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Evidence for a Cell Surface Adenosine Receptor on Coronary Myocytes and Atrial Muscle Cells

Studies with an Adenosine Derivative of High Molecular Weight*

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Summary. We have studied the site of action of adenosine with the aid of a large molecular weight adenosine derivative which is confined to the extracellular space. A stable protein-AMP-conjugate was formed using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide to covalently couple AMP by P-N linkage to lysine residues of a carbonic anhydrase preparation. The conjugate was characterized by disc-SDS-electrophoresis and exhibited a mean molecular weight of about 30000. When infused into the coronary arteries of isolated guinea pig hearts the protein-AMP-conjugate induced vasodilation which was similar in magnitude and time course to that elicited by free AMP or adenosine. The dilatory response of the coronaries was caused by the protein-AMP-conjugate itself and not by free AMP or by adenosine liberated from the conjugate. This conclusion is based on the facts that: i) The electrophoretic mobility of the conjugate remained unchanged after its passage through the heart. ii) Addition of 5′-nucleotidase and adenosine deaminase to the protein-AMP-conjugate prior to its infusion into the coronaries did not alter the vasoactive effects. iii) Perfusion of the isolated hearts with equipotent concentrations of ^{14}C-AMP, ^{14}C-adenosine or protein-^{14}C-AMP-conjugate resulted in a significant incorporation of radioactivity into cardiac adenine nucleotides only in the case of labeled AMP and adenosine. Besides its effects on the coronaries, the protein-AMP-conjugate also rapidly abolished the calcium-dependent action potential in the atrial muscle. Since the biological effects observed are most likely caused by the adenosine moiety of AMP our results provide evidence that AMP as well as adenosine act via a receptor site on the surface of coronary myocytes and atrial muscle cells.

Key words: Isolated heart – Vascular permeability – Myocardial blood flow – Adenosine – Adenosine receptor.

INTRODUCTION

It has been proposed that adenosine, a potent vasodilating compound [9], plays an important role in the regulation of coronary blood flow [2, 6]. Furthermore, adenosine inhibits contractile force of atrial muscle [9]; this effect is probably caused by an inhibition of the transmembrane Ca^{2+}-influx [22]. Concerning the primary site of adenosine action it has been postulated that this might be the membrane of coronary myocytes [4, 16] and atrial muscle cells [22]. This postulate is supported by findings which indicate that also ATP, ADP and AMP are biologically active, although phosphorylated purine compounds probably do not penetrate the cellular membranes because of their negative charge. On the other hand, adenosine is known to readily permeate the myocardial cell membrane [17] and to be rapidly metabolized [30]. It is therefore conceivable that adenosine might induce its effect primarily by an intracellular mechanism.

In order to distinguish between the possible modes of adenosine action, the biological effects of a newly synthesized soluble protein-AMP-conjugate were tested. Because of the large size of the protein chosen (carbonic anhydrase, MW 30000 [19]) the conjugate most likely remains only in the extracellular space. It should therefore be possible to decide whether adenosine acts via an intracellular mechanism or by a direct effect on surface receptor sites.

MATERIALS AND METHODS

Bovine carbonic anhydrase (EC 4.2.1.1), bovine chymotrypsinogen, ovalbumin, bovine albumin, acrylamide, N,N′-methylenbisacryl-

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amide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were purchased from Serva, Heidelberg. Adenosine, AMP, adenosine deaminase (EC 3.5.4.4), aldolase (EC 4.1.2.13) and bovine LDH (EC 1.1.1.27) were obtained from Boehringer, Mannheim. 5'-Nucleotidase was purchased from Sigma, München. (U-^{14}C)-AMP, specific activity 570 mCi/mmol, was obtained from the Radiochemical Centre, Amersham. PEI-cellulose thin-layer plates (Polygram, CEL 300-PEI) were purchased from Macherey-Nagel, Düren. Activated charcoal (Aktivkohle, No. 2186) and all other analytical reagent grade chemicals were obtained from Merck, Darmstadt.

Preparation of the Protein-AMP-Conjugate. Carbonic anhydrase was purified on a sephadex G-50 column (2.5 cm × 40 cm; elution with 0.1 N sodium phosphate buffer, pH 7.0 at 50 ml/h), dialyzed against H_2O for 24 h at 4° C (Visking dialysis bags) and concentrated using an Amicon diaflow cell (filter PM 10). AMP was coupled to carbonic anhydrase according to the method of Halloran and Parker [10]. 25 mg of the purified carbonic anhydrase were dissolved in 0.5 ml of water to which 65 mg AMP were then added and the pH was adjusted to 7.0. After addition of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, the reaction mixture was kept at room temperature for 24 h in the dark. Subsequent to centrifugation at 10000 × g for 2 min, the protein-AMP-conjugate was freed from the small molecular weight components of the reaction mixture by passage through a sephadex G-25 column (0.9 cm × 25 cm; elution with 0.1 N sodium phosphate buffer, pH 7.0). The conjugate was then dialyzed at 0° C and finally concentrated to 1 ml (protein concentration about 10 mg/ml) by use of an Amicon diaflow cell (filter PM 10). The concentrated protein-AMP-conjugate was immediately used for all further experiments.

When radioactively labeled AMP was coupled to carbonic anhydrase, 25 µCi of (U-^{14}C)-AMP were evaporated to dryness and the residue taken up in 0.5 ml H_2O containing 25 mg AMP. Coupling of the nucleotide to carbonic anhydrase was carried out as already described.

In order to determine the molar ratio between bound AMP and protein, two methods were applied. In one case binding of AMP was calculated by relating the radioactivity of the protein-^{14}C-AMP-conjugate to the specific activity of AMP in the reaction mixture. In the other case, calculation was based on the phosphate content of the conjugate as determined by the method of Gerlach and Deuticke [7]. Protein measurements were performed with the biuret test.

Electrophoretic Techniques. Polyacrylamide gel electrophoresis of native and modified carbonic anhydrase was performed in an Ultraphor (Colora/ISCO) electrophoresis apparatus. A discontinuous buffer system according to Ornstein and Davis [18] in the modification of Nees [15] was applied.

For SDS-electrophoresis a discontinuous SDS-buffer system according to Neville [14] in the modification of Jennissen [11] was used. In order to determine the protein content of the different fractions separated, the gel was stained with amido-black and the visible protein bands were cut out and eluted with 0.1 N NaOH at 40° C. After centrifugation the blue colour of the supernatant was quantitated spectrophotometrically at 800 µm.

Animal Experiments. Isolated guinea pig hearts were perfused according to the Langendorff technique with a modified Krebs-Henseleit solution, equilibrated with 95% O_2 – 5% CO_2 (methodological details see [3]). Coronary flow was monitored with a Statham M-4001 electromagnetic flowmeter and an IVM flow probe (type K-2B) which was incorporated into the aortic cannula. All compounds were administered by an infusion pump (Infors) directly into the aortic inflow. Left ventricular tension was monitored directly from a pressure transducer connected to a small rubber balloon inserted into the left ventricle. Developed tension was electronically differentiated to yield heart rate and dP/dt (Elektromanometer and Physio-Differentiator, Hugo Sachs Elektronik). The hemodynamic parameters were recorded on a Beckman dynograph (type R-411).

In experiments in which the hearts were perfused with ^{14}C-adenosine, ^{14}C-AMP and protein-^{14}C-AMP-conjugate, stop freezing of cardiac tissue to the temperature of liquid nitrogen and extraction of the tissue with 0.5 N perchloric acid was performed as has been described in detail elsewhere [21]. In brief, tissue extracts were neutralized with KOH, filtered through an Amicon diaflow cell (filter PM 10) and were then treated with activated charcoal. After elution of the purines from the charcoal with a mixture of 10% pyridine and 50% ethanol, the eluates were applied as streaks on PEI-cellulose thin layer plates together with carrier amounts of ATP, ADP and AMP (0.3 µmoles each). The plates were developed first with distilled water and, after drying, in a second run with 1.25 N NaCl as described by Jones et al. [12]. After separation, the UV-light absorbing bands representing the different nucleotides were scraped off and assayed for radioactivity [21].

Preparation of the isolated guinea pig atrium and intracellular microelectrode recording was performed as recently described [22]. The tissue chamber contained 2.0 ml of the modified Krebs-Henseleit solution which was equilibrated with 95% O_2 – 5% CO_2 and was recirculated by a roller pump. After 30 min of incubation, the medium was replaced by a Krebs-Henseleit solution containing 22 mM K^+. Norepinephrine at a final concentration of 5×10^{-5} M was then added, thereby restoring electrical activity. All compounds were added directly to the bath.

Radioactivity Measurements. Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer (model 3380). Each vial contained 10 ml scintillation fluid (0.1 g POPOP, 5.5 g POP, 750 ml toluene, 250 ml Triton X-100).

RESULTS

Characterization of the Protein-AMP-Conjugate. Electrophoretic separation of the commercially available carbonic anhydrase, purified on sephadex G-50, revealed three distinst protein bands (Fig. 1A). The upper two represent the isoenzymes A and B of carbonic anhydrase (same molecular weight of 30000, but different charge [19]), the lower band constitutes a protein contaminant. This carbonic anhydrase preparation was modified with AMP according to the methods described. The electrophoretic pattern of the resulting protein-AMP-conjugate is given in Figure 1B and demonstrates, that the mobility of the AMP modified carbonic anhydrase is considerably enhanced. This must be attributed to the binding of negatively charged AMP residues to the protein. It also should be noted, that the initially observed protein contaminant was no longer detectable in the modified protein preparation. Perfusion of the isolated heart with the protein-AMP-conjugate did not change the electrophoretic pattern of the conjugate (Fig. 1D). Furthermore, no proteins could be detected in the coronary effluent perfusate collected under control conditions (Fig. 1C).

In order to investigate whether the protein-AMP-conjugate used through out this study, might contain

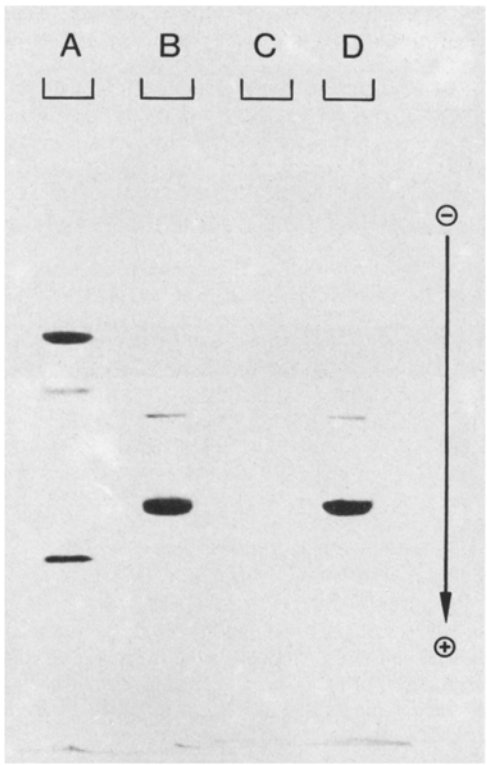

Fig. 1 a–d. Gel-electrophoresis in 7% polyacrylamide. Conditions of separation: Gel-thickness 2 mm. Ratio of acrylamide to acrylamidomethane 37.5:1. Voltage during stacking 50 V. Voltage during electrophoresis along the gel 100 V. The gel was stained with amido-black. (A) Carbonic anhydrase after purification on Sephadex G-50; protein applied: 200 µg. (B) Protein-AMP-conjugate, protein applied: 200 µg. (C) Coronary effluent perfusate. Perfusate (200 ml) collected under control conditions was concentrated (Amicon diaflow cell, filter PM 10) and the residue was applied to the gel slot. (D) Protein-AMP-conjugate after its passage through the heart at a concentration (40 µg/ml) which doubled coronary flow. Perfusate (200 ml) containing the protein-AMP-conjugate was concentrated as described under (C); protein applied: 200 µg

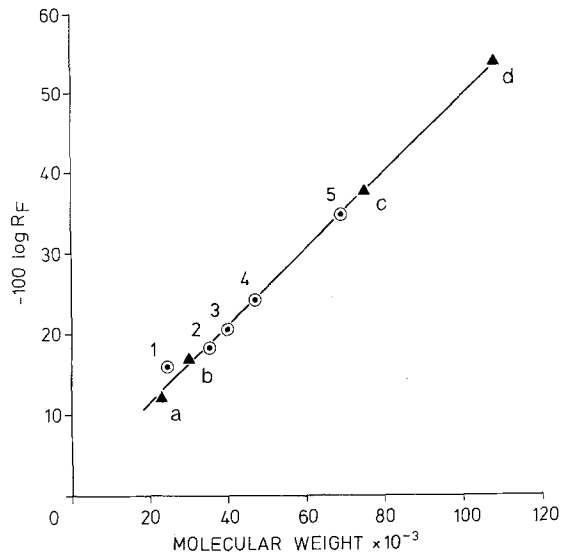

Fig. 2. SDS-gel-electrophoresis for determination of molecular weight distribution of the protein-AMP-conjugate. Degree of cross-linkage, dimensions of the gel and applied voltage were the same as given in legend to Figure 1. Calibration proteins: 1. chymotrypsinogen, MW 25000; 2. LDH, MW 35000; 3. ovalbumin MW 40000; 4. aldolase, MW 48000; 5. albumin, MW 69000. *a–d*: protein components of the protein-AMP-conjugate

low molecular weight proteins, it was further characterized by SDS-electrophoresis. As is evident from Figure 2, the protein-AMP-conjugate contained only high molecular weight proteins (MW: 23000, 30000, 75000, 108000). Determination of the protein content of the different fractions revealed that the two isoenzymes of carbonic anhydrase (MW: 30000) constitute more than 80% of the protein mixture. The occurrence of small amounts of proteins of high molecular weight might be attributed to cross-linkage of carbonic anhydrase molecules induced by the coupling reagent.

Based on the finding that carbonic anhydrase was by far the major protein fraction of the conjugate, we determined that about 2 moles AMP were bound to 1 mole of protein. This value was derived from radioactivity measurements of protein-^{14}C-AMP and closely corresponded to data obtained by quantitative phosphorus analyses.

The chemical stability of the protein-AMP-conjugate was assessed in a separate experimental series. From the results given in Figure 3 it is evident that at pH 7 and after 1 h of incubation, formation of free AMP and adenosine was below 1% of the total AMP. At pH 9, AMP and adenosine were liberated at higher rates, whereas at pH 4 only AMP was generated.

Effect of the Protein-AMP-Conjugate on Coronary Flow. Infusion of the protein-AMP-conjugate into the coronaries of isolated guinea pig hearts induced hemodynamic changes, which were qualitatively similar to those observed with AMP (Fig. 4). Application of respective amounts of pure carbonic anhydrase, however, did not alter coronary flow. When compared on a molar basis, the potency of free AMP was about 6 times greater than that of AMP bound to the protein. It is interesting to note that neither the protein-AMP-conjugate nor free AMP significantly altered heart rate, ventricular pressure and dP/dt.

Theophylline has been shown to inhibit coronary dilation of exogenously applied adenosine [1,3,20]. Also in our experiments the dilatory action of the protein-AMP-conjugate proved to be greatly attenuated in presence of theophylline (4×10^{-5} M).

In order to demonstrate that coronary vasodilation induced by the protein-AMP-conjugate was not

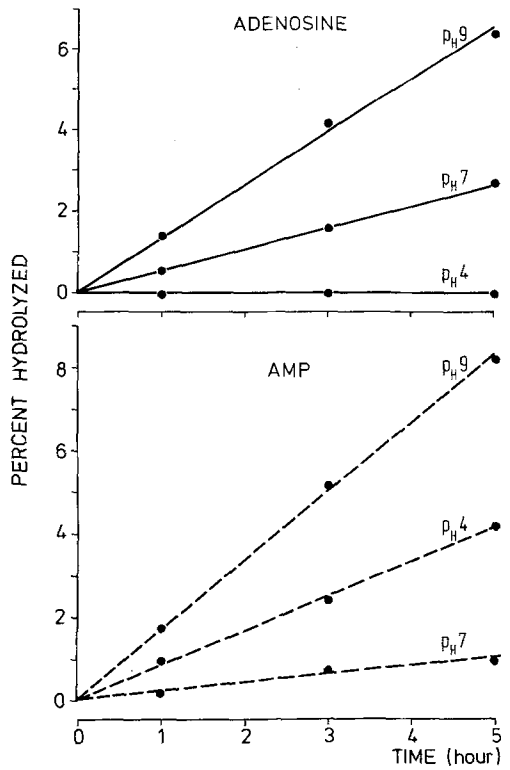

Fig. 3. pH-dependent hydrolysis of adenosine and AMP from the protein-AMP-conjugate at 37° C. Hydrolysis was assessed by measuring the formation of ^{14}C-adenosine and ^{14}C-AMP from protein-^{14}C-AMP (protein concentration: 7.5 mg/ml, specific activity of AMP: 0.15 mCi/mmole) in 0.1 N pyridine acetate buffer, pH 4 and 0.1 N triethylammonium formate buffer adjusted to pH 7 and pH 9. For analyses, samples were rapidly frozen with carrier amounts of adenosine and AMP, freeze-dried, applied on PEI-cellulose thin layer plates and developed in 0.5 N LiCl for 1.5 h. The respective areas on the plates containing AMP and adenosine were then scraped off and counted for radioactivity [21]

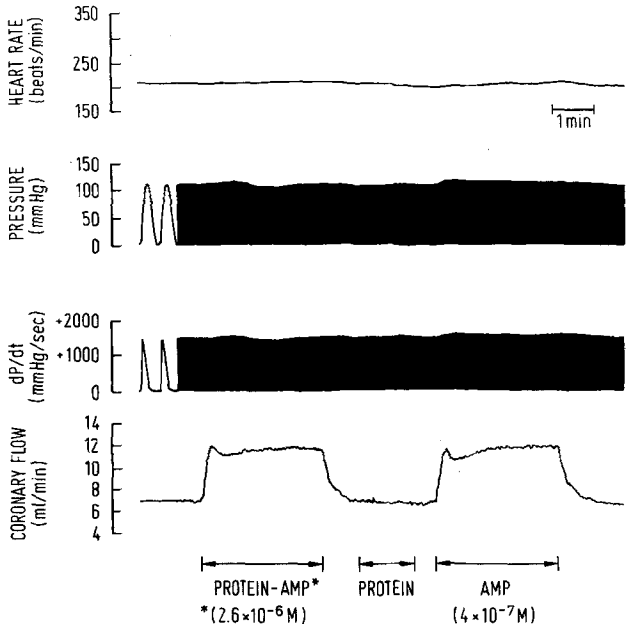

Fig. 4. Effect of infusion of protein-AMP-conjugate (protein-AMP), of carbonic anhydrase (protein) and of AMP on the hemodynamic parameters of the isolated guinea pig heart. Rate of protein infusion: 0.5 mg/min. In the case of protein-AMP, the given concentration refers to AMP bound to the protein

due to AMP or adenosine liberated from the protein, the following experiments were performed:

1. 5'-Nucleotidase and adenosine deaminase were added to the protein-AMP-conjugate prior to its infusion into the coronary arteries. Any free adenosine or AMP should be degraded by the presence of these enzymes. As can be seen from Figure 5, addition of the two enzymes did not diminish the coronary dilating effect of the protein-AMP-conjugate. On the other hand, coronary dilation elicited by free AMP and adenosine was rapidly abolished under these conditions.

2. After perfusion of isolated hearts with equipotent concentrations of ^{14}C-adenosine, ^{14}C-AMP and protein-^{14}C-AMP substantial incorporation of radioactivity into myocardial ATP and ADP was measured only in the case of labeled adenosine and ADP (Table 1). Only negligible radioactivity values could be detected in the adenine nucleotides after application of protein-^{14}C-AMP.

Effect of the Protein-AMP-Conjugate on the Calcium-Dependent Atrial Action Potential. In guinea pig atrium, the protein-AMP-conjugate effectively reduced the rate of rise and the amplitude of the slow action potential (Fig. 6). This effect occurred within several seconds and was completely reversible as indicated by the reappearance of the action potentials after wash-out of the protein-AMP-conjugate. Unmodified carbonic anhydrase did not influence the slow action potential.

DISCUSSION

Binding of small, but biologically active molecules to high molecular weight substances, which cannot penetrate cellular membranes has been introduced as an elegant tool in studies on the site of action of hormones [28, 29]. Recent results, however, indicate that the apparent biological activity of covalently linked growth hormone [23], insulin [8] and catecholamines [31] could be attributed in part to their gradual release from the supporting matrix. Therefore, demonstration of the stability of the compounds synthesized is essential in all these studies.

In the present investigation we coupled covalently adenosine monophosphate to carbonic anhydrase (MW: 30000) and found that infusion of the protein-AMP-conjugate into the coronaries of isolated hearts

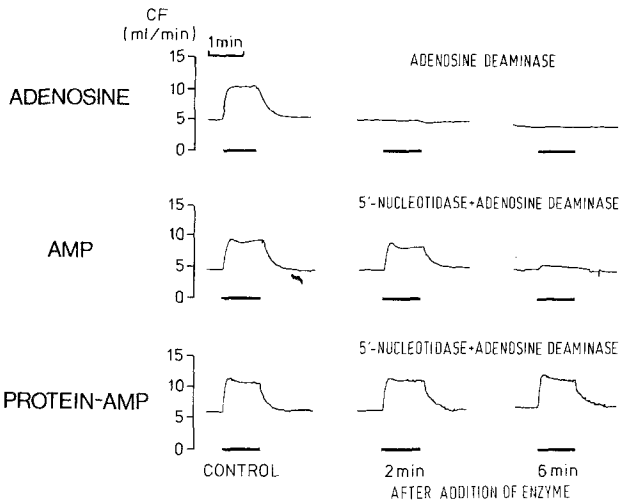

Fig. 5. Coronary dilating effects of adenosine, AMP and covalently bound AMP (protein-AMP) as influenced by adenosine deaminase and 5'-nucleotidase. In controls, adenosine (10 µg/ml), AMP (10 µg/ml) and protein-AMP (38 mg protein/ml) were infused at a rate to yield similar vascular responses. Rate of infusion < 50 µl/min. Enzymes were added to each solution to yield a final concentration of 2×10^{-3} U/ml for adenosine deaminase and 5×10^{-4} U/ml for 5'-nucleotidase

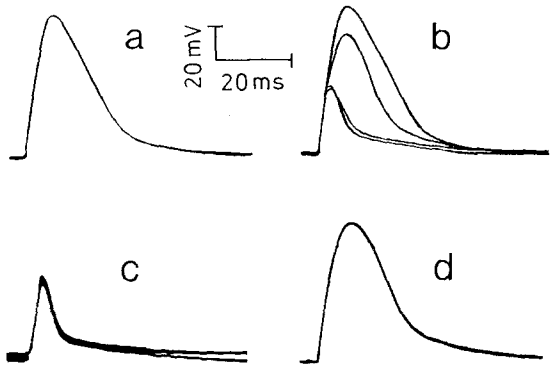

Fig. 6 a–d. Depressant effect of protein-AMP-conjugate on the slow action potential in guinea pig atria. The incubation medium, equilibrated with 95% O_2 – 5% CO_2, contained 22 mM K^+ and 5×10^{-5} M norepinephrine (for details see Methods and [22]). (a) Control action potential. (b) Successive tracings following additions of protein-AMP-conjugate (1.3 mg protein/ml, final concentration of protein-bound AMP: 7.6×10^{-6} M). (c) 3 min after addition of protein-AMP-conjugate. (d) 1 min after wash-out of protein-AMP-conjugate. All recordings from the same cell. Intensity of stimulus 90 V, duration 1 ms. Frequency of stimulation 0.5 Hz

Table 1. Incorporation of radioactivity into myocardial adenine nucleotides after perfusion for 15 min with (U-^{14}C)-AMP and protein-(U-^{14}C)-AMP. Specific activities of all purines: 0.15 µCi/µmol

	n	Coronary flow (% change)	ATP	ADP (cpm/g)	AMP
(U-^{14}C)-Adenosine (1.5×10^{-7} M)	2	46	178	30	3
(U-^{14}C)-AMP (4.0×10^{-7} M)	2	42	228	32	5
Protein-(U-^{14}C)-AMP (2.6×10^{-6} M)	4	54	3	2	3

induced a dose-dependent coronary vasodilation. All of our data indicate that the effects of the conjugate cannot be due to liberation of AMP or adenosine from the conjugate. In vitro experiments revealed that within 1 h at pH 7 less than 1% of the protein-bound AMP was hydrolyzed to yield AMP and adenosine. This rate, however, is much too low to account for the rapid onset of the coronary effects of the protein-AMP-conjugate. The possibility that under the conditions of the biological experiment more adenosine or AMP were formed, could also be excluded. The dilatory action of the conjugate was not altered after addition of 5'-nucleotidase and adenosine deaminase, which degrade free AMP and adenosine to vasoinactive inosine. Furthermore, no radioactivity was found to be incorporated into myocardial adenine nucleotides, when the hearts were perfused with protein-^{14}C-AMP. The protein-AMP-conjugate must therefore be considered to be a stable compound which is responsible for the effects observed.

According to the studies of Halloran and Parker [10] it is most likely that AMP in the protein-AMP-conjugate is predominantly – if not exclusively – linked via its phosphate to the ε-amino-group of lysine residues of the protein. The position of attachment of the AMP molecule leaves the ribose and the 6-amino group of the purine ring intact. These parts of the molecule are known from studies on the structure activity relationship of adenosine derivatives [5, 26] to be essential for their hemodynamic action. Therefore, the biological activity of the protein-AMP-conjugate appears to be mediated by the adenosine moiety of the bound AMP.

Because of the high molecular weight of the protein-AMP-conjugate which was confirmed by SDS-electrophoresis it is highly unlikely that this compound can permeate cellular membranes. Thus our data indicate that AMP linked to the protein induces coronary vasodilation by an action of its adenosine moiety on the membrane of the coronary myocyte, presumably by an interaction with an adenosine re-

ceptor. This conclusion supports and extends the studies of Bünger et al. [4]. It also confirms the work of Olsson et al. [16], who recently showed that adenosine covalently linked via N_6 of the purine ring to periodate oxidized stachyose dilates the coronaries. Interestingly, the effect of this compound could be antagonized by theophylline also linked to stachyose. These authors, however, did not provide information on the stability of the conjugates under their experimental conditions and they did not fully rule out the possibility that the adenosine stachyose complex with a molecular weight of only 1000 could penetrate the membrane of the coronary myocyte.

When the dilating activity of our protein-AMP-conjugate is compared with that of AMP, it is evident that about 6 times higher concentrations of protein-bound AMP were required to elicit the same dilatory effect as free AMP. This difference may be related to the finding that about 2 moles of AMP are bound to 1 mole of protein and that for steric reasons not each of the bound AMP molecules has equal chances to interact with the putative adenosine receptor.

In addition to the coronary dilating effect of the protein-AMP-conjugate we could also demonstrate that the conjugate inhibited the slow atrial action potential, which has been shown to be carried by Ca^{2+}-ions [22]. It thus appears that surface receptors for adenosine also exist in the atrial myocardium. However, as yet no information is available concerning the mechanism, by which adenosine might interact with the transmembrane Ca^{2+}-influx.

Despite the difference in the molecular size of AMP and protein-AMP-conjugate the onset of increase in coronary flow was almost identical with both substances. This finding indicates that the conjugate can readily affect the smooth muscle cells in our experimental preparation. Diffusion of macromolecules through the endothelium of capillaries are assumed to proceed via pores ($d \simeq 90$ or 500 Å), slits ($d \simeq 40-50$ Å) or vesicles [13, 24, 25, 27]. Because of the dimensions of the carbonic anhydrase molecule ($40 \times 50 \times 45$ Å [19]), the protein-AMP-conjugate can be expected to pass through endothelial cells, provided that similar permeability properties also pertain to the resistance vessels of isolated hearts. However, it is conceivable that the conjugate may affect the coronary myocytes from the vascular side, without permeating the endothelium. This possibility is presently explored in this laboratory by studying the dilating effects of protein-AMP-conjugates of different size and/or molecular weight.

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