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Effect of aprotinin on the metabolism of adenosine in cultured heart cells

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Aprotinin, a polypeptide, inhibits the proteolytic activity of trypsin, chymotrypsin, plasmin, kallikrein and lysosomal enzymes, including the cathepsins [1]. Recently, we reported [2] that aprotinin possesses significant beneficial effects toward restoration of higher energy phosphate compounds in ischemic dog myocardium. The improved metabolic status was accompanied by increased coronary flow and a corresponding fall in resistance. The present investigation was undertaken in order to gain insight into the cellular mechanism(s) underlying these observed changes and specifically the increase in the level of adenosine compared with control animals.

Since adenosine, an important vasoactive metabolite, may play an important role in the regulation of coronary blood flow [3], it is possible that aprotinin may produce its beneficial effects by exerting an action on the metabolism of adenosine, thereby affecting the delivery of adenosine to the coronary resistance vessels. Thus, the effects of aprotinin on the uptake and release of adenosine from myocardial cells were studied. These studies were performed using an isolated heart cell culture system since in this system it is possible to accurately measure the uptake from or release of radioactive adenosine into the incubation medium. In addition, these cultured cells represent a fairly pure cardiac muscle preparation which minimizes the possibility of interference by other cell types present in the myocardium.

Cardiac cells were isolated from 16-day-old chick embryonic hearts using the procedure described by Mustafa *et al.* [4]. The viability of the preparation was assessed by examining the monolayer cultures by phase contrast microscopy. Cells in culture, observed after 24 hr of plating, beat spontaneously, and about 85 per cent of the cells were revealed to be beating cardiac muscle cells [5]. Cultures older than 48 hr were not used because the ratio of fibroblasts to muscle cells increased as the culture aged.

Effect of aprotinin on release of radioactive adenosine from cultured heart cells. Following the change of growth medium 24 hr after plating, 0.05 ml [U-^{14}C]-adenosine (sp. act. 2.71 mCi/m-mole; 26,665 cpm; Amersham/Searle, Arlington Heights, Ill, U.S.A.) was added to each culture dish (5 ml) along with unlabeled adenosine (10^{-4} M) in order to label the nucleotide pool(s) of the cells. After incubating for up to 48 hr, these cultures were used to assess the release of radioactivity under hypoxic conditions. The medium was decanted and the adhering cell layer washed several times with modified Hanks solution to remove cell debris and growth medium, which also contained labeled adenosine and its metabolites. An assay mixture containing 0.1 ml aprotinin (an appropriate amount in saline) and 0.9 ml of modified Hanks solution (pH 7.4 without glucose) was layered over the cells. In control experiments, aprotinin was replaced by an additional 0.1 ml of modified Hanks solution. The dishes were mixed by swirling and were then incubated at 37° for 30 min in an incubator under 95% N_2 and 5% CO_2 to accelerate the breakdown of adenine nucleotides. At the end of the incubation, the thin layer of cells was scraped from the dishes with a plastic spatula and the cells were immediately transferred to polyethylene centrifuge tubes and immersed in ice. The samples were now processed for radioactivity according to the procedure described elsewhere [4]. The variation in quenching from sample to sample was negligible. The protein precipitate obtained from the cell fraction was used for protein measurement by the method of Lowry *et al.* [6].

Incorporation of radioactive adenosine into cardiac cells at varying periods of incubation. The cultures (grown for 48 hr or less) were washed thoroughly without disturbing the cell layer, using the method described above. The assay was carried out in the culture dishes. The incubation mixture consisted of 0.1 ml [U-^{14}C]-adenosine (sp. act. 2.71 mCi/m-mole) and modified Hanks solution containing phosphate

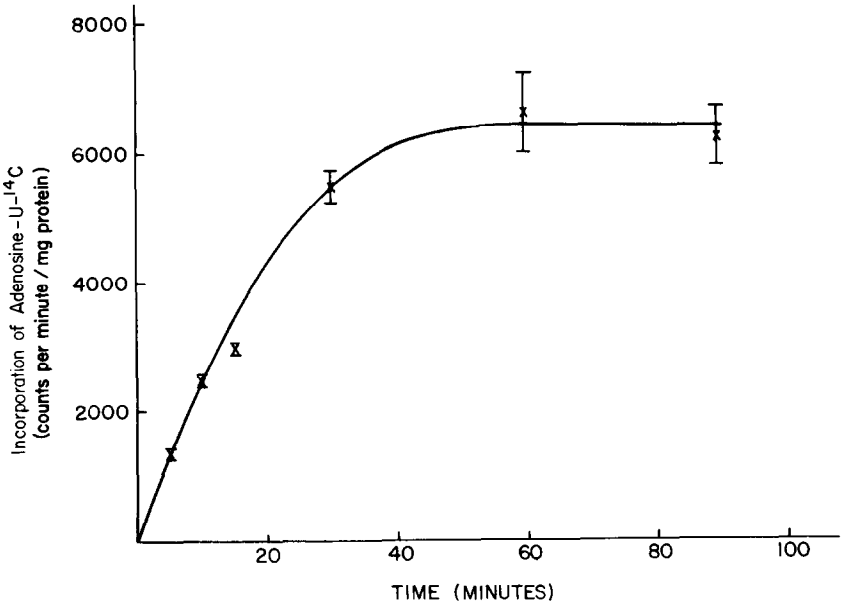

Fig. 1. Relationship between the intracellular uptake of radioactive adenosine and the incubation time in cultured cardiac cells. The assay mixture (2 ml) contained 0.1 ml of radioactive adenosine (sp. act. 2.71 mCi/m-mole) and modified Hanks having no glucose (pH 7.4).

buffer (pH 7.4) with no glucose in a final volume of 2.0 ml. The dishes were then incubated at 37° for the desired time with shaking. The cells and medium were separated and processed for radioactivity as described [4].

Effect of aprotinin on the relationship between cellular adenosine uptake and extracellular adenosine concentration. The washed cultures described earlier were incubated at 37° for 30 min. The final concentration of adenosine ranged between 1 and 30 μM and individual solutions contained 9.22 pmoles adenosine[U-^{14}C]. At the end of incubation, the samples were processed for radioactivity according to the procedure described [4].

Release of adenosine in the presence of aprotinin. Aprotinin at concentrations ranging from 2000 to 10,000 KIU was incubated with cells under hypoxic conditions (95% N_2 + 5% CO_2). No significant effects of aprotinin on the release of radioactivity into the incubation medium were observed in these experiments. Further, the cell fraction, at the end of incubation, was counted for radioactivity and found to be no different from controls. Thus, aprotinin had no significant effects on the release of adenosine from the cardiac cells.

Incorporation of radioactive adenosine into the cardiac cells after varying periods of incubation. Cultured heart cells were incubated with radioactive adenosine for 5, 10, 15, 30, 60 and 90 min. The results of these experiments, expressed as counts per min (cpm) per mg of protein incorporated into the cell fraction after varying periods of incubation, are given in Fig. 1. There is a linear rise in the cpm taken into the cells up to a period of 30 min, indicating a net uptake of adenosine. The activity reached a steady state level at 60 min.

Effect of aprotonin on the relationship between cellular adenosine uptake and extracellular adenosine concentration. Cultured heart cells were incubated with varying concentrations of adenosine both in the presence and absence of aprotinin. The results of these experiments are given in Fig. 2.

In control experiments, the uptake of adenosine followed saturable kinetics with a V_{max} of 0.90 nmoles/mg of protein and a K_m value of 3–6 μM. The effects of aprotinin on the uptake of adenosine were also studied using different concentrations of aprotinin, namely 4000 and 10,000 KIU respectively (Fig. 2). The uptake of adenosine was significantly depressed by 4000 KIU aprotinin and the V_{max} lowered from 0.9 to 0.50 nmole/mg of protein with no significant change in the K_m value. Uptake of adenosine at lower concentrations (1–3 μM) was slightly affected at 4000 KIU of the drug but markedly affected at higher concentrations of adenosine (10–20 μM). In the latter case, the uptake of adenosine was lowered to almost half of the control value. When the concentration of aprotinin was increased to 10,000 KIU aprotinin, the V_{max} was lowered to 0.18 nmole/mg of protein, a greater than 2-fold depression compared to 4000 KIU aprotonin, indicating that the effect was dose dependent. At 4000 and 10,000 KIU concentrations of aprotinin, there were no significant effects on the K_m value of the uptake process. These data suggest a noncompetitive inhibition of the uptake of adenosine with aprotinin.

In order to further confirm the type of inhibition, a separate experiment was performed in which 5 and 10 μM adenosine were incubated in the presence of 2, 4, 8 and 16 $\times$ 10^3 KIU concentrations of aprotinin. The data obtained are shown in Fig. 3. Since the curves with both concentrations of adenosine have the same intercept on the abscissa, this confirms noncompetitive inhibition. A K_i value of 24,200 KIU was obtained from this plot.

Inadequate perfusion of cardiac tissue can result in metabolic alterations leading to irreversible changes in cardiac muscle fibers [7]. Among the prominent metabolic changes associated with varying periods of cardiac ischemia are: (1) a reduction of glycogen stores [8]; (2) a breakdown of energy-rich compounds [9]; (3) ion imbalances [10]; (4) local release of catecholamines [11]; (5) accumulation of lactic acid [12]; and (6) activation of lysosomal proteases [13]. A number of workers have proposed [14] a role for lysosomal proteases in the development of irreversible cardiac cell damage during ischemic conditions. Thus, the rationale behind the use of aprotonin in the treatment of a wide variety of low flow states, including acute myocardial ischemia [2], lies in its ability to inhibit the release of proteases from the lysosomes.

Using an isolated cardiac cell system as a model for mammalian myocardium, it can be seen that aprotinin blocked the uptake of adenosine into the myocardial cells at all concentrations studied. An almost 50 per cent inhibition in the uptake of radioactive adenosine was noted at 4000 KIU.

In the present experiments we did not measure the degradative products of adenosine in the medium or cell fraction similar to those reported by Huang and Drummond [15]. Also, since the data exhibited Michaelis–Menten kinetics, we are apparently justified in equating the incorporation of radioactivity with the uptake of adenosine into the cellular fraction, especially since the label in the medium showed a parallel decrease with time. Thus, the incorporation of radioactive adenosine into the cell fraction most likely

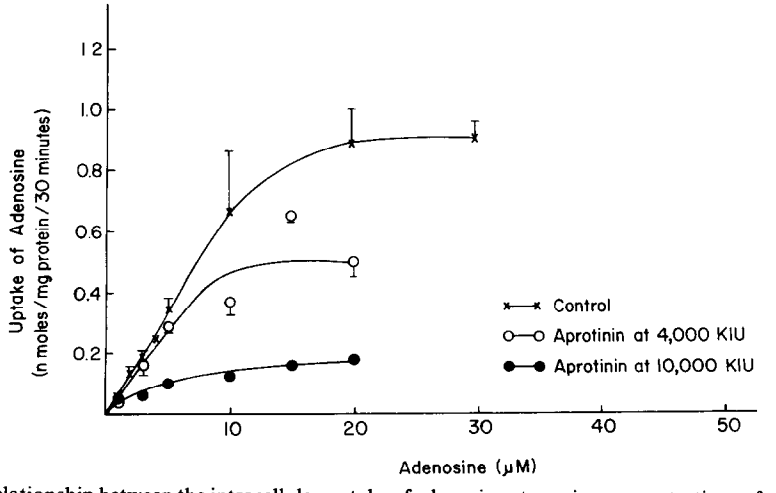

Fig. 2. Relationship between the intracellular uptake of adenosine at varying concentrations of extracellular adenosine in the presence and absence of aprotinin. Various concentrations of adenosine had a varying specific radioactivity. Other details are the same as in Fig. 1.

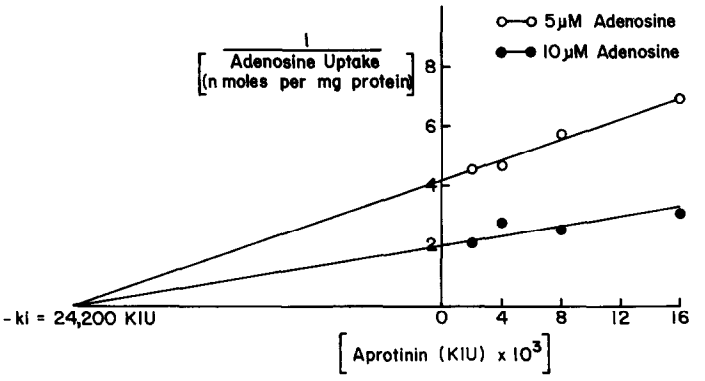

Fig. 3. Dixon plot showing the relationship between the inverse uptake of adenosine in 30 min and varying concentrations of aprotinin at 5 and 10 μM concentrations of extracellular adenosine. Other details are the same as in Fig. 1.

represents the uptake of adenosine. The uptake of adenosine at varying concentrations of extracellular adenosine increased linearly up to 10 μM and reached a steady state between 20 and 30 μM. Also, we have observed (S. J. Mustafa) an increase in the uptake of adenosine after the plateau phase, indicative of a simple diffusion at adenosine concentrations (extracellularly) of 50 μM or greater. Similar results have been reported in human red blood cell ghosts [16]. In the present experiments the uptake of adenosine is lower than reported earlier [17] with chick embryonic heart cells. One explanation for this observed difference is that, in the present experiments, levels of inosine and hypoxanthine, the degradation products of adenosine, were not measured. In the earlier report [17], the freshly isolated cells from 16-day-old chick embryonic hearts were used which may contain cells other than cardiac muscle as well as damaged cells which could account for higher uptakes. In the present investigation, freshly isolated cells were cultured for about 2 days, the growth medium was changed at the end of 24 hr and the cultures were washed thoroughly before the assay, which would tend to remove damaged cells and leave behind only healthy cells which are attached to culture dishes. Cultured cells, when added to the growth medium, have a chance to regenerate their membranes and to improve the levels of ATP while fresh cells have depressed ATP values [18]. These differences, in addition to not accounting for inosine and hypoxanthine in the cell or medium fractions, could well account for the higher uptake observed earlier. The mean value of the K_m of uptake of adenosine was noted at 3–6 μM, which is close to that previously reported [17]. These data indicate that the uptake of adenosine at low substrate concentrations into the cardiac cells probably takes place through a carrier-mediated process.

Additionally, in a separate experiment, when we measured the uptake of adenosine at substrate concentrations greater than 30 μM, the values obtained in the presence and absence of aprotinin were different. This finding indicates that even higher concentrations of adenosine cannot overcome this irreversible noncompetitive inhibition due to aprotinin. Also, the inhibition in the uptake of adenosine due to aprotinin at 10,000 KIU was rapid in action and was achieved in less than 5 min. Aprotinin seemed to have an effect only on the carrier-mediated portion of the uptake. The K_m of uptake was unchanged due to aprotinin, which is characteristic of a noncompetitive inhibition. It is of interest that there are no other compounds which have been shown to display this type of inhibition in the uptake of adenosine. Although aprotinin blocked the transport of adenosine, it is not a potent inhibitor since it had a high K_i value.

With respect to the specific mechanism of inhibition, it remains unclear how aprotinin blocks the uptake of adenosine into cardiac cells. In this regard, there were no significant effects of aprotinin on adenosine deaminase, adenosine kinase, and 5′-nucleotidase at concentrations comparable to those employed in the uptake experiments [2]. Thus, the ability of aprotinin to block the uptake of adenosine is not mediated via inhibition of adenosine kinase or adenosine deaminase. Additionally, aprotinin had no effect on the release of adenosine, thus excluding the possibility of more adenosine being provided extracellularly. The inhibitory action of aprotinin could result in higher extracellular concentrations of adenosine, thus exposing coronary resistance vessels to this higher concentration and producing an enhanced vasodilation and overall increases in coronary blood flow. Such increases in coronary flow may result in better perfusion of ischemic myocardium, and better preservation of high energy phosphate compounds in the infarcted portion of the myocardium [2].

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Inhibition of histamine-sensitive adenylate cyclase from the guinea pig gastric mucosa by nolinium bromide

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Recently discovered H_2-receptor antagonists [1, 2] not only have pharmacological properties which are distinct from those of classical antihistaminics (H_1-receptor antagonists) but also have a very characteristic chemical composition [2, 3]. H_2-histamine antagonists are basically structural analogs of histamine; these compounds all contain an imidazole ring with a polar, although uncharged, side chain [2, 3] (Fig. 1). On the other hand, H_1-antagonists are distinguished by the presence of aryl rings and resemble histamine only by the presence of an ethylene group with ammonium (or other similarly charged groups) at the termination of the side chain [2–4] (Fig. 1). The unique structural features of H_2-receptor antagonists are believed to be a prerequisite for their specific pharmacological effects, including their ability to block histamine-stimulated gastric acid secretion [3, 4].

There is considerable evidence indicating that in numerous vertebrate species [4, 5], including man [6], the HCl secreting action of histamine is elicited via mediation of cAMP as a second messenger. For example, in guinea pig, man, dog, rabbit and rat gastric mucosa [4–6] the stimulation of adenylate cyclase and/or the accumulation of cyclic 3′,5′-adenosine monophosphate (cAMP) in this tissue elicited by histamine is specifically blocked by H_2-receptor antagonists, thus supporting the notion that the fundic gastric mucosa adenylate cyclase is associated with H_2-receptors [4, 5].

Nolinium bromide [2-(3,4-dichlorophenylaminoanilino)-quinolizinium bromide] (Fig. 1), a recently synthesized compound with known antispasmodic properties [7, 8], was also found to be an effective inhibitor of gastric HCl secretion [7, 8]. Because of the potential importance of adenylate cyclase in the regulation of gastric HCl secretion, and because of considerable structural diversity between nolinium bromide (Nolinium Br) and classical H_2-receptor antagonists (Fig. 1) we addressed ourselves to the question of whether Nolinium Br may exert its HCl antisecretory effects through inhibition of histamine-stimulated adenylate cyclase. We examined the effects of Nolinium Br on basal adenylate cyclase activity and the activity stimulated by histamine, by prostaglandin PGE_2 and by the non-hormonal stimulatory agents such as 5′-guanyl-iminodiphosphate [Gpp(NH)p] and sodium fluoride (NaF).

Adenylate cyclase activity was measured in the crude membrane fraction for the guinea pig fundic mucosa, as described in detail in our previous communications [9, 10]. The protein content of our enzyme preparations was measured by the method of Lowry *et al.* [11]; the specific activity of adenylate cyclase was expressed in pmoles/min/mg of protein [9, 10]. Stimulation of adenylate cyclase was expressed as per cent increase ($\Delta\% \pm$ S.E.M.) over basal activity of the enzyme.

As in previous experiments, gastric fundic mucosa adenylate cyclase was stimulated markedly by 10^{-4} M histamine ($\Delta\% + 340 \pm 69$; n = 7), by 3×10^{-5} M PGE_2 ($\Delta\% + 149 \pm 44$, n = 4), by 10^{-4} M Gpp(NH)p ($\Delta\% + 444 \pm 53$, n = 4) and by sodium fluoride ($\Delta\% + 741 \pm 86$, n = 7). In the presence of 2×10^{-4} M Nolinium Br the stimulation of adenylate cyclase by 10^{-4} M histamine was markedly reduced to $\Delta\% + 94 \pm 27\%$ ($P < 0.05$, paired *t*-test; n = 7). On the other hand, Nolinium Br in the same concentration had no effect on the basal activity of adenylate cyclase or on the enzyme activity stimulated by PGE_2, Gpp(NH)p or by NaF.

Inhibition of histamine-stimulated adenylate cyclase activity with Nolinium Br was dose-dependent (Fig. 2, upper panel) over a range of concentrations similar to the inhibitory concentrations observed for metiamide [9, 12, 13]. The effects of Nolinium Br were also tested over varying concentrations of histamine. In the presence of Nolinium Br the slope of the dose–response curve of adenylate cyclase stimulation by histamine was displaced to the right, and the maximal stimulation by histamine was reduced (Fig. 2, lower panel).

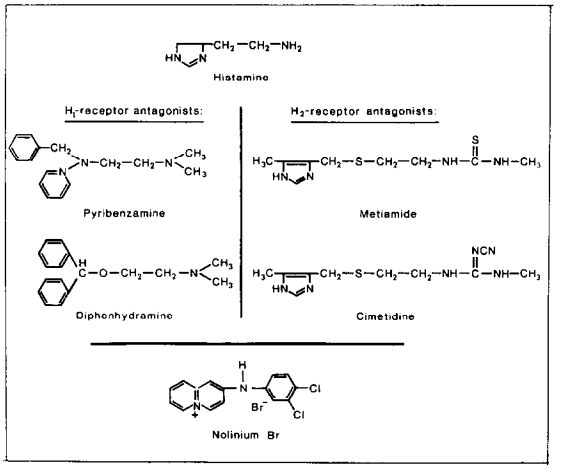

Fig. 1. Comparison of the chemical structures of histamine, typical H_2-antagonists (metiamide and cimetidine), typical H_1-antagonists (pyribenzamine and diphenhydramine) and nolinium bromide.