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Inhibition of muscarinic K^+ current in guinea-pig atrial myocytes by PD 81,723, an allosteric enhancer of adenosine binding to A_1 receptors

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1 PD 81,723 has been shown to enhance binding of adenosine to A_1 receptors by stabilizing G protein-receptor coupling ('allosteric enhancement'). Evidence has been provided that in the perfused hearts and isolated atria PD 81,723 causes a sensitization to adenosine via this mechanism.

2 We have studied the effect of PD 81,723 in guinea-pig isolated atrial myocytes by use of whole-cell measurement of the muscarinic K^+ current ($I_{K(ACh)}$) activated by different G_i-coupled receptors (A_1, M_2, sphingolipid). PD 81,273 caused inhibition of $I_{K(ACh)}$ ($IC_{50} \simeq 5\ \mu M$) activated by either of the three receptors. Receptor-independent $I_{K(ACh)}$ in cells loaded with GTP-γ-S and background $I_{K(ACh)}$, which contributes to the resting conductance of atrial myocytes, were equally sensitive to PD 81,723. At no combination of concentrations of adenosine and PD 81,723 could an enhancing effect be detected.

3 The compound was active from the outside only. Loading of the cells with PD 81,723 (50 μM) via the patch pipette did not affect either $I_{K(ACh)}$ or its sensitivity to adenosine. We suggest that PD 81,723 acts as an inhibitor of inward rectifying K^+ channels; this is supported by the finding that ventricular I_{K1}, which shares a large degree of homology with the proteins (GIRK1/GIRK4) forming $I_{K(ACh)}$ but is not G protein-gated, was also blocked by this compound.

4 It is concluded that the functional effects of PD 81,723 described in the literature are not mediated by the A_1 adenosine receptor-G_i-$I_{K(ACh)}$ pathway.

Keywords: Atrial myocyte; cardiac cell; muscarinic K^+ current; adenosine receptor; muscarinic receptor; allosteric enhancer; PD 81,723; G protein; inward rectifier; K^+ current

Introduction

The purine nucleoside adenosine exerts numerous physiological actions in a variety of tissues. Most of these are mediated by different G protein-coupled receptors (see Collis & Hourani, 1993; Olah *et al*., 1995 for review). PD 81,723, a 2-amino-3-benzoylthiophene compound, has been shown to increase selectively ligand binding to the A_1 subtype of adenosine receptors (A_1R) (Amoah-Apraku *et al*., 1993; Bhattacharya & Linden, 1995). This and related compounds therefore have been termed allosteric enhancers. Functional effects of PD 81,723 on adenosine-induced negative dromotropism which are in line with such an allosteric enhancement of ligand-receptor interaction have been described in perfused hearts (Amoah-Apraku *et al*., 1993; Kollias-Baker *et al*., 1994a). In cardiac myocytes A_1R are coupled via (a) pertussis toxin sensitive G protein(s) (G_i/G_k) to adenylyl cyclase, which is inhibited if prestimulated e.g. by β-adrenoceptors (Wilken *et al*., 1990). Another target of A_1R or the coupling G protein respectively is muscarinic K^+ current ($I_{K(ACh)}$). This current, which is expressed primarily (in most species exclusively) in supraventricular cells including those of the sino-atrial and atrio-ventricular nodes belongs to the class of strongly inward rectifying K^+ channels. It is composed of two proteins (GIRK1 and GIRK4; Krapivinsky *et al*., 1995). Activation of G_i-coupled receptors in supraventricular cells causes increased opening activity of this channel. The muscarinic (M_2) receptor which binds acetylcholine (ACh) released from parasympathetic nerve endings is coupled to this channel and regulates most of its physiological activity. $I_{K(ACh)}$ is a major molecular target mediating vagal activity and contributes to regulation of the heartbeat.

In the present study the effect of PD on $I_{K(ACh)}$ elicited by adenosine, ACh and sphingosyl-phosphorylcholine (SPC), an agonist at a novel G_i-coupled sphingolipid receptor in cardiac cells (Bünemann *et al*., 1996) was investigated by means of conventional whole-cell voltage clamp. $I_{K(ACh)}$ was inhibited by PD 81,723 at concentrations ± one order of magnitude of the concentrations which had been used previously. Inhibition of $I_{K(ACh)}$ was independent of the activating receptor/agonist and was also seen, if $I_{K(ACh)}$ was activated by loading of cells with guanosine-5′-*O*-(γ-thiotriphosphate) (GTP-γ-S). No evidence for an enhancement of the action of adenosine was found in this cellular paradigm. We concluded that the A_1R-$G_{i(K)}$-$I_{K(ACh)}$ pathway is not involved in potentiation of adenosine-induced effects in the heart.

Methods

Isolation and culture of atrial myocytes

Guinea-pigs of either sex (200 to 250 g) were killed by cervical dislocation. The method of enzymatic isolation of atrial myocytes has been described in detail previously (Banach *et al*., 1993). The culture medium was bicarbonate-buffered M199 (Gibco, Dreieich Germany) containing gentamycin and kanamycin (Sigma, Deisenhofen, Germany); culture medium was not supplemented with foetal calf serum (FCS) in order to prevent a time-dependent loss in sensitivity to adenosine (Bünemann & Pott, 1995). Cells were plated at several hundred cells per dish on 36 mm culture dishes; 50 to 70% of the myocytes attached within 16 to 24 h. Cells were cultured and used experimentally from the day of isolation up to 7 days in culture. No differences were found between freshly isolated and cultured myocytes in the phenomena to be studied. Medium was changed every second day.

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Solutions

If not stated otherwise the culture medium was replaced 30 min before an experiment by a solution containing (mM): NaCl 120, KCl 20, $CaCl_2$ 2.0, $MgCl_2$ 1.0, HEPES/NaOH 10.0, pH 7.4. The solution for filling the patch-clamp pipettes for whole-cell voltage clamp experiments contained (mM): K-aspartate 100, KCl 40, NaCl 10, MgATP 5.0, EGTA 2.0, GTP 0.01 and HEPES/KOH 10.0, pH 7.4.

Current measurement

Membrane currents were measured in the whole-cell mode (Hamill *et al*., 1981). Pipettes were fabricated from borosilicate glass with filament (Clark, Pangbourne, U.K.) on a horizontal puller (DMZ, Munich, Germany) and were filled with the solution listed above. The liquid junction potential of the pipettes were compensated. The d.c. resistance of the filled pipettes ranged from 2 to 6 MΩ. Current measurements were performed by means of a patch clamp amplifier (List LM/EPC 7, Darmstadt, Germany). Signals were passed through an analogue filter (corner frequency of 1 to 3 KHz) and were stored on the hard-disc of an IBM compatible AT-computer, equipped with a hardware/software package (ISO2 by MFK, Frankfurt/Main, Germany) for voltage control, data acquisition and data evaluation. Experiments were performed at ambient temperature (22–24°C). If not otherwise stated, cells were voltage-clamped at a holding potential of −90 mV, i.e. negative to E_K (−50 mV). K^+ channel currents under this condition are in the inward direction. This experimental condition was chosen because of the strong inward-rectifying properties of $I_{K(ACh)}$. Ramp-shaped changes of E_m from −130 to +60 mV were used to measure current-voltage relations and to check that the access resistance had not changed. Rapid superfusion of the cells for application and withdrawal of different solutions was performed by means of a solenoid-operated flow system which permitted switching between up to 6 different solutions. The half time of exchange of solution was estimated as 200–400 ms by a change from standard (high K^+) to Cs^+ (5 mM) containing solution; this resulted in an instantaneous block of $I_{K(ACh)}$.

Materials

Standard salts were from Merck (Darmstadt, Germany). EGTA, HEPES, MgATP, GTP, acetylcholine/chloride, adenosine and SPC were from Sigma (Deisenhofen, Germany). PD 81,723 was kindly provided by Dr R.F. Bruns, Lilly Research Laboratories, (Indianapolis, U.S.A.). PD 81,723 (2-amino-4,5-dimethyl-3-thienyl-[3(trifluoromethyl)-phenyl]methanone) was dissolved in dimethylsulphoxide (DMSO) as a 100 mM stock solution. The highest concentration of DMSO (0.1% v/v) had no direct effect on the current under study nor did it affect the effects of ACh or adenosine. The identity of the sample with the published structure of the compound was verified by means of nuclear magnetic resonance (n.m.r.) in the Department of Organic Chemistry, Ruhr-University Bochum.

Results

In guinea-pig atrial myocytes freshly isolated or kept in serum-free culture for several days a saturating concentration of adenosine (100 μM) elicited $I_{K(ACh)}$, the amplitude of which on average was about 70% of the current activated by a saturating concentration of ACh (10 μM; Bünemann & Pott, 1995). Figure 1A shows a representative current trace recorded from a myocyte that was challenged by saturating concentrations of ACh (10 μM) and adenosine (100 μM). The voltage-dependence of the current activated by either agonist showed the strong inward rectification characteristic of $I_{K(ACh)}$ (B, C). In the presence of PD 81,723 (50 μM) peak currents evoked by ACh and adenosine were reduced by 92% and 94%, respectively. The *I*/V-curves of the inhibited current obtained by subtracting voltage ramp-generated *I*/V-curves labelled (a) and (d) (ACh) and (b) and (f) (adenosine) exhibited identical characteristics as the agonist-induced current (b, c) suggesting that PD 81,723 inhibited $I_{K(ACh)}$ irrespective of the activated receptor. The action of the compound on $I_{K(ACh)}$ was reversible within some tens of seconds. In the presence of PD 81,723 not only adenosine and ACh activated currents, but also, as can be seen in Figure 1A, the inward holding current in the absence of agonist was reduced – in the example shown by 166 pA. The *I*/V-characteristics of the inhibited 'background' current, again obtained by subtraction (c – e) displayed the same strongly inward-rectifying characteristics as $I_{K(ACh)}$ (D). Background current in atrial myocytes is partly carried by agonist-independent openings of $I_{K(ACh)}$ channels (Sakmann *et al*., 1983; Okabe *et al*., 1991). This basal activity of $I_{K(ACh)}$ has been shown to reflect basal activation of $G_{i(K)}$ molecules by non-liganded receptors (Kaibara *et al*., 1991).

Independence of the effect of PD 81,723 on the nature of the receptor was further confirmed by means of sphingosyl-phosphorylcholine (SPC) a sphingolipid which has been shown recently to activate $I_{K(ACh)}$ via a novel G_i-coupled sphingolipid receptor (Bünemann *et al*., 1996). In a previous study, by use of simultaneous activation of the M_2 and the SPC receptor, it has been demonstrated that they converge on the same population of channels (Banach *et al*., 1993). Similar data have been published for the M_2/A_1 receptors (Kurachi *et al*., 1987). In the experiment shown in Figure 2, which is representative of a total of six different cells, a myocyte was superfused with SPC (100 nM), resulting in activation of a steady-state inward $I_{K(ACh)}$ of about 1.2 nA. In the continuous presence of the sphingolipid PD 81,723 caused a reversible inhibition of the inward current and a reduction of the baseline current, which is in line with the assumption that both SPC-activated and basal $I_{K(ACh)}$ were affected by the compound. As in Figure 1, the I/V-curves of the receptor-induced and the inhibited current were identical (B). To analyse further at which level of the receptor-G_i-$I_{K(ACh)}$ pathway PD 81,723 exerts its inhibitory action, cells were loaded with GTP-γ-S (500 μM) in the pipette filling solution. After a brief (5 min) loading period the cell was superfused with ACh-containng (10 μM) solution, resulting in activation of $I_{K(ACh)}$ to its maximal steady-state level (not shown). This level was maintained after washing off the agonist. Figure 3 shows a current recording from a representative myocyte. Inward 'holding' current (background plus GTP-γ-S activated) was 1.9 nA at −90 mV. Upon superfusion with PD 81,723-containing solution (50 μM) a reversible inhibition of inward current by 1.4 nA was recorded. From this result, which qualitatively is representative of seven cells studied by means of this protocol, we hypothesize that the inhibitory action of PD 81,723 on $I_{K(ACh)}$ does not result from interference with receptor-G protein interaction, but reflects either an action on the $I_{K(ACh)}$ channel itself or on $G_{\beta\gamma}$-GIRK1 interaction.

In the experiments described so far PD 81,723 was used at a concentration of 50 μM which has been shown yield the optimal enhancement in binding studies (Bhattacharya & Linden, 1995). Functional effects in the heart have been detected below 10 μM (Amoah-Apraku *et al*., 1993; Kollias-Baker *et al*., 1994a). It is conceivable therefore that the potentiating effect of PD 81,723 on adenosine-evoked $I_{K(ACh)}$ is masked by the inhibitory action of this compound at higher concentrations. We therefore measured the effect of PD 81,723 (500 nM, 10 μM and 100 μM) on adenosine-$I_{K(ACh)}$ concentration-response curves. The results are summarized in Figure 4. All three concentrations of PD 81,723 resulted in a reduction of adenosine-induced current. At 50 nM PD 81,723 no change in adenosine-evoked $I_{K(ACh)}$ was seen (not shown).

In summary, at no combination of concentrations of adenosine and PD 81,723 tested was a higher sensitivity compared to PD 81,723-free conditions found.

The experimental conditions used so far, namely a holding potential negative to E_K were selected because of the strong inward-rectifying properties of $I_{K(ACh)}$. As can be estimated

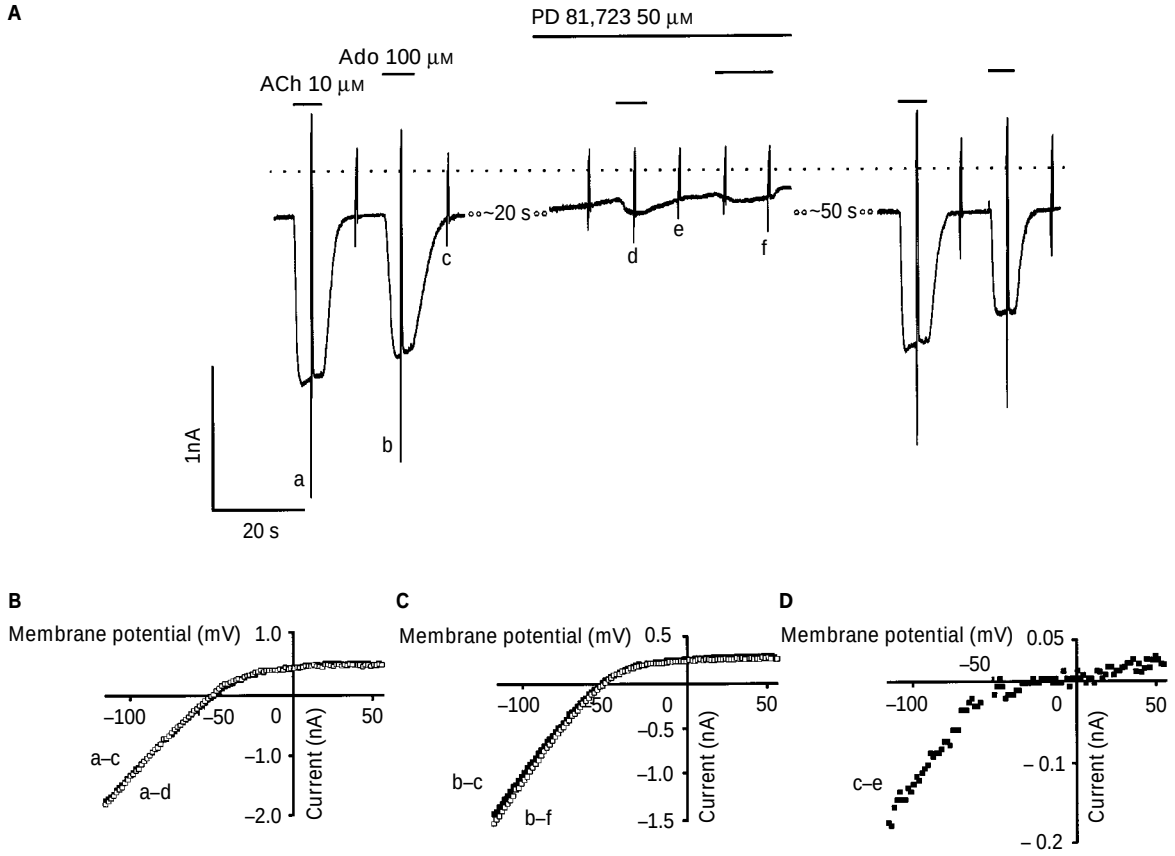

Figure 1 Inhibition of $I_{K(ACh)}$ by PD 81,723. (A) Inward $I_{K(ACh)}$ was measured as described in Methods. Superfusion with solutions containing 10 μM ACh, 100 μM adenosine (Ado), 50 μM PD 81,723 or PD 81,723 plus one of the agonists is indicated by the horizontal bars. Rapid current deflections in this and subsequent figures represent changes in membrane current caused by voltage ramps from -130 to $+60$ mV. Peaks of these signals have been cut off. (B) ACh-evoked current (solid symbols) obtained by subtraction I/V curve labelled c in (A) from I/V-curve labelled a. The solid symbols represent the current-voltage relation of the PD 81,723 inhibited current in the presence of ACh (a–d). (C) Same as in (A) for Ado-evoked current. (D) Background current inhibited by PD 81,723 obtained by subtraction (c–e).

from the I/V-curves (e.g. Figure 1B) the current signal which can be measured at -90 mV (40 mV negative to E_K) was substantially larger than a signal at a membrane potential 40 mV positive to E_K. It is conceivable that the inhibition by PD 81,723 is limited to inward $I_{K(ACh)}$, which is the unphysiological current direction, since a membrane potential more negative than E_K is never encountered by a cell. Therefore in a series of experiments an extracellular solution containing 5 mM K^+ was used and a holding potential of -40 mV ($E_K = -85$ mV), resulting in an outward driving force for K^+ currents. Representative current traces from two cells showing inhibition of outward $I_{K(ACh)}$ are illustrated in Figure 5. It was a consistent finding that outward $I_{K(ACh)}$ showed a more pronounced desensitization for both ACh and adenosine as activating ligands. This suggests that fast desensitization of $I_{K(ACh)}$, the mechanism of which at present is not fully understood (cf. Kurachi *et al*., 1987; Kim, 1993; Shui *et al*., 1995) seems to have a voltage-dependent component. These findings demonstrate that both ACh- and adenosine-activated outward currents were not different from inward currents in terms of their inhibition by PD 81,723. In the examples shown, peak and late currents caused by ACh and adenosine were inhibited by about 90%, respectively, by 50 μM PD 81,723, which qualitatively corresponds to the data shown in Figure 4.

As PD 81,723 is a very lipophilic compound it should cross the membrane rapidly upon extracellular application. Its site of action therefore could be localized either on the outer face of the membrane or intracellularly. In order to differentiate between an intracellular and an extracellular site of action, cells were loaded with PD 81,723, by including the compound (50 μM) in the filling solution of the recording pipette. A representative result is illustrated in Figure 6. Inward $I_{K(ACh)}$ was repetitively activated by brief superfusion with 10 μM ACh. The first response was elicited within 20 s after getting access to the cell. In experiments with GTP-γ-S effects can be detected within about 1 min, a steady state being reached within about 5 min. At 20 s contamination by intracellular PD 81,723, if any, should be small. Furthermore, current density of $I_{K(ACh)}$ was in a normal range throughout the experiment. $I_{K(ACh)}$ remained constant for more than 30 min. A slight inward shift of the holding current by about 90 pA was probably due to an increase in leak conductance, which is sometimes observed during long-lasting recordings. The same concentration of PD 81,723 applied extracellularly (in the continuous presence of ACh) after 30 min of cell dialysis with PD 81,723 caused complete inhibition of $I_{K(ACh)}$. In line with Figure 1, an inhibition of background $I_{K(ACh)}$ could also be identified. Comparable results were obtained in all 5 cells tested by means of such a protocol. This finding suggests that an intracellular site of action of PD 81,723, such as the G protein or one of its subunits, is very unlikely. As PD 81,723 does not inhibit the system at the receptor level, the $I_{K(ACh)}$ channel or one of its subunits respectively is the most likely site of action. If PD 81,723 acts as a blocker of the $I_{K(ACh)}$ channel, it is likely to act also on other K^+ channels with similar structural properties. A major K^+ channel expressed in ventricular myocytes is the inward rectifying I_{K1} which represents the major background current pathway. The channel protein (IRK1) shares a large degree of homology to GIRK1, one of the subunits of $I_{K(ACh)}$, which in guinea-pig

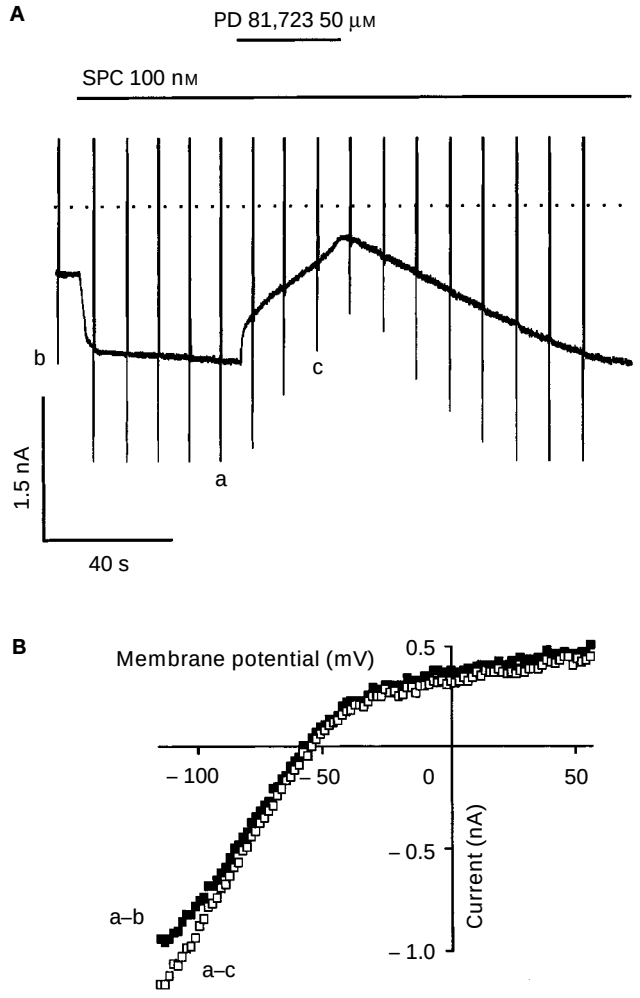

Figure 2 Inhibition of SPC-induced $I_{K(ACh)}$ by PD 81,723. (A) Continuous recording of membrane current at −90 mV holding potential. Superfusion with solutions containing SPC (100 nM) and SPC plus PD 81,723 (50 μM) is indicated by horizontal bars. (B) I/V curves of SPC-induced and PD 81,723-inhibited current obtained by subtraction as indicated by labels a–c.

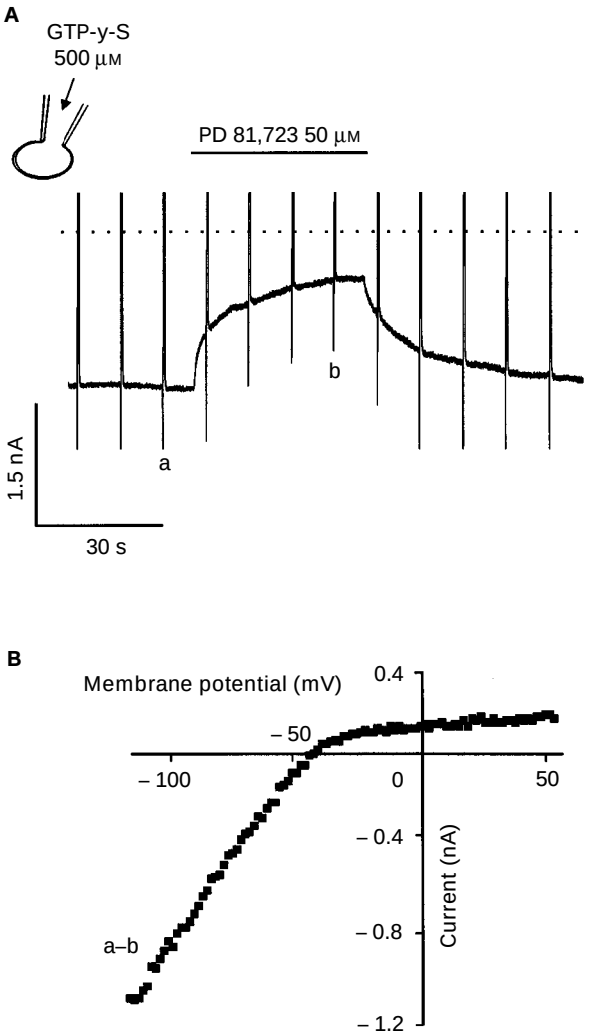

Figure 3 Inhibition of $I_{K(ACh)}$ induced by loading of a cell with GTP-γ-S (500 μM) in the recording pipette. (A) Recording of membrane current. (B) I/V curve of PD 81,723-inhibited current.

ventricular myocytes is not functionally expressed. However, functional expression of $I_{K(ACh)}$ has been demonstrated in ferret ventricular myocytes, where it can be activated by ACh and adenosine (Ito *et al*., 1995). In ferret ventricular cells PD 81,723 also acts as an inhibitor of $I_{K(ACh)}$ (L. Belardinelli, personal communication). Figure 7 represents a current recording from a ventricular myocyte studied under the same conditions as the atrial cells. PD 81,723 (50 μM) caused a reduction of the inward current (at −90 mV) from 940 pA to 370 pA (A). By subtraction of the I/V-curves in the presence and absence of the drug the I/V-curve of the inhibited current was obtained (B). It was a strongly inward-rectifying current, which in ventricular myocytes represents I_{K1} (e.g. Carmeliet 1992). Comparable results were obtained in all 8 ventricular myocytes from three different hearts. This suggests that PD 81,723 acts as a K^+-channel blocker. To what extent its action is specific to the family of inward-rectifying channels has to be further investigated.

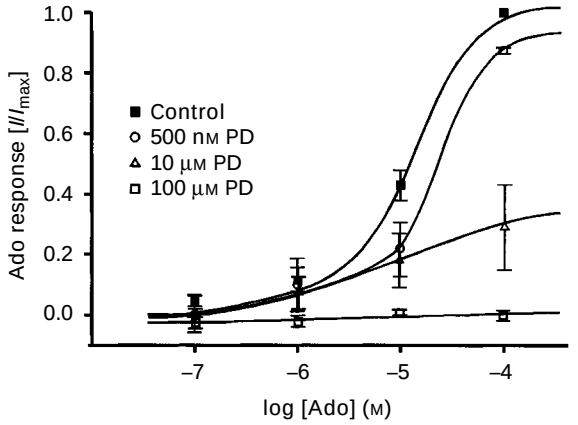

Figure 4 Effect of PD 81,723 on adenosine (Ado)-$I_{K(ACh)}$ concentration-response curves. Ado-evoked currents were normalized to the maximum response in each cell in response to 100 μM in the absence of PD 81,723 (PD); mean values (n=4 to 6); vertical lines show s.d.

Discussion

The major results of the present investigation can be summarized as follows: PD 81,723, a compound which has been described as an allosteric enhancer of effects of adenosine mediated by the A_1 subtype of adenosine receptor inhibited atrial $I_{K(ACh)}$ in a receptor-independent way. PD 81,723 acted from the outside only. In ventricular myocytes it inhibited with similar potency the inward-rectifying I_{K1} which is structurally related to the $I_{K(ACh)}$ subunits GIRK1 and GIRK4 (see Deal *et al*., 1996 for review). In atrial myocytes at no combination of concentrations of adenosine, and PD 81,723 could a poten-

tiation be found, as expected from previously published data on this substance. These findings render a contribution of the A_1R-G_i-$I_{K(ACh)}$ pathway to the adenosine-enhancing effects of PD 81,723 in perfused and *in situ* hearts very unlikely. (Kollias-Baker *et al*., 1994a).

A_1 receptors are coupled to pertussis toxin sensitive (G_i-type) G proteins, which inhibit adenylylcyclase in many types of cells. In the heart and certain neurones (Lesage *et al*., 1994; Koyama *et al*., 1994; Karschin *et al*., 1994) G_i in addition controls G protein-gated inward rectifying K^+ channels, of which atrial $I_{K(ACh)}$ represents the prototype (Pfaffinger *et al*., 1985). The inward-rectifier-type of K^+ channels are formed by proteins (IRK1, GIRKs, $_{rc}K_{ATP-1}$) which are distinctly different from the shaker or eag types of K^+ channels (see Deal *et al*., 1996 for review). In the present study we found that PD 81,723 inhibited currents generated by two different cardiac inward-rectifying channels. At present we have not yet tested PD 81,723 on other K^+ channels. Therefore it is premature to speculate on selectivity of PD 81,723 for inward rectifier-type channels. However, there is hardly any doubt that the inhibitory effect of PD 81,723 on $I_{K(ACh)}$ can be localized to one of the channel proteins forming $I_{K(ACh)}$, since (i) inhibition was independent of the nature of the activating receptor, (ii) PD 81,723 acted from the outside only. The components of the signalling pathway accessible from the outside are, apart from

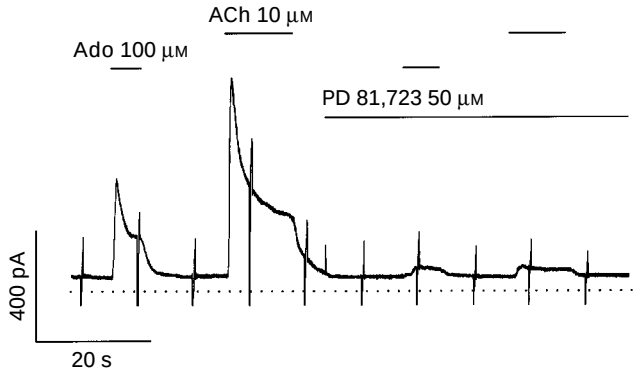

Figure 5 Inhibition of outward $I_{K(ACh)}$ evoked by 100 μM adenosine (Ado) and 10 μM ACh (data from two different cells) by PD 81,723. An extracellular K^+ concentration of 5 mM was used in this set of experiments; holding potential was −40 mV.

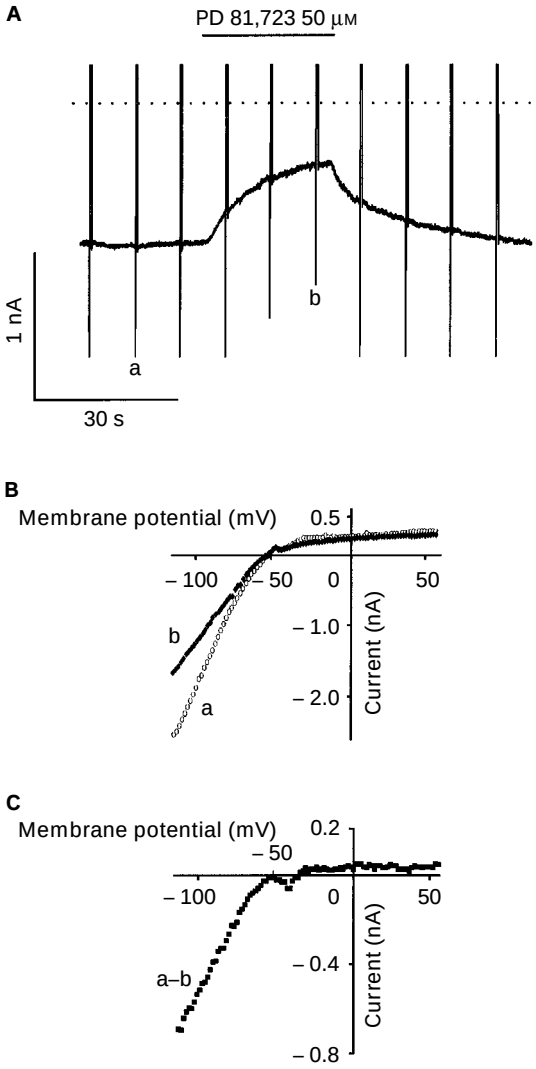

Figure 7 Inhibition of ventricular inward-rectifying K^+ current (I_{K1}) by PD 81,723. (A) Continuous recording of membrane current at −90 mV holding potential. (B) Current-voltage relations (a) control, (b) in presence of PD 81,723 (note that the peaks of current ramps in (A) had been cut off). (C) Voltage-dependence of current inhibited by PD 81,723 obtained by subtraction.

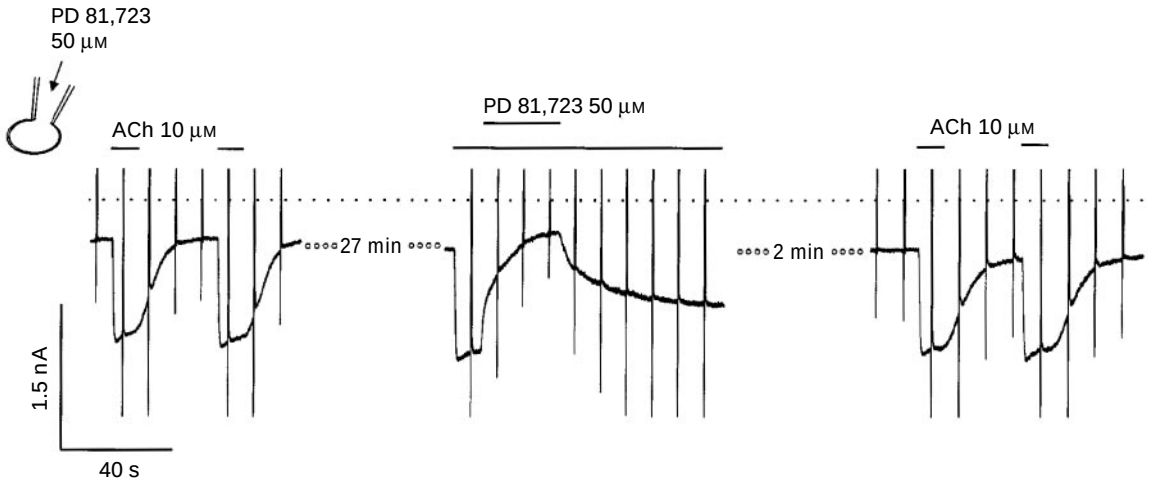

Figure 6 Intracellular PD 81,723 failed to inhibit $I_{K(ACh)}$. Representative recording of membrane current from one atrial myocyte. The leftmost response to ACh was elicited 20 s after access was obtained to the cell by rupture of the membrane patch under the tip of the recording pipette.

the receptor(s), the channel proteins. (iii) Current through structurally similar but functionally different inward rectifying K^+ channels in ventricular myocytes was also sensitive to PD 81,723. As gating of that channel does not involve interaction with $G_{\beta\gamma}$, interference of PD 81,723 with the $G_{\beta\gamma}$-GIRK1 interaction can be excluded as a mechanism underlying its inhibition of $I_{K(ACh)}$.

Previous publications have introduced PD 81,723 as a compound which selectively enhances actions of adenosine mediated by A_1 receptors, presumably by an allosteric effect that enhances binding of the agonist to the receptor. From radioligand binding studies stabilization of the ligated A_1R-G protein complex has been suggested as the underlying mechanism (Kollias-Baker *et al*., 1994a; Bhattacharya & Linden, 1995). In studies on guinea-pig electrically driven, perfused hearts an augmentation of PD 81,723 of the adenosine-induced negative dromotropic effect (prolongation of the stimulus-His bundle interval) has been found. Analogous results were obtained with driven *in situ* hearts. In this model atrial conduction time under hypoxia was prolonged by PD 81,723. The authors suggested that the increased interstitial concentration of adenosine under cardiac hypoxia becomes more effective due to the enhancing action of PD 81,723 (Kollias-Baker *et al*., 1994b). In spontaneously beating right atria Mudumbi *et al*. (1996) found a sensitization by PD 81,723 of the negative chronotropic effect of adenosine. Taken together, the binding studies and these functional investigations support the concept that PD 81,723 acts as an allosteric enhancer. However, in these studies no direct test of the final target of A_1 adenosine receptor activation ($I_{K(ACh)}$, adenylyl cyclase) was done. More recently in a dog infarct model it was shown that PD 81,723 lowers threshold for ischaemic preconditioning, an effect which was sensitive to glibenclamide, an inhibitor of $I_{K(ACh)}$ channels (Mizumura *et al*., 1996). This is in line with the finding that in guinea-pig ventricular myocytes $I_{K(ATP)}$ channels can be activated via a G protein by adenosine (Ito *et al*., 1994). To what extent K^+ channel inhibition contributes to the functional effects described in the literature at present is unknown. It is conceivable that a moderate depolarization due to inhibition of supraventricular 'background' K^+ channels (preferentially $I_{K(ACh)}$) and/or their ventricular equivalent (I_{K1}) results in a decrease in dV/dt_{max}, which in turn could reduce conduction velocity. To address this question further, experiments on the action of PD 81,723 itself over a wide range of concentrations in perfused hearts are required.

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References

AMOAH-APRAKU, B., XU, J., LU, J.Y., PELLEG, A., BRUNS, R.F. & BELARDINELLI, L. (1993). Selective potentiation by an A_1 adenosine receptor enhancer of the negative dromotropic action of adenosine in the guinea pig heart. *J. Pharmacol. Exp. Ther*., **266,** 611–617.

BANACH, K., HÜSER, J., LIPP, P., WELLNER, M.C. & POTT, L. (1993). Activation of muscarinic K^+ current in guinea-pig atrial myocytes by a serum factor. *J. Physiol*., **461,** 263–281.

BHATTACHARYA, S. & LINDEN, J. (1995). The allosteric enhancer, PD 81,723, stabilizes human A_1 adenosine receptor coupling to G proteins. *Biochim. Biophys. Acta: Molec. Cell. Res*., **1265,** 15–21.

BÜNEMANN, M., LILIOM, K., BRANDTS, B., POTT, L., TSENG, J.-L., DESIDERIO, G.M., SUN, G., MILLER, D. & TIGYI, G. (1996). A novel receptor with high affinity for lysosphingomyelin and sphingosine 1-phosphate in atrial myocytes. *EMBO J*., **15,** 5527–5534.

BÜNEMANN, M. & POTT, L. (1995). Down-regulation of A_1 adenosine receptors coupled to muscarinic K^+ current in cultured guinea-pig atrial myocytes. *J. Physiol*., **482,** 81–92.

CARMELIET, E. (1992). Potassium channels in cardiac cells. *Cardiovasc. Drug Ther*., **6,** 305–312.

COLLIS, M.G. & HOURANI, S.M.O. (1993). Adenosine receptor subtypes. *Trends Pharmacol. Sci*., **14,** 360–366.

DEAL, K.K., ENGLAND, S.K. & TAMKUN, M.M. (1996). Molecular physiology of cardiac potassium channels. *Physiol. Rev*., **76,** 49–67.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch*., **391,** 85–100.

ITO, H., HOSOYA, Y., INANOBE, A., TOMOIKE, H. & ENDOH, M. (1995). Acetylcholine and adenosine activate the G protein-gated muscarinic K^+ channel in ferret ventricular myocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol*., **351,** 610–617.

ITO, H., VEREECKE, J. & CARMELIET, E. (1994). Mode of regulation by G protein of the ATP-sensitive K^+ channel in guinea-pig ventricular cell membrane. *J. Physiol*., **478,** 101–107.

KAIBARA, M., NAKAJIMA, T., IRISAWA, H. & GILES, W. (1991). Regulation of spontaneous opening of muscarinic K^+ channels in rabbit atrium. *J. Physiol*., **433,** 589–613.

KARSCHIN, C., SCHREIBMAYER, W., DASCAL, N., LESTER, H., DAVIDSON, N. & KARSCHIN, A. (1994). Distribution and localization of a G protein-coupled inwardly rectifying K^+ channel in the rat. *FEBS Lett*., **348,** 139–144.

KIM, D. (1993). Mechanism of rapid desensitization of muscarinic K^+ current in adult rat and guinea pig atrial cells. *Circ. Res*., **73,** 89–97.

KOLLIAS-BAKER, C., RUBLE, J., DENNIS, D., BRUNS, R.F., LINDEN, J. & BELARDINELLI, L. (1994a). Allosteric enhancer PD 81,723 acts by novel mechanism to potentiate cardiac actions of adenosine. *Circ. Res*., **75,** 961–971.

KOLLIAS-BAKER, C., XU, J., PELLEG, A. & BELARDINELLI, L. (1994b). Novel approach for enhancing atrioventricular nodal conduction delay mediated by endogenous adenosine. *Circ. Res*., **75,** 972–980.

KOYAMA, H., MORISHIGE, K.-I., TAKAHASHI, N., ZANELLI, J.S., FASS, D.N. & KURACHI, Y. (1994). Molecular cloning, functional expression and localization of a novel inward rectifier potassium channel in the rat brain. *FEBS Lett*., **341,** 303–307.

KRAPIVINSKY, G., GORDON, E.A., WICKMAN, K., VELIMIROVIC, B., KRAPIVINSKY, L. & CLAPHAM, D.E. (1995). The G-protein-gated atrial K^+ channel I_{KACh} is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature*, **374,** 135–141.

KURACHI, Y., NAKAJIMA, T. & SUGIMOTO, T. (1987). Short-term desensitization of muscarinic K^+ channel current in isolated atrial myocytes and possible role of GTP-binding proteins. *Pflügers Arch*., **410,** 227–233.

LESAGE, F., DUPRAT, F., FINK, M., GUILLEMARE, E., COPPOLA, T., LAZDUNSKI, M. & HUGNOT, J.-P. (1994). Cloning provides evidence for a family of inward rectifier and G-protein coupled K^+ channels in the brain. *FEBS Letts*, **353,** 37–42.

MIZUMURA, T., AUCHAMPACH, J.A., LINDEN, J., BRUNS, R.F. & GROSS, G.J. (1996). PD 81,723, an allosteric enhancer of the A_1 adenosine receptor, lowers the threshold for ischemic preconditioning in dogs. *Circ. Res*., **79,** 415–423.

MUDUMBI, R.V., MONTAMAT, S.C., BRUNS, R.F. & VESTAL, R.E. (1996). Cardiac functional responses to adenosine by PD 81,723, an allosteric enhancer of the adenosine A_1 receptor. *Am. J. Physiol. (Heart Circ. Physiol.)*, **33,** H1017–H1022.

OKABE, K., YATANI, A. & BROWN, A.M. (1991). The nature and origin of spontaneous noise in G protein- gated ion channels. *J. Gen. Physiol*., **97,** 1279–1293.

OLAH, M.E., REN, H. & STILES, G.H. (1995). Adenosine receptors: protein and gene structure. *Arch. Int. Pharmacodyn. Thér*., **329,** 135–149.

PFAFFINGER, P.J., MARTIN, J.M., HUNTER, D.D., NATHANSON, N.M. & HILLE, B. (1985). GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature*, **317,** 536–538.

SAKMANN, B., NOMA, A. & TRAUTWEIN, W. (1983). Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature*, **303,** 250–253.

SHUI, Z., BOYETT, M.R., ZANG, W.J., HAGA, T. & KAMEYAMA, K. (1995). Receptor kinase-dependent desensitization of the muscarinic K^+ current in rat atrial cells. *J. Physiol.*, **487,** 359–366.

WILKEN, A., TAWFIK-SCHLIEPER, H., KLOTZ, K.-N. & SCHWABE, U. (1990). Pharmacological characterization of the adenylate cyclase-coupled adenosine receptor in isolated guinea pig atrial myocytes. *Mol. Pharmacol.*, **37,** 916–920.

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