrial and ventricular preparations have been indicated for receptor-operated responses, e.g. adenosine response (Chiba & Himori, 1975; Burnstock & Meghji, 1981) or acetylcholine response (Inoue *et al.*, 1983), no differences have been demonstrated for the adenylate cyclase system.

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