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Mediation by nitric oxide of the indirect effects of adenosine on calcium current in rabbit heart pacemaker cells

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1 Adenosine (ADO) is a potent negative chronotropic agent in the mammalian myocardium. We have used single myocytes from rabbit sino-atrial node (SAN) to examine whether nitric oxide (NO) is a significant mediator of the effects of ADO on the pacemaker activity, or the underlying Ca^{2+} and K^+ currents.

2 SAN pacemaker cells were isolated from rabbit hearts by enzymatic dispersion, and Ca^{2+} and K^+ currents were recorded by the nystatin-perforated patch voltage clamp method. ADO was applied in the presence of the β-adrenoceptor agonist, isoprenaline (Iso) to mimic the adrenergic tone which the SAN is subjected to *in vivo*

3 Control experiments confirmed that isolated SAN cells responded to ADO (10–100 μM) with the expected (i) small increase in background inwardly rectifying K^+ current, $I_{K\text{-}ADOi}$ and (ii) pronounced decrease in L-type Ca^{2+} current, $I_{Ca\text{-}L}$. These effects were mimicked by a selective A_1 purinoceptor agonist, N^6-cyclopentyladenosine (CPA, 10 μM); and were inhibited following bath application of the antagonist, DPCPX (10 μM), which selectively blocks A_1 purinoceptors. DMPX (10 μM), a blocker of A_2 purinoceptor, had no effect on the actions of ADO.

4 A nitric oxide synthase inhibitor, L-NMMA (100 μM), abolished the inhibitory effect of ADO on $I_{Ca\text{-}L}$ but did not alter activation of $I_{K\text{-}ADO}$. After L-NMMA washoff, it was possible to obtain the normal response (inhibition) of $I_{Ca\text{-}L}$ to ADO in the same cell.

5 To evaluate whether the observed effect of nitric oxide (NO) on $I_{Ca\text{-}L}$ was mediated by an increase in guanylyl cyclase (GC) activity and cyclic GMP formation, the guanylyl cyclase inhibitor, LY 83583 (40 μM) was applied prior to ADO. Under these conditions, the inhibitory effect of ADO on $I_{Ca\text{-}L}$ was abolished, but the activation of $I_{K\text{-}ADO}$ was still observed.

6 In combination, these findings strongly suggest that in mammalian primary pacemaker tissue which is under adrenergic tone, the effects of ADO on $I_{Ca\text{-}L}$ are mediated by NO.

Keywords: Adenosine; L-type calcium current; cardiac pacemaker cells; nitric oxide; adrenergic tone

Introduction

Adenosine is a well-established modulator of electrophysiological responses in the cardiovascular and nervous systems (Stone, 1981; Berlardinelli *et al.*, 1989; 1995; Belardinelli & Lerman, 1991; Shen & Kurachi, 1995; Olah & Stiles, 1995; Stiles, 1990). In cardiac pacemaker cells, adenosine binds to A_1 purinoceptors, leading to a decrease in firing rate and slowing of conduction (Pelleg & Belardinelli, 1993). Some of these effects of adenosine are augmented under conditions of early ischaemia, and may help to protect the brain and heart from further damage (Rudolphi *et al.*, 1992; Parratt, 1993). For example, in the heart, adenosine is essential for a process termed preconditioning whereby short and mild ischaemic episodes limit the deleterious effects of later more severe insults (Liu *et al.*, 1991; Eckert *et al.*, 1993; Cordeiro *et al.*, 1995 Hoshida *et al.*, 1995). Adenosine is also used to treat some supraventricular tachycardias (DiMarco *et al.*, 1983; Rankin *et al.*, 1992).

The effects of adenosine on ionic currents in cardiac pacemaker tissue (Belardinelli *et al.*, 1988; Belardinelli & Lerman, 1991; Qu *et al.*, 1993) are very similar to those of the parasympathetic neurotransmitter, acetylcholine (Irisawa *et al.*, 1993). They include a small hyperpolarization caused by a GTP-binding (G_i) protein-mediated activation of a K^+ current, $I_{K\text{-}ADO}$. This is referred to as the direct action of ADO. A second very important effect of ADO (also mediated by G_i) occurs in the presence of β-adrenoceptor stimulation, and consists of a reduction in intracellular cyclic AMP levels (Olsson & Pearson, 1990; Stiles, 1990; Olah & Stiles 1995). This strongly attenuates the L-type calcium current, $I_{Ca\text{-}L}$, due to a reduction in the cyclic AMP-dependent protein kinase-catalysed phosphorylation of Ca^{2+} channels, and is referred to as the indirect effect (Pelleg & Belardinelli, 1993; Han *et al.*, 1994; Belardinelli *et al.*, 1995). Recently, we have demonstrated an obligatory role for nitric oxide (NO) formation and activation of guanylyl cyclase in the inhibition of $I_{Ca\text{-}L}$ by acetylcholine in rabbit sino-atrial node (Han *et al.*, 1994; 1995). In the present experiments we have investigated whether NO is also involved in the direct and/or indirect effect of ADO on these cardiac pacemaker cells.

Methods

All experiments were performed on spontaneously beating myocytes from the central region of the rabbit sinoatrial node (SAN). Rabbits were anaesthetized with phenobarbitone (65 ml kg^{-1}, administered i.v.). Following cervical dislocation, hearts were removed and placed in a Langendorff perfusion apparatus for retrograde coronary perfusion at 37°C, with 80 cmH_2O pressure. Blood was removed by perfusing with normal (Ca^{2+}-containing) Tyrodes solution. This was followed by Ca^{2+}-free Tyrode perfusion for 10 min, and then by perfusion with a Ca^{2+}-free Tyrode containing 0.02 mg ml^{-1} collagenase (Yakult Honsha Ltd., Tokyo, 500 units mg^{-1}) and 0.01 mg ml^{-1} protease (Sigma Chem. Co. Ltd., type XIV), for 7–8 min. The sinoatrial node was removed by dissection and incubated in a solution containing 1 mg ml^{-1} collagenase and 0.5 mg ml^{-1} elastase (Sigma Co. Ltd.) (see Han *et al.*, 1995 for

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details). Isolated, spontaneously beating cells were collected over the next 10–40 min, and were stored in a KB solution (cf. Isenberg & Klockner, 1982). Aliquots of cells were placed in a 0.5 ml chamber and superfused with control and test solutions maintained at 32°C. Control solutions contained (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, Na_2HPO_4 0.4, HEPES 5, glucose 10, pH 7.4. All drug-containing solutions were prepared daily. Action potentials and underlying ionic currents were recorded by the nystatin-perforated patch whole-cell recording method (Horn & Marty, 1988) as described previously (Han *et al.*, 1995). The pipette solution contained (in mM): KCl 140, NaCl 6, $MgCl_2$ 1, HEPES 5, adjusted to pH 7.2 with KOH. Nystatin (0.4 mg/ml) was added to this solution. Following seal formation, electrical access to the cell interior was gained through the permeabilizing action of nystatin. Only cells in which the access resistance was less than 10 megohms were used. Voltage and current records were collected from spontaneously beating, spindle-shaped cells. Data were collected and stored on a personal computer for subsequent analyses. Results were statistically evaluated by measuring mean ± standard errors of changes in current magnitudes. Where appropriate, Student's *t* test was used to evaluate the significance of differences between experimental groups.

Results

As expected, in cells from the rabbit sino-atrial node (SAN), β-adrenoceptor stimulation with isoprenaline (Iso, 1 μM) accelerated the rate of firing. Subsequent addition of adenosine (ADO, 20 μM) caused a slowing of this spontaneous pacemaker activity, and hyperpolarization of the maximum

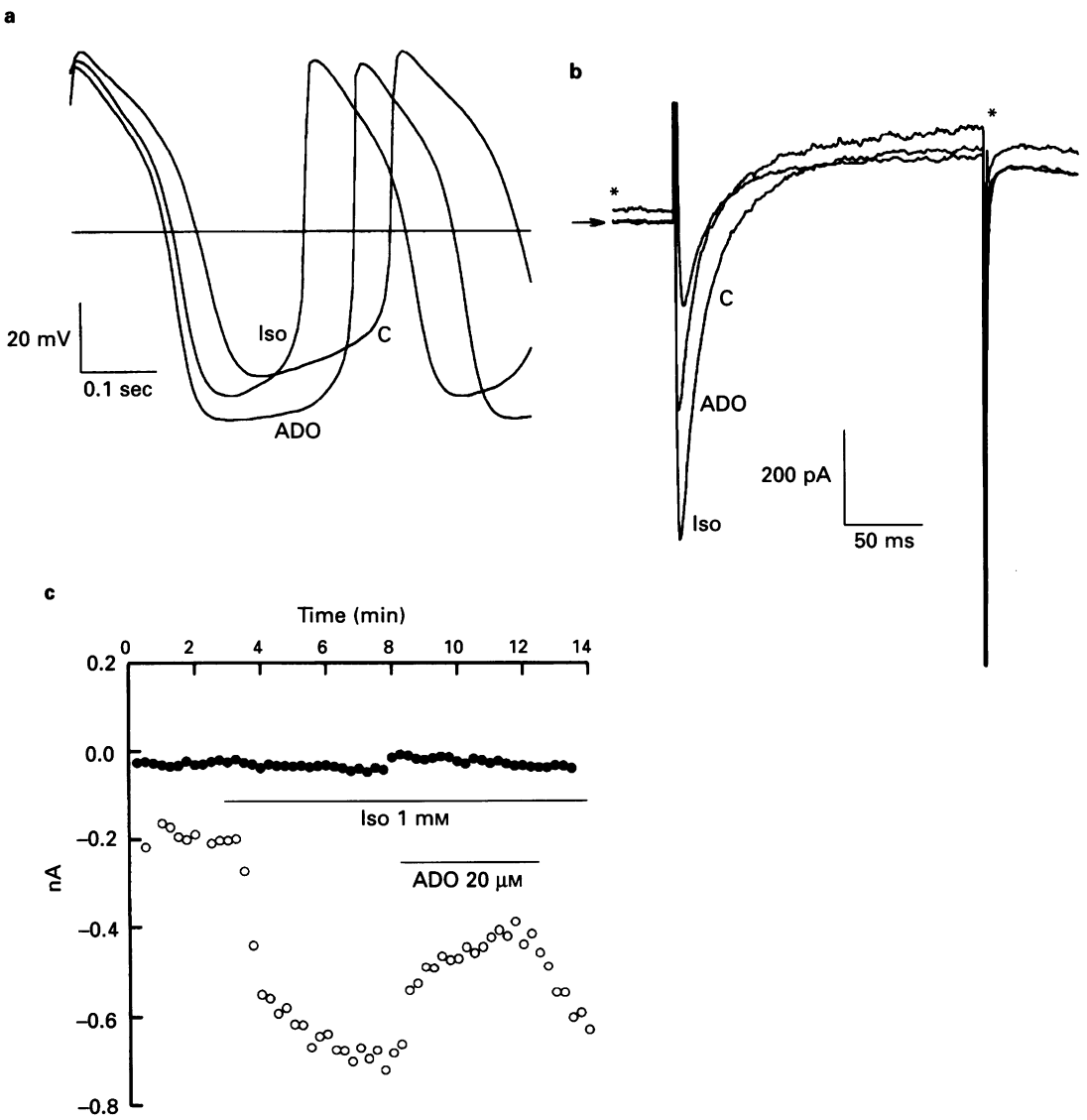

Figure 1 The effects of adenosine on spontaneous action potentials (a) and membrane currents (b,c) in a rabbit sino-atrial node (SAN) cell. The nystatin-perforated patch method was used to record spontaneous action potentials and underlying ionic currents. Panel (a) shows superimposed action potentials in control conditions (C), after addition of 1 μM isoprenaline (Iso), and then in Iso plus 20 μM adenosine (ADO). ADO slowed the heart rate in this and all other cells studied ($n = 5$) and also hyperpolarized the maximum diastolic potential in most experiments. Panel (b) shows the effects of ADO on membrane currents. The cell was held at −40 mV, and a 300 ms depolarizing pulse to 0 mV was applied every 15 s. The superimposed current traces show the L-type calcium current, $I_{Ca\text{-}L}$, in control conditions (C), in the presence of 1 μM Iso, and after addition of 20 μM ADO. Note the small outward shift of the holding current, which is caused by activation of a K^+ current, $I_{K\text{-}ADO}$ (denoted by *). The arrow in this and subsequent figures marks the zero current level. (c) Time course of changes in $I_{Ca\text{-}L}$ (○) and $I_{K\text{-}ADO}$ (●). Iso (1 μM) augmented $I_{Ca\text{-}L}$; subsequent addition of 20 μM adenosine (ADO) attenuated this effect, as well as activating a small $I_{K\text{-}ADO}$. The effect of ADO in $I_{Ca\text{-}L}$ was reversible upon washout. This and all other recordings were made at 32°C.

diastolic potential (Figure 1a). In 9 cells, in which the maximum diastolic potential (in 1 μM Iso) was -56.5 ± 1.7 mV (mean$\pm$s.e.mean), ADO produced a mean hyperpolarization of 3.9 ± 1.1 mV. The ionic mechanism(s) for these electrophysiological effects were examined by recording the underlying ionic currents in the presence of Iso. Each SAN cell was voltage clamped at -40 mV and 300 ms depolarizing pulses to 0 mV were applied to activate $I_{Ca\text{-}L}$. Superfusion of Iso (1 μM) caused a substantial (1.5 to 2 fold) increase in $I_{Ca\text{-}L}$. Subsequent addition of ADO (10–100 μM), in the presence of Iso significantly decreased $I_{Ca\text{-}L}$ as illustrated in Figure 1b.

The effectiveness of ADO in attenuating the action of Iso was assessed by measuring the reduction (in %) of the Iso-induced augmentation of the Ca^{2+} current, $I_{Ca\text{-}L}$. Since the effects of 10–20 μM ($n=20$) and 100 μM ADO ($n=13$) did not differ significantly, these results were pooled In 33 cells, the mean reduction of $I_{Ca\text{-}L}$ was 59.4 ± 4.4% (mean$\pm$s.e.mean). In 15 out of 33 successful experiments, adenosine also activated a time-independent (background) current, $I_{K\text{-}ADO}$, which was recorded as a small outward shift in the holding current (Figure 1b,c). The mean shift (for all 33 cells) was 11.9 ± 30 pA. The fact that this current change was not always observed may be due to the fact that it is very small, and therefore at, or below, the limit of our detection with this recording method (5-10 pA). The time course of the changes in $I_{K\text{-}ADO}$ and $I_{Ca\text{-}L}$ following addition of Iso and then ADO are shown in Figure 1c.

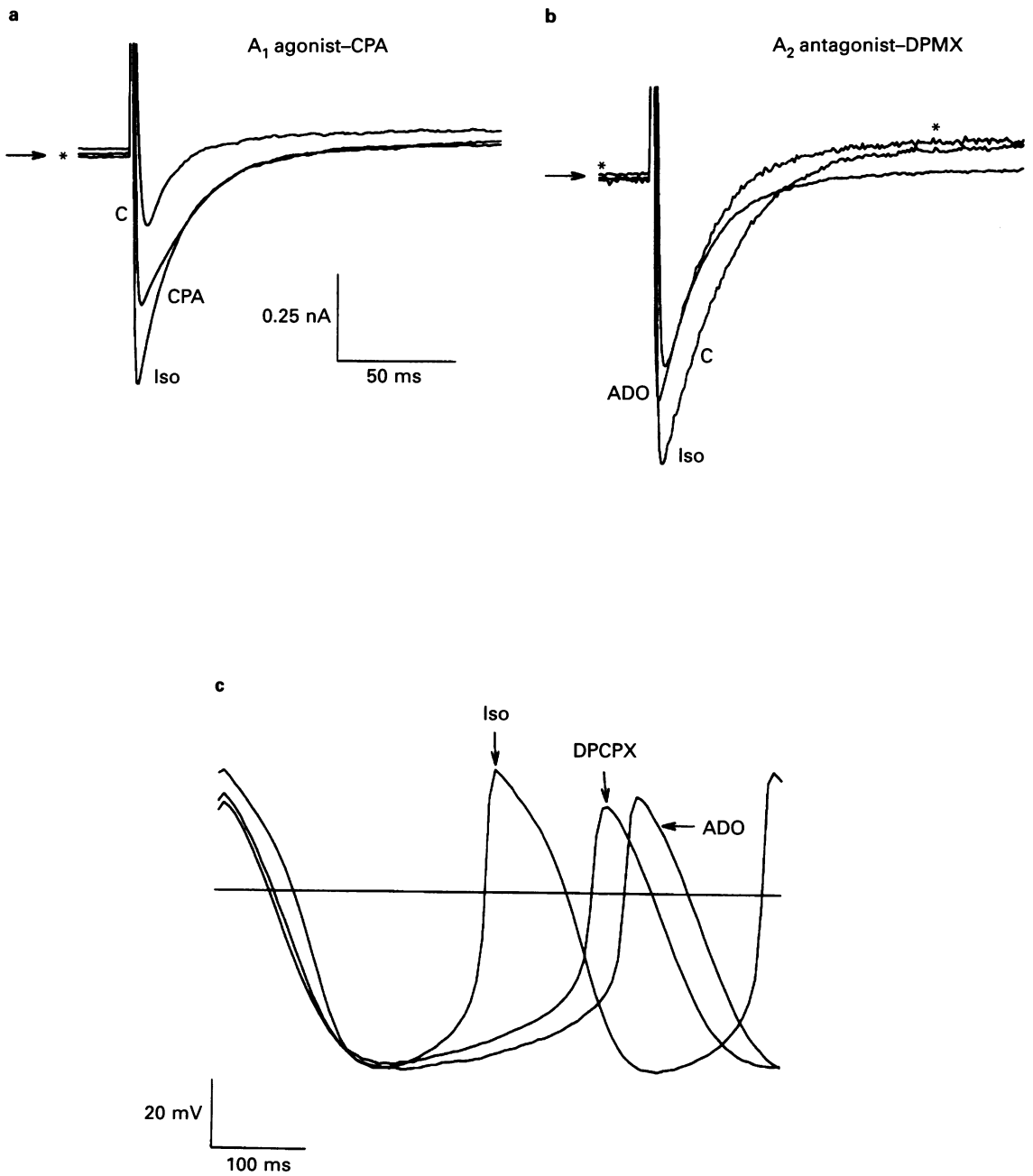

Figure 2 Pharmacological specificity of adenosine (ADO) action on $I_{Ca\text{-}L}$ in SAN cells. Results from several different cells are shown. (a) The A_1 agonist, N^6-cyclopentyladenosine (CPA, 10 μM) reduced $I_{Ca\text{-}L}$ in the presence of 1 μM Iso. Superimposed current traces, obtained in response to voltage steps (4 min^{-1}) from -40 to 0 mV are shown in panels a,b. (b) ADO (20 μM) attenuated $I_{Ca\text{-}L}$ and activated $I_{K(ADO)}$ in the presence of the A_2 antagonist, DPMX (10 μM). (c) Superimposed spontaneous action potentials are shown: Iso (1 μM) increased the firing rate compared with the control rate (not shown) in these cells. ADO (20 μM) significantly slowed the Iso-enhanced pacemaker rate. The addition of DPCPX (10 μM), an A_1 purinoceptor antagonist, partially blocked the action of ADO as illustrated by the faster beating rate.

The pharmacological profile of the effects of ADO in these pacemaker cells was also determined. Both the inhibitory effect of ADO on $I_{Ca\text{-}L}$, and the activation of $I_{K\text{-}ADO}$, were mimicked by the selective A_1 agonist, N^6-cyclopentyl-adenosine (CPA). In 8 cells, 3–10 μM CPA reduced the Iso-induced augmentation of $I_{Ca\text{-}L}$ by 53.1 ± 5.7%. The mean $I_{K\text{-}ADO}$ was 4.0 ± 2.7 pA. An example of this pattern of results is shown in Figure 2a. Further evidence that ADO acts through A_1 purinoceptor in SAN was obtained by use of the A_2 purinoceptor antagonist, 3,7-dimethy-1-propargyl-xanthine (DMPX). In 6 cells, addition of 100 μM ADO in the presence of 10 μM DPMX produced a reduction of the Iso-augmented current by

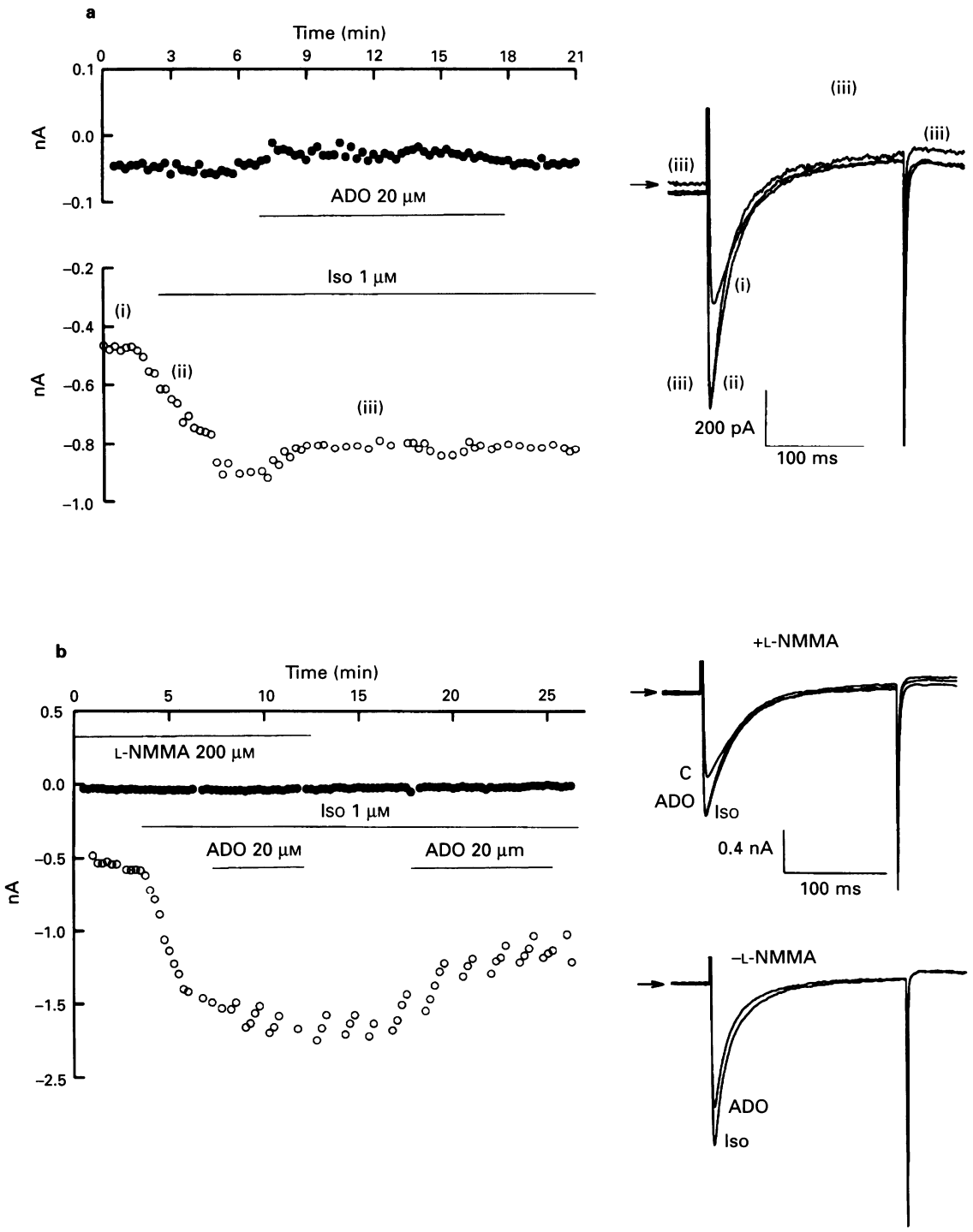

Figure 3 Inhibition of adenosine action on $I_{Ca\text{-}L}$ by the NO synthase inhibitor L-NMMA. $I_{Ca\text{-}L}$ (○) and holding current (●) amplitudes are plotted against time. (a) After preincubation of the SAN cells for 20 min in 100 μM L-NMMA, 1 μM Iso still increased $I_{Ca\text{-}L}$. Subsequent addition of 20 μM adenosine failed to attenuate $I_{Ca\text{-}L}$, although $I_{K(ADO)}$ was activated. The superimposed current traces on the right were recorded at times corresponding to points (i) control (ii) after Iso; and (iii) after ADO and Iso. Note the outward current $I_{K\text{-}ADO}$ was activated by adenosine (iii) in the presence of L-NMMA, when the inhibitory effect on $I_{Ca\text{-}L}$ was blocked. (b) Examples from 2 cells exposed to ADO in the presence of L-NMMA, and again following washout. On the left is the time course of current changes in response to 1 μM Iso and 20 μM ADO. No attenuation of $I_{Ca\text{-}L}$ was observed following preincubation in 200 μM L-NMMA, but the inhibitory effect of ADO was restored following L-NMMA washout. On the right are superimposed current traces from a different cell subjected to the same protocols. Results in the presence of and following removal of L-NMMA are shown at the top and bottom, respectively.

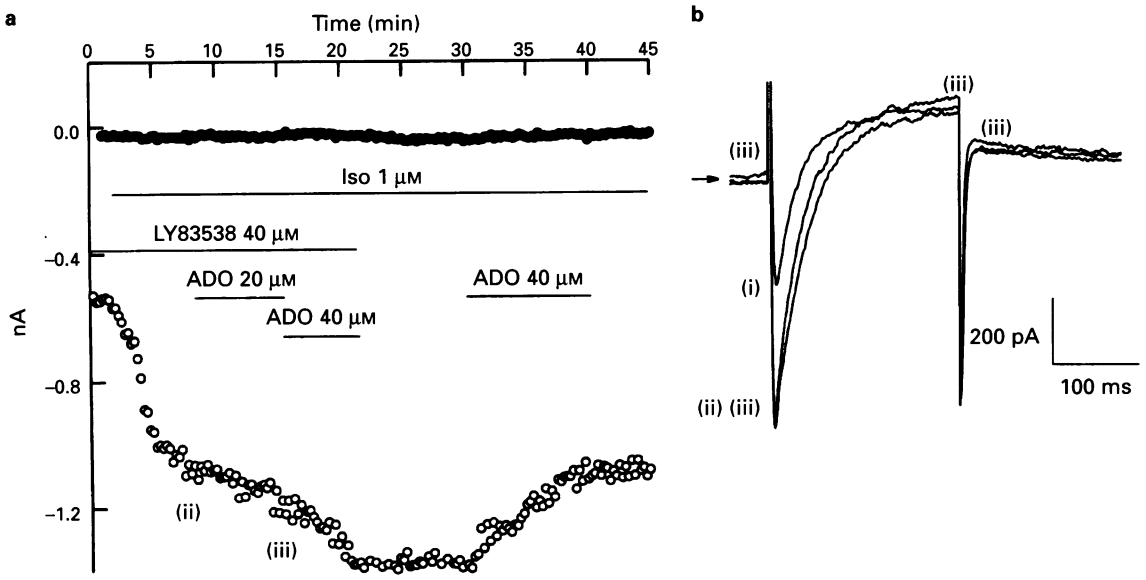

Figure 4 The effects of the guanylyl cyclase inhibitor, LY83583, on the ADO-induced inhibition of $I_{Ca\text{-}L}$. The cell was held at −40 mV, and depolarized to 0 mV for 300 ms every 15 s. In the presence of 40 μM LY83583, Iso increased $I_{Ca\text{-}L}$, (○) but subsequent addition of 20 or 40 μM ADO had no inhibitory effect. After LY83583 and ADO were washed off, a second exposure to 40 μM ADO produced the characteristic attenuation of $I_{Ca\text{-}L}$. The super-imposed current traces on the right were obtained from a different control cell (i), after Iso was added in the presence of LY83583 (ii), and then following addition of 40 μM ADO (iii). Note $I_{K\text{-}ADO}$ was still activated by ADO (iii) when there was no significant reduction in $I_{Ca\text{-}L}$.

53.1±11.5%. The mean outward $I_{K\text{-}ADO}$ was 7.8±3.8 pA. These results are not statistically different from those with ADO alone, demonstrating that block of the A_2 purinoceptor population has no effect on this action of ADO. Figure 2b shows an example of this pattern of responses. On the other hand, the effects of ADO on the action potential and pacemaker activity could be blocked significantly by the A_1 purinoceptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 μM; $n=6$). As shown in Figure 2c, after ADO (20 μM) had slowed the spontaneous beating rate from the level reached in Iso, DPCPX (10 μM) increased the spontaneous beating rate, but not the Iso-induced rate.

In both the cardiovascular and nervous systems, a biochemical second messenger cascade involving agonist-induced formation of nitric oxide (NO) from L-arginine has been identified and extensively investigated (Bredt & Snyder, 1990; Moncada *et al.*, 1991; Balligand *et al.*, 1993). Recently, we have shown that NO mediates the indirect inhibitory effect of muscarinic agonists on $I_{Ca\text{-}L}$ in rabbit SAN pacemaker cells (Han *et al*, 1994; 1995). To determine whether NO formation is involved in the A_1-mediated purinoceptor responses in rabbit SAN, the enzyme which is responsible for NO synthesis, NO synthase, was inhibited. After pre-incubation of SAN cells with N^G-nitromethyl L-arginine (L-NMMA, 100 μM), there was no longer an ADO-induced inhibition of $I_{Ca\text{-}L}$, although the direct action of ADO on $I_{K\text{-}ADO}$, was still observed. ADO caused no measurable attenuation of $I_{Ca\text{-}L}$ in 13 out of 20 cells which were preincubated with 100 μM L-NMMA (20-40 min). In the other 7 cells there was a small reduction of $I_{Ca\text{-}L}$, perhaps due to incomplete inhibition of NO synthase. In all 20 SAN cells, the mean reduction of the Iso-augmented $I_{Ca\text{-}L}$ was only 7.9±2.8%. Figure 3a shows an example of this pattern of results.

In 7 of these cells, it was possible to wash out the ADO and L-NMMA, and then expose the cell to ADO for a second time, in the absence of NO synthase inhibition. Under these conditions, in all 7 cells, ADO produced a larger reduction in $I_{Ca\text{-}L}$ following washout than was observed in the first exposure. Examples from two cells are shown in Figure 3b (right). The inhibition of NO synthase did not block the activation of $I_{K\text{-}ADO}$ (Figure 3a). A measurable outward current was activated by ADO in 14 of 20 cells, with a mean size of 19.7±6.4 pA ($n=20$). The maintained direct action of ADO on $I_{K\text{-}ADO}$ indicates that L-NMMA was not acting in a nonspecific fashion by, e.g. interfering with ADO binding. We have shown previously (Han *et al.*, 1994; 1995), that altering NO levels by application of a NO-donor N-[ethoxycarbonyl]-3-[4-morpholinyl-sydnonimine] (SIN-1, 100 μM), significantly reduces the Iso-stimulated $I_{Ca\text{-}L}$ in SAN pacemaker cells. Very similar effects were observed in this set of experiments with ADO/SIN-1 combinations (results not shown).

In many tissues (Schmidt *et al*, 1993), NO activates soluble guanylyl cyclase, resulting in an increase in intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP); this response can be inhibited by 6-anilino-5,8-quinolinedione, LY83583 (Schmidt *et al.*, 1984). In our experiments, after LY83583 (40 μM) was added to the superfusate, the Iso-induced augmentation of $I_{Ca\text{-}L}$ was still consistently observed. However, in 18 cells, there was no detectable reduction (<1%) of the Iso-stimulated $I_{Ca\text{-}L}$ by 20 μM or 40 μM ADO. After LY83538 and ADO were washed off, a second exposure to 40 μM ADO produced the characteristic inhibition of $I_{Ca\text{-}L}$ (Figure 4). LY83538 did not interfere with the activation of $I_{K(ADO)}$ by ADO. The mean outward current activated by ADO in the presence of LY83538 was 10.6±4.0 pA ($n=18$), which is comparable to the value for $I_{K\text{-}ADO}$ under control conditions. LY83583 also consistently blocked the action of SIN-1 on $I_{Ca\text{-}L}$, providing further evidence that the NO-mediated inhibition of $I_{Ca\text{-}L}$ involves formation of cyclic GMP (results not shown).

In summary, inhibition of both NO formation (L-NMMA) and NO-induced enhancement of cyclic GMP formation (LY 83583) can abolish the inhibitory action of ADO on $I_{Ca\text{-}L}$, but are without effect on $I_{K(ADO)}$. The histograms in Figure 5 summarize these results.

Discussion

Summary of results and relation to previous studies

Our results show that in rabbit SAN pacemaker cells, adenosine (ADO) binds to A_1 purinoceptors and attenuates $I_{Ca\text{-}L}$ by activating a biochemical pathway which requires NO and

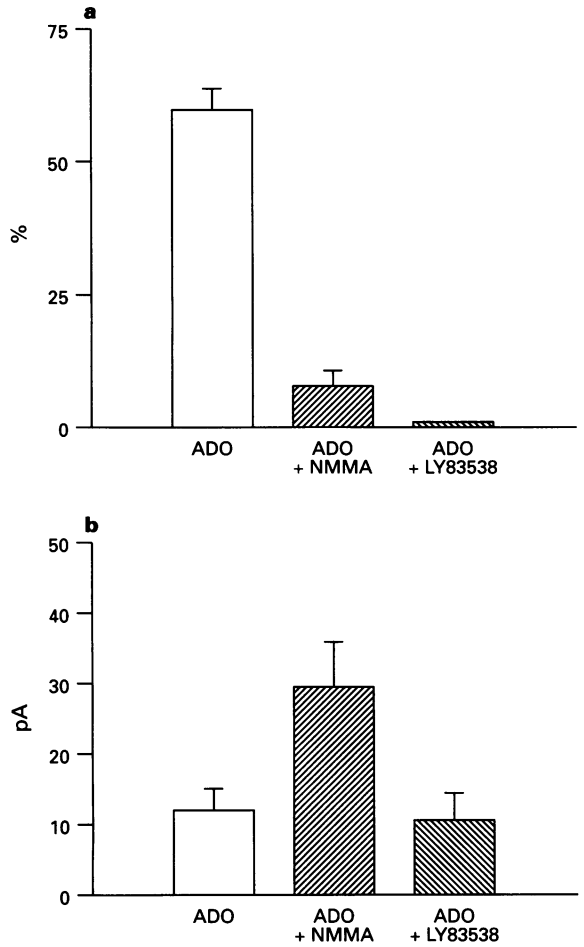

Figure 5 Summary of ADO actions in control SAN cells ($n=33$), following inhibition of NO formation by pre-treatment with L-NMMA ($n=20$), and following inhibition of guanylyl cyclase by LY83538 ($n=18$). (a) Mean (±s.e.mean) values for the attenuation (in %) of the Iso-augmented $I_{Ca\text{-}L}$. (b) The mean holding current (±s.e.mean in pA) $I_{K\text{-}ADO}$ activated by ADO in these 3 groups of SAN cells.

cyclic GMP formation. This effect occurs in the presence of stimulated β-adrenoceptors (adrenergic tone) and is therefore likely to be of physiological importance. Adrenergic tone is present in all mammals (Belardinelli *et al.*, 1988; 1989; Irisawa *et al.*, 1983), although both the direct and the indirect actions of ADO in the heart show prominent species-dependence (Pelleg & Belardinelli, 1993). Additional experimental work is needed to determine whether similar NO-mediated indirect effects on $I_{Ca\text{-}L}$ are involved in the negative inotropic response to ADO in mammalian atrium (Kabell *et al.*, 1994; Wang & Belardinelli, 1994; Nawrath *et al*, 1995) and/or ventricle (cf. Zakharov *et al.*, 1996). It has recently been shown that NO mediates ADO-mediated attenuation of $I_{Ca\text{-}L}$ in rabbit AV node cells (Martynyuk *et al.*, 1996), and the ionic basis for its negative dromotropic effect has been examined (Wang *et al.*, 1996).

ADO-induced reduction in cyclic AMP levels (which is required for attenuation of $I_{Ca\text{-}L}$) could be caused by a direct inhibition of adenylyl cyclase. However, the involvement of NO and cyclic GMP raise other possibilities, as suggested by our earlier work on muscarinic inhibition of $I_{Ca\text{-}L}$ (Han *et al.*, 1995), and the results of several other groups (Fischmeister & Hartzell, 1987; Mery *et al.*, 1993, 1995; Levi *et al.*, 1994). Two important intracellular targets for cyclic GMP are the cyclic GMP-activated protein kinase (PKG) and the cyclic GMP-stimulated phosphodiesterase (PDE II, Schmidt *et al.*, 1993). It appears that in mammalian ventricle PKG is the mediator of cyclic GMP actions (Mubagwa *et al.*, 1993; Levi *et al.*, 1994), whereas in frog ventricle, PDE II serves this function (Fischmeister & Hartzell, 1987; Mery *et al.*, 1993). Previously, we (Han *et al.*, 1995, 1996) suggested that in rabbit SAN cells, PDE activation also plays a major role in mediating the muscarinic inhibition of $I_{Ca\text{-}L}$. It is now known that selective inhibition of PDE type III is without effect in myocytes from rabbit A-V node (Han *et al.*, 1996), whereas PDE II inhibition inhibits the muscarinic attenuation of $I_{Ca\text{-}L}$ in frog ventricle (Mery *et al.*, 1995).

Until very recently NO had not been implicated as an intracellular messenger in the effects of ADO on Ca^{2+} influx in the cardiovascular system. However, a similar pattern of results has been reported recently for AV node cells (Martynyuk *et al.*, 1995). A somewhat similar mechanism for the action of ADO on 5-hydroxytryptamine transport in basophilic cells (Miller & Hoffman, 1994) has also been postulated.

Implications and limitations of this study

We used relatively high concentrations of ADO (10 to 100 μM) to maximize the activation of the outward current, $I_{K(ADO)}$, since this current change served as an important control in each recording by demonstrating that neither L-NMMA nor LY83538 was simply blocking the A_1 purinoceptors. The physiological concentrations of adenosine which have been measured are considerably lower than the ones we used (Olsson & Pearson, 1990). This may mean that the NO-mediated effects on $I_{Ca\text{-}L}$ apply mainly to conditions of metabolic impairment, when ADO levels increase markedly.

In principle, it is now possible to measure directly NO production from isolated single cells (Malinski & Taha, 1992). This information, if it would be obtained in conjunction with our electrophysiological recordings, would provide a more rigorous test of our working hypothesis than the pharmacological approaches now available to us.

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