# Down-regulation of $A_1$ adenosine receptors coupled to muscarinic $K^+$ current in cultured guinea-pig atrial myocytes

Moritz Bünemann and Lutz Pott*

*Institut für Physiologie, Ruhr-Universität Bochum, D-44780 Bochum, Germany*

1. Muscarinic $K^+$ current ($I_{K(ACh)}$) was measured in cultured atrial myocytes from hearts of adult guinea-pigs using whole-cell voltage clamp. $I_{K(ACh)}$ was activated by superfusion with solutions containing either acetylcholine (ACh) or adenosine (Ado), in saturating concentrations of 2 $\mu$M (ACh) and 1 mM (Ado), respectively.

2. In freshly isolated cells the amplitude of the current activated by Ado ($I_{K(Ado)}$) was 58% (mean) of the current that was induced by ACh. In serum-free culture this relation, but also the absolute density of $I_{K(ACh)}$, remained fairly constant for up to 8 days.

3. If the culture medium was supplemented with fetal calf serum (FCS, 5%) the relation $I_{K(Ado)}/I_{K(ACh)}$ gradually decayed, reaching a value of less than 0·1 on days 7–8, whereas the response to ACh remained stable over this period of time.

4. After treatment of cells with FCS-containing medium, no recovery was observed upon FCS withdrawal for up to 4 days.

5. The effect of FCS on responsiveness to Ado was half-maximal at about 1% (v/v). The active principle can be dialysed (mol. mass exclusion: 10 kDa). It is not identical with an albumin-associated factor that has been shown to be a potent activator of atrial $I_{K(ACh)}$ upon acute superfusion. Loss of responsiveness to Ado was paralleled by a reduction of binding sites to the $A_1$ adenosine receptor-specific radioligand 8-cyclopentyl-1,3-dipropylxanthine ([$^3$H]CPX).

6. It is concluded that FCS contains a factor that causes down-regulation of $A_1$ Ado receptors. The signalling pathway that leads to an increased opening activity of $I_{K(ACh)}$ channels and other receptors, such as the $M_2$ muscarinic receptor, linked to this signalling pathway are not affected by this factor.

Apart from its role in energy metabolism, adenosine exerts various effects on different types of cells by interacting with specific surface receptors (Olah & Stiles, 1992; Liang, 1992). Profound effects on the cardiovascular system were first described by Drury & Szent-Györgyi (1929). In supraventricular myocytes adenosine (Ado) activates a $K^+$ current ($I_{K(ACh)}$) by binding to the $A_1$ receptor subtype (Belardinelli, Giles & West, 1988; Tawfik-Schlieper, Klotz, Kreye & Schwabe, 1989). Control of this current by acetylcholine (ACh) via muscarinic ($M_2$) receptors is a major mechanism of negative chronotropism exerted by vagal activity. Occupancy of both the $A_1$ adenosine receptor and the $M_2$ muscarinic receptor by an appropriate agonist is transmitted to the $I_{K(ACh)}$ channel by activation of a heterotrimeric G protein ($G_i$, $G_k$) in a membrane-delimited fashion, i.e. without a soluble second messenger being involved (Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985; Kurachi, Nakajima & Sugimoto, 1987).

In recent years an increasing amount of experimental work on receptor-controlled cellular mechanisms is being performed in *in vitro* systems using acutely isolated cells, primary cultures or cell lines. Despite the inestimable value of cell culture systems for cellular physiology and pharmacology and related fields, one has to be aware that any cultured cell is exposed to an artificial environment, the composition of which generally is optimized for maximal proliferation, morphological differentiation, or at least long-term survival. This is often achieved by using commercially available culture medium supplemented with fetal calf serum (FCS). The artificial set of regulatory inputs ranging from a lack of certain factors on one side to an over-representation of other factors on the other, is likely to have profound effects on the molecular make-up of a cell. In the present investigation we show that adult (terminally differentiated) atrial myocytes from guinea-pig hearts loose the responsiveness of $I_{K(ACh)}$ to Ado within a

* To whom correspondence should be addressed.

few days, if cultured in serum-supplemented but not in serum-free medium, whereas sensitivity of the cells to ACh, again revealed by measurements of $I_{K(ACh)}$, is hardly affected by the presence of serum. Loss of $A_1$ responsiveness is paralleled by a decrease in the number of specific $A_1$ binding sites as revealed by a radioligand binding assay using the selective $A_1$ antagonist 8-cyclopentyl-1,3-dipropylxanthine ([$^3$H]CPX).

## METHODS

### Isolation and culture of atrial myocytes

Single myocytes from hearts of guinea-pigs (200–250 g) killed by cervical dislocation after stunning, were isolated via a Langendorff perfusion method. Details of the method have been described previously (e.g. Bechem & Pott, 1985). The culture medium was bicarbonate-buffered M199 (Gibco, Dreieich, Germany) containing gentamycin and kanamycin (each at 25 $\mu$g ml$^{-1}$; Sigma, Deisenhofen, Germany). The cells were plated at a low density (several hundred cells per dish) on 36 mm culture dishes. As cardiac myocytes represent terminally differentiated cells, no proliferation occurs. The cultures were placed in an incubator at 37 °C, 95% humidity and 5% $CO_2$. In order to improve attachment of the myocytes, particularly in FCS-free media, which is essential for their survival, the cell suspension was plated as a thin layer, preventing floating of the cells. Under these conditions 50–70% of the cells attached within 16–24 h. After this period the culture dishes were filled with 2 ml of medium. Cells were cultured and used experimentally for up to 8 days. The medium was changed every second day.

### Solutions

For the measurements, the culture medium was replaced 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): potassium aspartate, 110; KCl, 20; MgATP, 5·0; $MgCl_2$, 1·0; EGTA, 2·0; GTP, 0·01; Hepes–NaOH, 10·0, pH 7·4. The resulting $K^+$ gradient corresponds to a Nernst potential for $K^+$ of −48 mV.

### Current measurement

Membrane currents were measured under voltage clamp by means of patch clamp pipettes (whole-cell mode; Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Pipettes were fabricated from borosilicate glass with filament (Clark, Pangbourne, UK) and were filled with the solution listed above. The DC 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). Signals were passed through an analog filter with a corner frequency of 1–3 kHz and were digitally stored on the hard disk of an IBM compatible AT-computer. The computer was equipped with a hardware–software package (ISO2 by MFK, Frankfurt, Germany) for voltage control (pulse and ramp generation), data aquisition, 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, and ramp-shaped changes of the membrane potential ($E_m$) from −130 to + 60 mV were used to measure current–voltage relations and monitor constancy of electrical access to the cell.

Rapid superfusion of the cells for application and withdrawal of different solutions was performed by means of a solenoid-operated flow system that permitted switching between up to five different solutions. The solutions flowed through capillary tubes into a common outlet. The cell under study was continuously superfused at approximately 0·25 ml min$^{-1}$ with a stream of solution of 200 $\mu$m in diameter close to the outlet. In order to exclude artifacts due to varying shear forces detected by the cell (e.g. Kim, 1993), they were continuously superfused with standard (agonist-free) solution between periods of exposure to an agonist. The half-time of a change in concentration seen by the cell was determined as 50–200 ms using a solution in which $[K^+]_o$ was replaced by $Cs^+$.

### Measurement of binding of tritiated 8-cyclopentyl-1,3-dipropylxanthine ([$^3$H]CPX)

[$^3$H]CPX (specific activity 80 Ci mmol$^{-1}$) was purchased from New England Nuclear (DuPont de Nemours, Bad Homburg, Germany). For the binding measurements cells were plated onto 60 mm culture dishes under otherwise identical conditions as described above. Cells were removed from the dishes by washing and incubation for 20 min in 5 ml of $Ca^{2+}$-free solution containing 1 mM EGTA. Thereafter cells were removed by agitation with a Pasteur pipette. The cells were concentrated in a volume of 550 $\mu$l using Centriprep 30 concentration tubes (Amicon, Witten, Germany). For protein assays 50 $\mu$l of this suspension was used. Two samples of 250 $\mu$l from each culture were used for binding assays. To this either 55 $\mu$l of 10% DMSO solution or 55 $\mu$l of the solution containing unlabelled CPX (RBI, Natick, MA, USA) was added (final concentration 100 $\mu$M) in order to determine unspecific binding. After incubation for 20 min, 36 $\mu$l of the [$^3$H]CPX solution were added, resulting in a final concentration of 10 nM. After another 20 min 300 $\mu$l samples were filtered (GF/B, Whatman, Maidstone, UK). The filters were washed 4 times with 2 ml isotonic buffer containing 10% DMSO. The filters were placed in 4 ml Hydroluma scintillation fluid (Baker, Deventer, The Netherlands) and counted on an LKB scintillation counter. Protein content was determined using the bicinchoninic acid (BCA) protein assay, (Pierce, Rockford, IL, USA).

Whenever possible data are presented as mean values ± standard deviation. Significance of differences was evaluated using Student's $t$ test. $P$ values $< 0{\cdot}05$ were considered statistically significant.

## RESULTS

The holding potential for recording agonist-evoked changes of muscarinic $K^+$ current throughout the present investigation was −90 mV. The Nernst potential for $K^+$ ions was −48 mV for the $K^+$ gradient of 20 mM $[K^+]_o$/130 mM $[K^+]_i$. As $I_{K(ACh)}$ displays strong inwardly rectifying properties (Sakmann, Noma & Trautwein, 1983; Horie & Irisawa, 1987), much larger current signals are obtained in the inward direction, i.e. at membrane potentials negative to the equilibrium potential for $K^+$ ($E_K$), as compared with the more physiological outward current. The responses to saturating concentrations of ACh (2 $\mu$M) and Ado (1 mM) recorded from a freshly isolated myocyte are traced in Fig. 1*A*. Membrane currents were recorded while the cell was clamped at a holding potential

of −90 mV. The rapid deflections represent current changes due to voltage ramps (−130 to +60 mV) that were routinely applied in order to monitor the *I–V* characteristics of the cell. The concentrations of Ado (1 mM) as well as ACh (2 μM) were selected to produce a saturating response, in order to obtain information on the maximal current that is available for either of the agonists. It was consistently found that $I_{K(ACh)}$ activation by the parasympathetic transmitter was much faster than by Ado (Thedford, Shuba, Pelzer & Belardinelli, 1993). This finding will be described and analysed in more detail elsewhere. In Fig. 1*B* current–voltage relations of ACh- and Ado-evoked currents have been superimposed (left graph). In the right graph the Ado-evoked current has been scaled to match the ACh-evoked current at −130 mV. The perfect match of both *I–V* curves supports the view that ACh and Ado activate the same type and population of $K^+$ channels (Kurachi *et al.* 1987). Despite this fact the terms $I_{K(ACh)}$ and $I_{K(Ado)}$ will be used, depending on the activating ligand or receptor, respectively.

The result illustrated in Fig. 1 was obtained from a cell that was in culture for less than 10 h. The behaviour illustrated at this time was not different from that of a freshly isolated cell. As the agonist concentrations used were in a

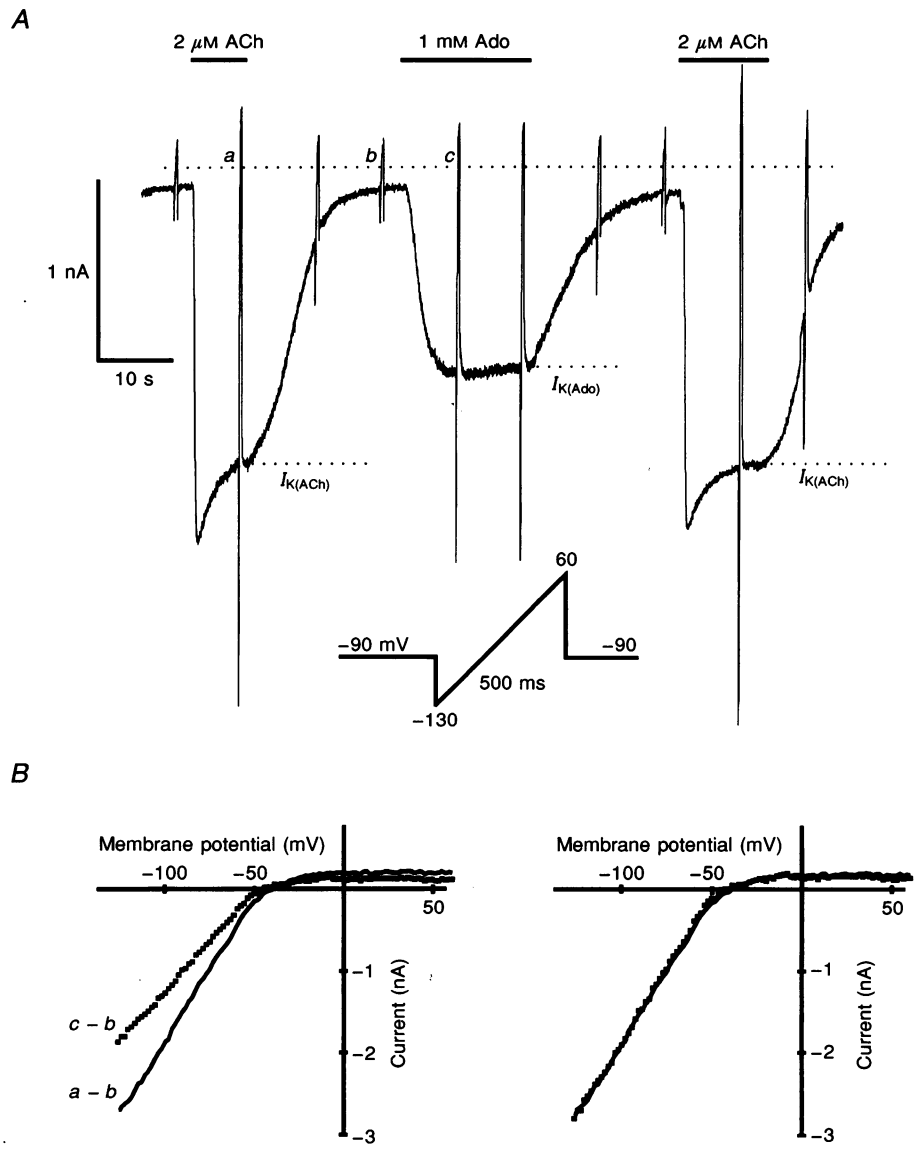


**Figure 1. Changes in membrane current induced by ACh and Ado**

*A*, recording of membrane current. Periods of superfusion by agonist-containing solutions are represented by horizontal bars. Membrane potential was −90 mV. Rapid deflections represent currents due to voltage ramps (inset) Cell capacitance was 12 pF. *B*, left: voltage dependence of $I_{K(ACh)}$ ($a - b$) and $I_{K(Ado)}$ ($c - b$, dotted graph); right: Ado-induced current (dotted trace) was multiplied by 1·4 and superimposed on the *I–V* curve of $I_{K(ACh)}$.

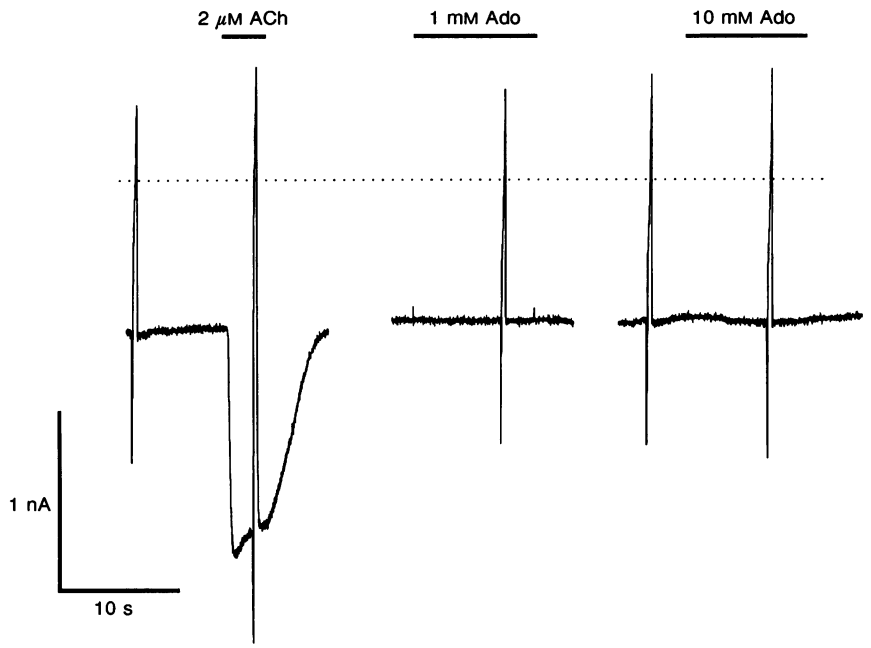


**Figure 2. Response of a myocyte cultured for 7 days in FCS-containing (5%) medium to ACh (2 μM) and Ado (1 and 10 mM)**

Cell capacitance was 13 pF. Identical recording conditions to those described in legend to Fig. 1.

saturating range, the current changes represent the maximum currents that could be evoked by either of the two agonists. The relation $I_{K(Ado)}/I_{K(ACh)}$ at −90 mV, i.e. in the linear range of the $I$–$V$ curve in this cell, was 0·63. In eight cells stored in FCS-free medium studied within 10 h of isolation a mean relation of 0·58 ± 0·11 was determined. No significant difference was found in cells which were stored in FCS-containing medium for this period of time.

In myocytes cultured for about 1 week in standard (FCS-containing) medium, responsiveness to Ado was almost completely lost. A representative example is illustrated in Fig. 2. Superfusion with ACh-containing solution resulted in a steady inward $I_{K(ACh)}$ (at −90 mV) of 1·07 nA (current density of 89·6 pA pF$^{-1}$) whereas in a freshly isolated cell (see Fig. 1), Ado, at concentrations of 1 and 10 mM, failed do induce any current. A concentration of 10 mM

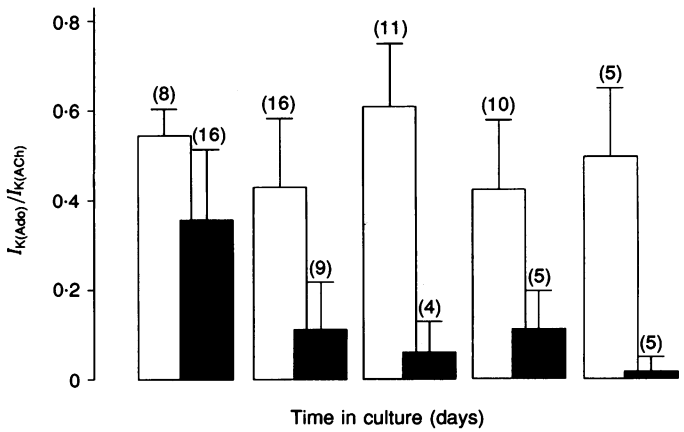


**Figure 3. Dependence of $I_{K(Ado)}$ on time in culture**

■, medium containing 5% FCS; □, FCS-free medium. Current amplitudes ($I_{K(Ado)}$) have been normalized to $I_{K(ACh)}$ measured in the same cell. Experimental protocols were similar to those illustrated in Fig. 1. Values in parentheses represent the number of cells investigated. For each condition cells from at least 3 different cultures and 2 different animals were tested. Data from the period termed day 1 were collected within 14–20 h of isolation of myocytes. Differences are significant ($P < 0·01$) for each pair of data.

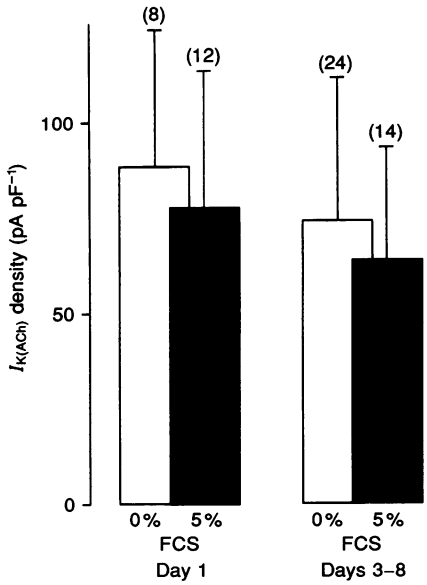


**Figure 4. $I_{K(ACh)}$ densities of myocytes cultured in medium with and without FCS**

As there was no significant difference in data from days 3 and 8, measurements from this period of time were pooled. Only the difference between 0% FCS (day 1) and 5% FCS (days 3–8) is statistically significant ($0{\cdot}01 < P < 0{\cdot}05$).

corresponds to about $10^3$-fold the $K_d$ for the Ado–receptor interaction (Kurachi, Nakajima & Sugimoto, 1986), suggesting that responsiveness of this myocyte to Ado was completely lost.

As the non-desensitizing current induced by 2 $\mu$M ACh represents the maximum available steady-state $I_{K(ACh)}$, this was used as a reference to study the loss of $A_1$ responsiveness in more detail. Myocytes were cultured for variable periods of time either in FCS-containing or FCS-free medium. The data are summarized in Fig. 3. In the presence of FCS there was a gradual decrease of Ado-evoked current with respect to the reference signal, whereas in FCS-free cultures – despite some variation – no such tendency was observed. The difference is statistically significant already at day 1 (these measurements include a period of time of 16–22 h in culture). After 1 week (day 7/8) there was hardly any response to Ado in FCS-cultured cells, whereas the fractional current was not significantly different from day 1 (or day 0, respectively) in FCS-free cultures. This clearly demonstrates that myocytes cultured in FCS-containing medium lose their $A_1$ receptor-mediated responsiveness to Ado. There was also a minor effect of the culture environment on available $I_{K(ACh)}$, which served as the reference signal. This is illustrated in Fig. 4, where densities of $I_{K(ACh)}$ measured in cells kept at the two different culture conditions have been compared.

A concentration–response relation of the effect of FCS on Ado-evoked $I_{K(ACh)}$ is depicted in Fig. 5. All data from this set of measurements were obtained on day 2 in culture. At this time the relation $I_{K(Ado)}/I_{K(ACh)}$ in the FCS-treated cultures was about 30% of the FCS-free cultures. Half-maximal reduction of Ado responsiveness at this time was observed with approximately 1% FCS.

In order to study reversibility of the FCS effect, cells were incubated with 5% serum for 3 days, which in this set of measurements resulted in a reduction of sensitivity to Ado to 10·7% (mean) compared with the FCS-free sister cultures. On day 3 a fraction of the FCS-treated cultures was carefully washed with FCS-free medium, and incubated for another 3 days. This treatment did not result in any recovery, i.e. sensitivity to Ado remained at 10·3% in this group of cultures. The summarized data are illustrated in Fig. 6. This result suggests that there is a

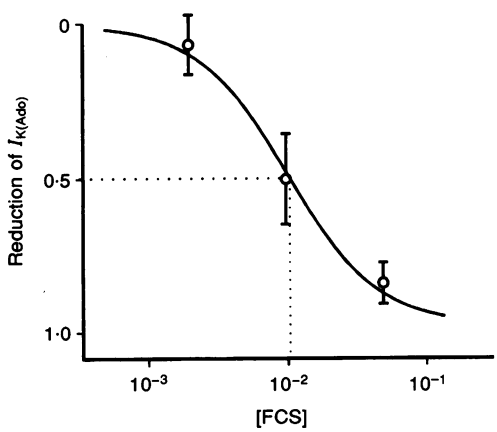


**Figure 5. Concentration–response curve for reduction of $I_{K(Ado)}$ by FCS**

All data were obtained from cells that were in culture for 2 days. Mean ± S.E.M. from 3 cells for each condition. $I_{K(Ado)}$ was normalized to $I_{K(ACh)}$ recorded in the same cell. These values are expressed with reference to a mean value of $I_{K(Ado)}/I_{K(ACh)}$ ($n = 3$) for cells that were kept for 2 days in FCS-free medium. The curve represents a formal fit using simple saturation kinetics ($\log EC_{50} = -1{\cdot}98$; Hill coefficient ($n_H$) = 1·35).

serum-associated signal that is responsible for down-regulation of $A_1$ responsiveness. Up-regulation, after omission of this hypothetical signal molecule, does not occur spontaneously.

In recent studies we have shown that sera from different species and various sources contain a factor that upon acute application, i.e. within a few seconds, activates $I_{K(ACh)}$ and inhibits $\beta$-receptor-stimulated adenylyl cyclase – recorded as inhibition of $\beta$-receptor-stimulated calcium current ($I_{Ca}$) – in atrial myocytes (Banach, Bünemann, Hüser & Pott, 1993*a*; Banach, Hüser, Lipp, Wellner & Pott, 1993*b*). Both effects can be detected at serum concentrations down to 0·001% ($EC_{50}$ ~ 1:1000) and are abolished in cells pre-treated with pertussis toxin. In the case of $I_{K(ACh)}$ activation a membrane-delimited G protein–channel interaction has been rendered likely for this serum-associated factor (Bünemann & Pott, 1993), comparable to $I_{K(ACh)}$ activation by ACh (or Ado). The active principle has not yet been completely identified. It seems to be a lipid compound which, in serum, is associated with albumin. Samples of non-delipidated albumin could mimick the effect, whereas samples of delipidated ('fatty-acid free') albumin could not (Bünemann & Pott, 1993). It is conceivable that chronic activation of the muscarinic signalling pathway by this factor, which is also present in FCS, could result in long-term desensitization of other receptors coupled to this pathway, such as the $A_1$ Ado receptor. In order to test the hypothesis of such a heterologous mechanism, cultures were incubated with serum-free medium containing non-delipidated human serum albumin (HSA) at a concentration (5·8 $\mu$M) corresponding to about 1% serum. Acute activation of $I_{K(ACh)}$ by this batch of HSA (Sigma A1653, lot 106F9374, 12 $\mu$M) is illustrated in Fig. 7*A*. In agreement with the studies quoted above, the amplitude of HSA-induced $I_{K(ACh)}$ reached 100% of the non-desensitizing fraction of $I_{K(ACh)}$ evoked by the parasympathetic transmitter (2 $\mu$M). Chronic exposure to HSA was performed in five cultures for

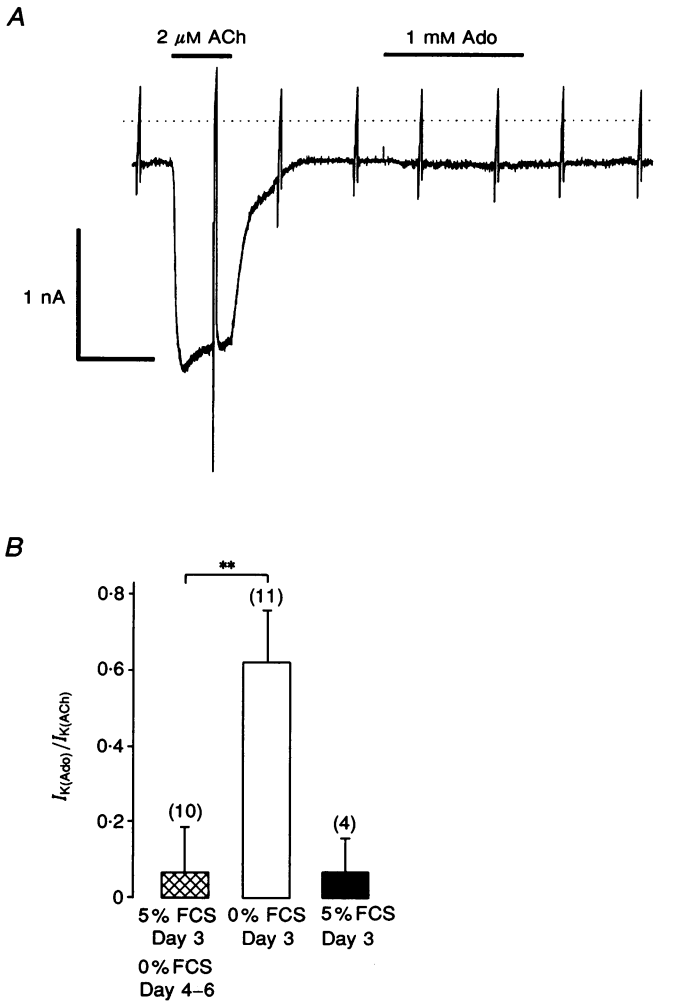


**Figure 6. $I_{K(Ado)}$ does not recover after FCS removal**

*A*, sample recording of membrane current of a cell that was cultured in medium containing 5% FCS for 3 days and, after careful washing, was incubated in FCS-free medium for another 3 days. *B*, summarized data from 10 cells treated in this way.

3 days. The results are summarized in Fig. 7*B*. No reduction of Ado-induced $I_{K(ACh)}$, compared with ACh as the activating ligand, was observed in cells cultured in the presence of HSA. This excludes the involvement of the albumin-associated lipid factor in the loss of Ado responsiveness. $I_{K(ACh)}$ activation by acute exposure of a cell to HSA or serum was independent of whether the cells were kept in FCS- (or HSA-) containing or FCS-free medium (not shown).

The down-regulatory action on $I_{K(Ado)}$ was not limited to FCS, of which all three different batches tested were found to be equally active. Qualitatively similar potencies to reduce $A_1$ responsiveness were found in newborn calf serum (NCS), which reduced Ado-activated $I_{K(ACh)}$ to 0·1 ± 0·076 ($n = 5$, all measurements taken on day 5), and horse serum (0·25 ± 0·17, $n = 10$; day 4)

In order to delineate the active principle further, FCS was dialysed. Figure 8*A* shows a representative result obtained from a cell that was kept for 3 days in FCS-free medium. This medium (50 ml) was treated by inserting a dialysing tube containing 2·5 ml of FCS for 20 h. Exposure to the treated medium containing the low-molecular weight components of FCS resulted in a reduction of $I_{K(Ado)}/I_{K(ACh)}$ to 0·14, comparable with the result obtained with medium containing 5% FCS. The dialysing tube had a molecular mass exclusion of 12000 Da. The data from eight

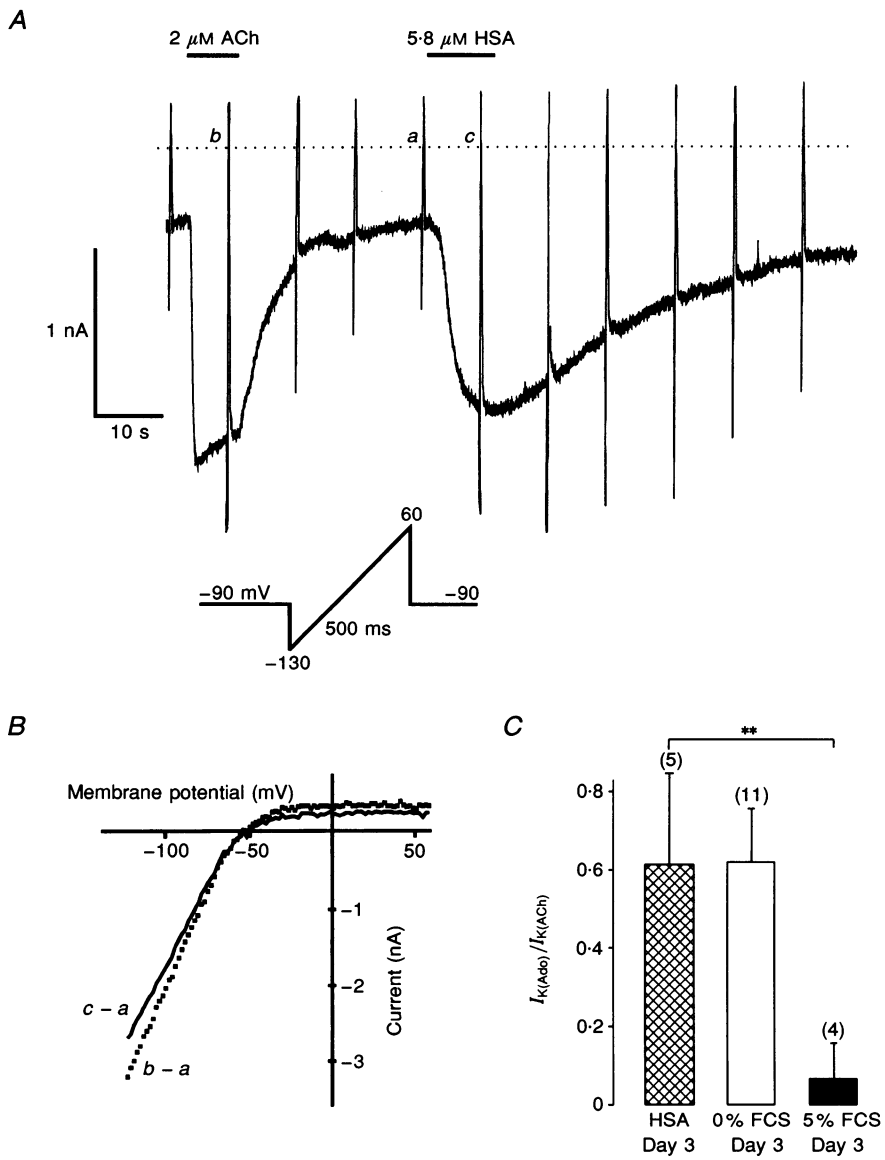


**Figure 7. Albumin activates $I_{K(ACh)}$ upon acute application but does not cause down-regulation of $I_{K(Ado)}$**

*A*, recording of currents evoked by ACh or human serum albumin (HSA, 5·8 μM). *B*, *I–V* curves of ACh- and HSA-evoked current. *C*, relative $I_{K(Ado)}$ in cells treated with 5·8 μM HSA in FCS-free medium for 3 days compared with cells kept in medium without supplement or 5% FCS.

cells (4 cultures) treated in this way and the corresponding controls are summarized in Fig. 8*B*. This finding excludes the contribution of large proteins such as, for example, growth factors, most of which have a molecular mass > 20 kDa, to the down-regulation of responsiveness to Ado.

It is conceivable that Ado itself is involved in causing down-regulation of the $A_1$ receptors. Using a high-performance liquid chromatography (HPLC) method unambiguous detection of Ado at a concentration of $10^{-9}$ M was possible. The Ado signal obtained by a solution containing 10% FCS could just be identified and was smaller than the peak representing $10^{-9}$ M Ado. Thus, in medium supplemented with 5% FCS the concentration of Ado is $< 5 \times 10^{-10}$ M. A comparable concentration was determined in medium conditioned by atrial myocytes for 3 days. This renders a contribution of Ado to down-regulation of its receptor unlikely. This does not exclude the possibility, however, that some other factor in serum could stimulate Ado release from the myocytes. Ado, in an autocrine or paracrine fashion, could tonically act on its receptors, resulting in homologous down-regulation without a detectable concentration of the compound in the bulk medium. As a defined concentration of Ado cannot be maintained in a cell culture, because of uptake and degradation, the synthetic $A_1$-selective agonist *R*-(−)-*N*-(2-phenylisopropyl)-adenosine (PIA) was used to study the effect of chronic exposure of the cell to an $A_1$ agonist. Supplementation of FCS-free culture medium with PIA at a concentration of 10 μM, which is far above the $K_d$ of 1·4 nM reported for the binding of this agonist in guinea-pig atrial tissue (Tawfik-Schlieper *et al.* 1989), resulted in an almost complete loss of responsiveness of myocytes to Ado within 4 days. Interestingly, the effect of PIA was strictly homologous, i.e. the response to ACh was not significantly affected. In contrast to the effect of FCS, which could not be reversed (Fig. 6), a significant recovery of Ado responsiveness was observed following omission of PIA from the culture medium. A representative current trace and summarized data from a series of measurements on cells that were either cultured in the presence of PIA for 4 days only or were allowed to recover for another 3 days in the absence of the $A_1$ agonist, are illustrated in Fig. 9. Although recovery was not complete after 3 days, an approximately 10-fold increase of Ado responsiveness, as compared with the sister cultures, was found. This suggests

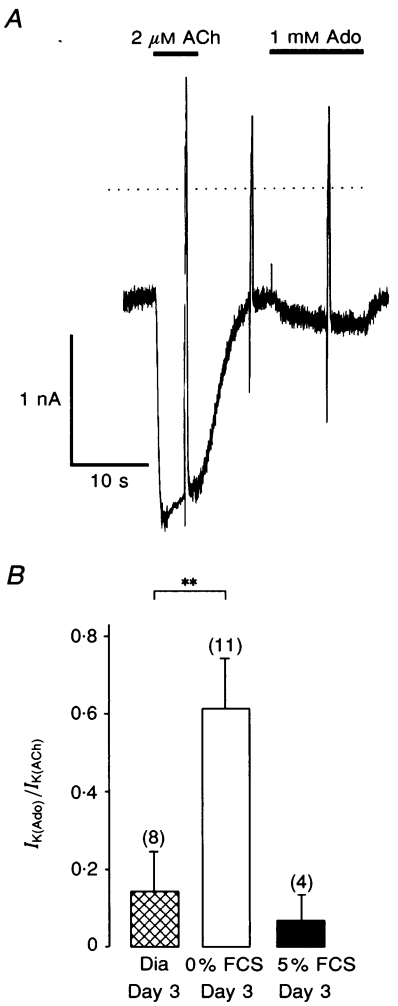


**Figure 8. Dialysate of FCS causes down-regulation of $I_{K(Ado)}$**
*A*, representative measurements of $I_{K(ACh)}$ and $I_{K(Ado)}$ from a myocyte that was kept for 3 days in a culture medium containing the dialysate of FCS as described in the text. (The ramp-induced inward current in the presence of ACh is cut off.) *B*, relative $I_{K(Ado)}$ of cells cultured for 3 days in FCS dialysate-containing medium (Dia), compared with FCS-free and 5% FCS-treated cells.

that the events underlying PIA-induced down-regulation are different from those involved in FCS-induced irreversible loss of Ado responsiveness. A contribution of Ado to down-regulation is also rendered unlikely by the observation that addition of the specific $A_1$ receptor antagonist CPX (2 $\mu$M) to FCS-containing medium did not prevent the loss of responsiveness to Ado (not shown).

In order to study whether the loss in $A_1$ responsiveness described above is related to a loss of $A_1$-type binding sites or reflects some modification of the signalling reactions downstream to agonist–receptor interaction, we measured binding of a highly $A_1$-selective ligand ([$^3$H]CPX; Liang, 1989) to atrial myocytes that were cultured for 5 days with and without FCS. Half-saturation of specific binding of the labelled ligand was observed at about 3 nM, which is in agreement with previous studies using [$^3$H]CPX in cultured chick embryonic cardiac cells. The binding data from cells cultured with (5%) and without FCS are summarized in Fig. 10. The mean density of specific [$^3$H]CPX binding sites in FCS-treated cultures was 32% of the binding sites determined in FCS-free sister cultures. Although the difference in the number of binding sites is less dramatic than the differences in available $I_{K(ACh)}$, these data clearly support the notion that the number of $A_1$

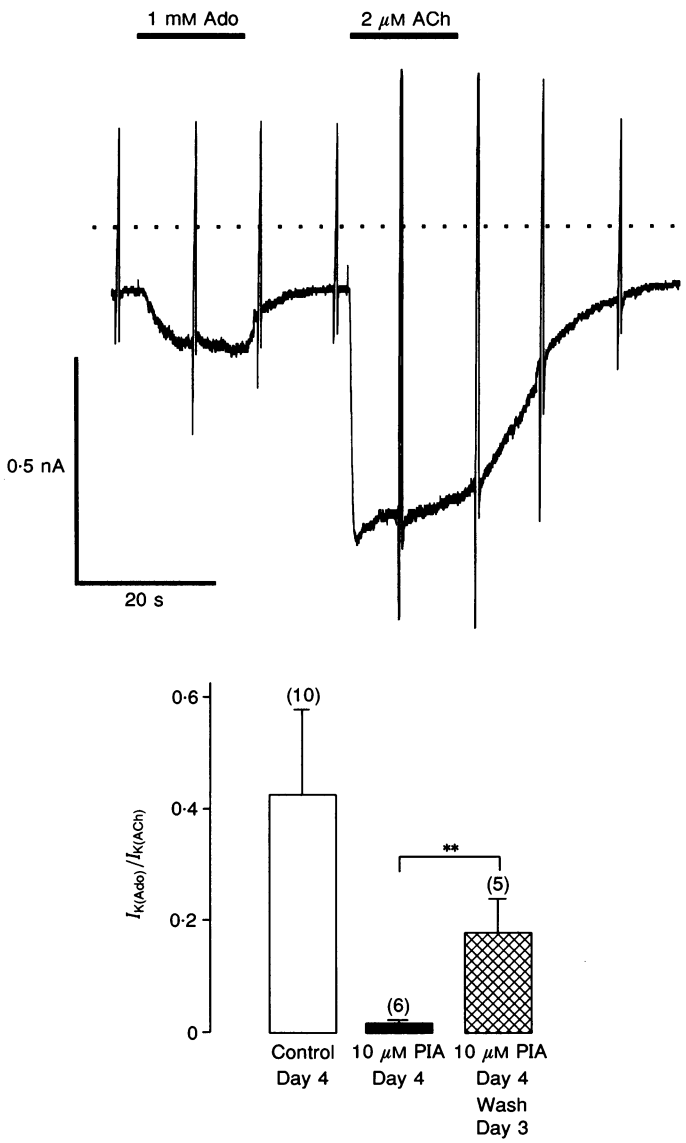


**Figure 9. Reversible down-regulation of $I_{K(Ado)}$ by PIA**

*A*, membrane currents evoked by Ado (1 mM) or ACh (2 $\mu$M) of a cell that was cultured for 4 days in PIA-containing medium (FCS free), followed by 3 days in a medium that contained neither PIA nor FCS. *B*, summarized data; cells were cultured for 4 days in FCS-free control medium (left), in PIA-containing medium (middle), or in PIA-containing medium followed by 3 days in control medium (right).

receptors is decreased in serum-supplemented culture media. As for the binding assay, we did not attempt to purify the sarcolemmal fraction. The possibility cannot be excluded, therefore, that these data in some way are contaminated by non-sarcolemmal binding sites such as internalized receptors. In this respect the binding data ought to be considered as a qualitative support of the electrophysiological data.

## DISCUSSION

Despite the fact that effects of Ado on cardiac function are well documented in various experimental models (e.g. Pappano & Mubagwa, 1992, for review), and their cellular mechanisms are quite well understood (Belardinelli, Linden & Berne, 1989), the role and contribution of this regulatory mechanism to normal control of the heart beat are much less defined than the role of ACh, the mediator of vagal activity.

In the heart the targets of $A_1$ receptor activation are identical to those controlled by $M_2$ receptors. In supraventricular tissue these are $\beta$-receptor-stimulated adenylyl cyclase which is inhibited (Wilken, Tawfik-Schlieper, Klotz & Schwabe, 1990), and $I_{K(ACh)}$ channels which are activated (Belardinelli & Isenberg, 1983). The latter effect on pacemaker and atrial cells most probably is the physiological basis of its clinical use as a treatment to terminate supraventricular tachyarrhythmias (DiMarco, Sellers, Lerman, Greenberg, Berne & Belardinelli, 1985).

Desensitization is a common phenomenon of receptor-controlled reactions. The term 'desensitization', however, has not been coined to one precisely defined reaction, but includes very different mechanisms such as rapid conformational changes on a millisecond time scale, for example in the case of the nicotinic ACh receptor (Katz & Thesleff, 1957), to slow processes on a time scale of hours or even days such as degradation of receptors or decreased levels of receptor mRNA in the $\beta$-receptor–adenylyl-cyclase system (reviewed e.g. by Hausdorff, Caron & Lefkowitz, 1990). The various types of desensitization have in common that they are initiated by exposure of the desensitizing type of receptor to an agonist (homologous desensitization) or alternatively, agonist exposure of a different type of receptor that is linked to the same signalling pathway (heterologous desensitization).

In the case of the atrial $M_2$ receptor–$I_{K(ACh)}$ system, a rapid form of desensitization has been described that takes place on the time scale of hundreds of milliseconds to seconds (Kurachi *et al.* 1987; Kim, 1993*b*; Zang, Yu, Honjo, Kirby & Boyett, 1993). The mechanism of this rapid desensitization has not so far been resolved unequivocally. Whereas Kurachi *et al.* suggest a mechanism localized on the G protein, Kim and Zang *et al.* provide evidence for the involvement of phosphorylation/dephosphorylation of the $I_{K(ACh)}$ channel protein. This rapid desensitization (compare Fig. 1 of the present study) has heterologous properties. Pre-exposure to Ado desensitizes the response to ACh (Kurachi *et al.* 1987) and vice versa. Activation of $I_{K(ACh)}$ by the albumin-associated factor described above, which does not proceed via $M_2$ receptors, also desensitizes a subsequent response to ACh (Banach *et al.* 1993*b*). In the present study the response to Ado did not show a rapid desensitizing component, which is in contrast to the findings described by Kurachi *et al.* (1987). This is likely to result from the lower temperature used in our experiments. Contribution of this kind of rapid desensitization to the long-term reduction of $I_{K(Ado)}$ is unlikely since the latter seems to be strictly limited to the $A_1$ receptor. Furthermore, the rapid type of desensitization is completely reversed within seconds (see Fig. 1 for $I_{K(ACh)}$) following removal of the desensitizing stimulus, whereas in our experiments down-regulation of the $A_1$ receptor was irreversible.

In different cardiac models long-term changes of sensitivity of Ado responsiveness by various conditions have been demonstrated, such as up-regulation following chronic administration of theophyllin to guinea-pigs (Wu, Linden, Visentin, Boykin & Belardinelli, 1989), down-regulation of $A_1$ binding sites, paralleled by up-regulation of muscarinic

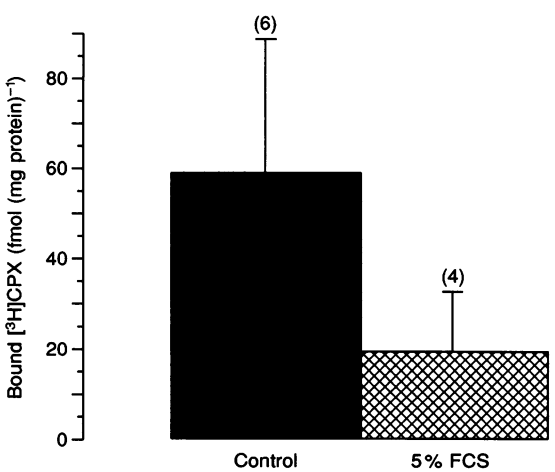


**Figure 10. FCS reduces specific binding of [³H]CPX**
Binding data (see Methods) from myocytes cultured for 5 days in FCS-free (control) and FCS-containing (5%) medium. $P < 0.05$.

receptors under chronic hypoxia in rat hearts (Kacimi, Richalet & Crozatier, 1993), or down-regulation in embryonic chick hearts after treatment with the stable $A_1$ agonist PIA (Shryock, Patel, Bellardinelli & Linden, 1989). In agreement with our finding that long-term treatment with PIA did not affect sensitivity to ACh, the latter effect has been described as homologous, i.e. density and responsiveness of muscarinic receptors, which are coupled via $G_i$ to the same signalling machinery, were unaffected by PIA treatment. In this respect, the action of serum seems to be similar to the action of chronic administration of an agonist, which represents the classical condition causing desensitization of a receptor (e.g. Huganir & Greengard, 1990). This similarity supports the view that sera contain an $A_1$ agonist. As the concentration of Ado itself in FCS is negligible, this must be a ligand of unknown nature. An alternative hypothesis would be that in the presence of FCS or the corresponding unknown factor, the release of Ado from the myocytes is stimulated, which in turn causes desensitization. In supernatants from low-density cultures of myocytes used here we could not detect Ado by means of an enzymatic assay, irrespective of whether the medium contained FCS (not shown). Although this argues against Ado as a candidate, it does not completely exclude the possibility. Ado, released from a myocyte in the low-density cultures used in the present investigation, could act locally in an autocrine fashion without being detectable in the bulk solution. A contribution of non-myocyte cells, such as fibroblasts, or a factor derived from such cells that tends to proliferate in FCS-containing but not in FCS-free media, to Ado receptor down-regulation can be excluded. Up to the third day, when down-regulation is already very significant, hardly any fibroblast-like cells are discernible in either condition. Thereafter proliferation of non-muscle cells becomes prominent in the FCS-containing cultures, leading to confluency after 10–15 days.

In order to answer the question of whether our results represent an agonist-induced desensitization/down-regulation of the $A_1$ adenosine receptor, we are currently working on the identification of the factor(s) in serum. As the physiological role of $I_{K(ACh)}$ activation by Ado has not hitherto been understood, any speculation of a physiological role of the down-regulation described here at present is premature. The present findings are representative for the cell culture situation, using supplementation with FCS or other sera. To what extent the unknown factor(s) that cause down-regulation of $A_1$ receptors in a cultured myocyte are also active in the *in situ* situation at present cannot be answered. The observation that in myocytes studied briefly after isolation, maximum $I_{K(Ado)}$ is always smaller than maximum $I_{K(ACh)}$, would be compatible with a partial down-regulation of $A_1$ receptors *in situ*. Whether such a chronic down-regulation or a genuine lower density of $A_1$ as compared with $M_2$ receptors is the basis of this finding has to be investigated further.

With regard to experimental work using cultured cells, the finding that one particular membrane protein is functionally lost in a cell that otherwise retains its highly differentiated properties with regard to membrane currents, $Ca^{2+}$ regulation, the ability to contract, and other receptor-controlled mechanisms, is rather unique (e.g. Mechmann & Pott, 1986; Bals, Bechem, Paffhausen & Pott, 1990; Lipp, Pott, Callewaert & Carmeliet, 1992).

Identification of the active principle is a prerequisite for any further interpretation in terms of a physiological role for our findings. Furthermore, knowledge of the identity of this factor and of its source could help the better understanding of the contribution of adenosine to regulation of cardiac function.


Bals, S., Bechem, M., Paffhausen, W. & Pott, L. (1990). Spontaneous and experimentally evoked $[Ca^{2+}]_i$-transients in cardiac myocytes measured by means of a fast fura-2 technique. *Cell Calcium* **11**, 385–396.

Banach, K., Bünemann, M., Hüser, J. & Pott, L. (1993*a*). Serum contains a potent factor that decreases $\beta$-adrenergic receptor-stimulated L-type $Ca^{2+}$ current in cardiac myocytes. *Pflügers Archiv* **62**, 1–6.

Banach, K., Hüser, J., Lipp, P., Wellner, M. C. & Pott, L. (1993*b*). Activation of muscarinic $K^+$ current in guinea-pig atrial myocytes by a serum factor. *Journal of Physiology* **461**, 263–281.

Bechem, M. & Pott, L. (1985). Removal of Ca current inactivation in dialysed guinea-pig atrial cardioballs by Ca chelators. *Pflügers Archiv* **404**, 10–20.

Belardinelli, L., Giles, W. R. & West, A. (1988). Ionic mechanisms of adenosine actions in pacemaker cells from rabbit heart. *Journal of Physiology* **405**, 615–633.

Belardinelli, L. & Isenberg, G. (1983). Isolated atrial myocytes: adenosine and acetylcholine increase potassium conductance. *American Journal of Physiology* **224**, H734–737.

Belardinelli, L., Linden, J. & Berne, M. (1989). The cardiac effects of adenosine. *Progress in Cardiovascular Diseases* **32**, 73–97.

Bünemann, M. & Pott, L. (1993). Membrane-delimited activation of muscarinic K current by an albumin-associated factor in guinea-pig atrial myocytes. *Pflügers Archiv* **425**, 329–334.

Dimarco, J. P., Sellers, T. D., Lerman, B. B., Greenberg, M. L., Berne, R. M. & Belardinelli, L. (1985). Diagnostic and therapeutic use of adenosine in patients with supraventricular tachyarrhythmias. *Journal of the American College of Cardiologists* **6**, 417–425.

Drury, A. N. & Szent-Györgyi, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *Journal of Physiology* **68**, 213–237.

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 Archiv* **391**, 85–100.

HAUSDORFF, W. P., CARON, M. G. & LEFKOWITZ, R. J. (1990). Turning off the signal: desensitization of $\beta$-adrenergic receptor function. *FASEB Journal* **4**, 2881–2889.

HORIE, M. & IRISAWA, H. (1987). Rectification of muscarinic $K^+$ current by magnesium ion in guinea pig atrial cells. *American Journal of Physiology* **253**, H210–214.

HUGANIR, R. L. & GREENGARD, P. (1990). Regulation of neutrotransmitter receptor desensitization by protein phosphorylation. *Neuron* **5**, 555–567.

KACIMI, R., RICHALET, J.-P. & CROZATIER, B. (1993). Hypoxia-induced differential modulation of adenosinergic and muscarinic receptors in rat heart. *Journal of Applied Physiology* **75**, 1123–1128.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor endplate. *Journal of Physiology* **138**, 63–80.

KIM, D. (1993*a*). Novel cation-selective mechanosensitive ion channel in the atrial cell membrane. *Circulation Research* **72**, 225–231.

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

KURACHI, Y., NAKAJIMA, T. & SUGIMOTO, T. (1986). On the mechanism of activation of muscarinic $K^+$ channels by adenosine in isolated atrial cells: Involvement of GTP-binding proteins. *Pflügers Archiv* **407**, 264–274.

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 Archiv* **410**, 227–233.

LIANG, B. T. (1989). Characterization of the adenosine receptor in cultured embryonic chick atrial myocytes: coupling to modulation of contractility and adenylate cyclase activity and identification by direct radioligand binding. *Journal of Pharmacology and Experimental Therapeutics* **249**, 775–784.

LIANG, B. T. (1992). Adenosine receptors and cardiovascular function. *Trends in Cardiovascular Medicine* **2**, 100–108.

LIPP, P., POTT, L., CALLEWAERT, G. & CARMELIET, E. (1992). Calcium transients caused by calcium entry are influenced by the sarcoplasmic reticulum in guinea-pig atrial myocytes. *Journal of Physiology* **454**, 321–338.

MECHMANN, S. & POTT, L. (1986). Identification of Na–Ca exchange current in single cardiac myocytes. *Nature* **319**, 597–599.

OLAH, M. E. & STILES, G. L. (1992). Adenosine receptors. *Annual Review of Physiology* **54**, 211–225.

PAPPANO, A. J. & MUBAGWA, K. (1992). Actions of muscarinic agents and adenosine on the heart. In *The Heart and Cardiovascular System*, ed. FOZZARD, H. A., HABER, E., JENNINGS, R. B., KATZ, A. M. & MORGAN, H. E., pp. 1765–1776. Raven Press, New York.

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.

SHRYOCK, J., PATEL, A., BELARDINELLI, L. & LINDEN, J. (1989). Downregulation and desensitization of $A_1$-adenosine receptors in embryonic chicken heart. *American Journal of Physiology* **256**, H321–327.

TAWFIK-SCHLIEPER, H., KLOTZ, K.-N., KREYE, V. A. W. & SCHWABE, U. (1989). Characterization of the $K^+$-channel-coupled adenosine receptor in guinea pig atria. *Naunyn-Schmiedeberg's Archives of Pharmacology* **340**, 684–688.

THEDFORD, S. E., SHUBA, Y. M., PELZER, D. J. & BELARDINELLI, L. (1993). The kinetics of activation of $I_{KACh,Ado}$ in atrial myocytes are related to receptor density and receptor–G-protein coupling efficiency. *Circulation Research* **88**, I-223.

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. *Molecular Pharmacology* **37**, 916–920.

WU, S.-N., LINDEN, J., VISENTIN, S., BOYKIN, M. & BELARDINELLI, L. (1989). Enhanced sensitivity of heart cells to adenosine and upregulation of receptor number after treatment of guinea pigs with theophylline. *Circulation Research* **65**, 1066–1077.

ZANG, W.-J., YU, X. J., HONJO, H., KIRBY, M. S. & BOYETT, M. R. (1993). On the role of G-protein activation and phosphorylation in desensitization to acetylcholine in guinea-pig atrial cells. *Journal of Physiology* **464**, 649–670.

## Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Po 212/6-1). The excellent technical assistance of Mrs Bing Liu is gratefully acknowledged.

*Received 16 February 1994; accepted 28 May 1994.*