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Modulation by extracellular ATP of L-type calcium channels in guinea-pig single sinoatrial nodal cell

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1 The effects of extracellular adenosine 5′-triphosphate ($[ATP]_o$) on the L-type Ca^{2+} channel currents in guinea-pig single sinoatrial nodal (SAN) cells, isolated by enzymatic dissociation, were investigated by use of whole-cell patch-clamp techniques.

2 The application of $[ATP]_o$ (2 μM – 1 mM) produced an inhibitory effect on the L-type Ca^{2+} channel current peak amplitude (10 mM Ba^{2+} as charge carrier) in a concentration-dependent and reversible manner with an IC_{50} of 100 μM and a Hill coefficient of 1.83.

3 The presence of the adenosine receptor antagonists, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 μM) and 8-phenyltheophylline (10 μM) did not affect the $[ATP]_o$-induced inhibition of the Ca^{2+} channel currents. Adenosine (100 μM) had little effect on the basal Ca^{2+} channel currents. Adenosine 500 μM, caused 23% inhibition of the Ca^{2+} channel current, which was abolished by 0.1 μM DPCPX.

4 The presence of the P_2-purinoceptor antagonists, suramin (1, 10 and 100 μM), reactive blue 2 (1 and 10 μM) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 50 and 100 μM) failed to affect the inhibitory action of $[ATP]_o$ on Ca^{2+} channel currents.

5 The relative rank order of potency of different nucleotides and nucleosides, at a concentration of 100 μM, on the inhibition of the Ca^{2+} channel currents is as follows: adenosine 5′-triphosphate (ATP) = α,β-methylene-ATP (α,βMeATP) > > 2-methylthioATP (2-MeSATP) $\geq$ adenosine 5′-O-(3-thiotriphosphate) (ATPγS) > > uridine 5′-triphosphate (UTP) = adenosine 5′-diphosphate (ADP) > adenosine 5′-monophosphate (AMP) $\geq$ adenosine.

6 These results suggest that $[ATP]_o$ may play an important role in the heart beat by inhibiting the L-type Ca^{2+} channel currents in single SAN cells. This inhibitory effect is not due to the formation of adenosine resulting from the enzymatic degradation of $[ATP]_o$. Based on the relative order of inhibitory potency of different nucleotides and nucleosides on the L-type Ca^{2+} channel currents and the ineffectiveness of the purinoceptor antagonists tested, a novel type of purinoceptor may be involved.

Keywords: Sinoatrial node (guinea-pig); extracellular ATP; Ca^{2+} channel currents

Introduction

In the mammalian heart, rhythmic activity arises from the discharge of cells in the sinoatrial nodal (SAN) region which is important for normal cardiac activity. The SAN region is highly innervated by both sympathetic and parasympathetic nerves. It now known that the sympathetic neurotransmitters, adenosine 5′-triphosphate (ATP) and noradrenaline (NA) are stored and released from the same vesicles within the nerve varicosity (Sneddon & Westfall, 1984; Stjärne, 1989; Burnstock, 1995). It is therefore possible that ATP released from the sympathetic nerve terminals can modulate the pacemaking activity of the heart. *In vivo*, it has been shown that $[ATP]_o$ has depressant effects on pacemaker activity and slows down the rate of the ventricular escape rhythm (Pelleg *et al.*, 1986) and it has been suggested that this inhibitory effect may be due to the formation of adenosine from the enzymatic breakdown of ATP.

Most studies in the literature examining the modulatory effects of $[ATP]_o$ on L-type Ca^{2+} channel currents have used myocytes isolated from atria and ventricles (Alvarez *et al.*, 1990; Qu *et al.*, 1992; 1993; Mantelli *et al.*, 1993; Scamps *et al.*, 1992; 1993; Scamps & Vassort, 1994) and both an increase and a decrease in the L-type Ca^{2+} channel current amplitude, has been reported. Unlike the atrial and ventricular myocardia, in the SAN the L-type Ca^{2+} channel current has been suggested to play an important role in the initiation of the action potential (Irisawa & Noma, 1984, Doerr *et al.*, 1989, Irisawa *et al.*, 1993) and therefore pacemaker activity. At present, no detailed electrophysiological information is available regarding the effects of $[ATP]_o$ on L-type Ca^{2+} channel currents of the mammalian cardiac pacemaker cells. Therefore, the effects of extracellular ATP ($[ATP]_o$) on the L-type Ca^{2+} channel currents of pacemaker cells obtained from SAN were investigated in the present study.

Based on the results obtained from experiments studying agonist specificity, the relative rank order of potency of different ATP analogues on various preparations and molecular structures, have provided evidence for the existence of, at least, five subtypes of P_2-purinoceptors (P_{2X}, P_{2Y}, P_{2T}, P_{2U} and P_{2Z}) (for review see Dubyak & El-Moatassim, 1993; Fredholm *et al.*, 1994) and the list for subclassification of purinoceptors is still growing. Both P_1 and P_2 purinoceptors have been found in guinea-pig atrial and ventricular preparations (Burnstock & Meghji, 1981; Dorigo *et al.*, 1988; Wilson & Broadley, 1989; Mantelli *et al.*, 1993) but nothing is known about the type of purinoceptors present in the sinoatrial node. To characterize what type of purinoceptors were involved in the present study, two different methods were employed. The effect of $[ATP]_o$ in the presence of different commercially available adenosine receptor (A_1 and A_2) antagonists (8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-phenyltheophylline (8-PT)) and purinoceptor antagonists (suramin, reactive blue 2 and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS)) was investigated. The rank order of potency of different nucleotides and nucleosides on the inhibition of L-type Ca^{2+} channel currents was also examined. Part of this study has been presented in abstract form (Qi & Kwan, 1995).

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Methods

Isolation of guinea-pig single sinoatrial nodal cells

Hearts from adult guinea-pigs (either sex, 220–250 g) were retrogradedly perfused with enzyme solutions as described previously (Arena & Kass, 1988). Briefly, the guinea-pig was killed by cervical dislocation, the heart excised and quickly mounted onto a Langendorff retrograde perfusion system. All procedures were approved by our University Animal Research Ethics Committee. The heart was successively perfused with Ca^{2+}-free Tyrode solution for 5–7 min and Ca^{2+}-free Tyrode solution containing 1 mg ml^{-1} of collagenase type II (Worthington Biochemicals Co., Freehold, NJ, U.S.A.) plus 0.1 mg ml^{-1} of protease type XIV (Sigma, St. Louis, MO, U.S.A.) for 7–8 min. This was then followed by a 10 min perfusion with 200 μM Ca^{2+} Tyrode solution. These solutions were perfused at a flow rate of 10 ml min^{-1} (37°C). After the perfusion procedure, the right atrium was identified, removed and placed in a Petri dish containing 200 μM Ca^{2+} Tyrode solution at room temperature (22°C).

Three criteria, as described by Anumonwo *et al.* (1992), were used for the identification of SAN cells. The SAN region was isolated as a small piece of tissue (2 mm × 2 mm) bordered by the crista terminalis and intra-atrial septum on two sides and by the cranial and caudal vena cava on the other two sides. SAN cells were then obtained by gentle agitation of this piece of tissue. The majority of the cells isolated were oval/spherical and only cells which had a smooth surface (no membrane blebs) were used in this study. All experiments were conducted within 8 h following cell isolation.

Electrophysiology

In this study, the L-type Ca^{2+} channel current was measured by the whole-cell patch-clamp techniques (Hamill *et al.*, 1981). The cells were placed in a recording chamber (0.5 ml) which was mounted on the stage of an inverted microscope (Nikon Diaphot TMD). Cells were voltage-clamped using Gold Seal Accu-fill 90 micropets (Clay Adams Inc., Parsippany, NJ, U.S.A.) glass capillaries. The resistance of the pipettes was 1–3 MΩ when filled with internal pipette solution. Series resistance compensation was used in all experiments and was adjusted to give the fastest possible capacitative transients without producing ringing. After the whole-cell patch-clamp configuration was established, normal Tyrode solution was substituted for the external bathing solution. Membrane currents were measured with and voltages were controlled by an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, U.S.A.). Currents were filtered at 5 kHz and sampled at 3–5 kHz. Data were stored and analysed on a Comtech 486 computer with the use of a pCLAMP software (Axon Instruments, Foster City, CA, U.S.A.). All experiments were performed at room temperature (20–22°C).

The L-type Ca^{2+} channel current was recorded with a holding potential of −40 mV, in the presence of 10 μM tetrodotoxin (TTX) and 132 mM N-methyl-D-glucamine (NMDG), to exclude any T-type Ca^{2+} channel current or the possibility of Ca^{2+} moving through the fast Na^+ channel. The inward currents recorded in our study were abolished by 10 nM nifidepine ($n=5$), a well known 1,4-dihydropyridine L-type Ca^{2+} channel blocker. For this reason we are confident that these inward currents recorded are L-type Ca^{2+} channel currents, according to the terminology suggested by Nilius *et al.*, (1985). Even though it has been shown that T-type Ca^{2+} channels are present in cardiac tissues (Tytgat *et al.*, 1990; Nuss & Houser, 1993; Mishra & Hermsmeyer, 1994), T-type calcium channel currents cannot be identified in our preparation even with 20 mM Ba^{2+} as the charge carrier and at the negative holding potential of −70 or −80 mV ($n=4$).

Delivery of drugs (ATP and its analogues, and different antagonists) to the cell was achieved via a home-made multi-barrelled perfusion pipette which is placed at ≈50–60 μm from the cell. Flow of solution from the perfusion pipette was electronically controlled by a solenoid valve coupled to a 4-channel valve driver (General Valve Corp., U.S.A.) and with complete solution change surrounding the cell achievable within 30–40 ms. Changing solution from one solution reservoir to another containing no drug did not cause any change in the Ca^{2+} channel current amplitude.

Solutions

Normal Tyrode solution in this study contained (in mM): NaCl 132, KCl 4.8, $CaCl_2$ 1, $MgCl_2$ 2, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES) 10 and glucose 5 (pH 7.4 adjusted with NaOH). Internal (pipette) solution contained (mM): CsCl 60, $CaCl_2$ 1, $MgCl_2$ 1, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) 11, aspartic acid 50, HEPES 10 and K_2ATP 5 (pH 7.4 adjusted with CsOH). External bathing solution for L-type Ca^{2+} channel current recording contained (mM): N-methyl-D-glucamine (NMDG) 132, CsCl 4.8, HEPES 5, $MgCl_2$ 2, glucose 5 and $BaCl_2$ 10 (pH 7.4 adjusted with HCl acid) plus 10 μM tetrodotoxin (TTX). In this study, 10 mM Ba^{2+} was used as the charge carrier for the L-type Ca^{2+} channel current to increase the signal to noise ratio and facilitate accurate measurements as well as blocking the K^+ channels. To avoid desensitization caused by $[ATP]_o$ and its analogues, only one concentration of a particular agent was tested on each cell. In the antagonist experiments in which the reproducibility of the $[ATP]_o$-induced effect was investigated, the cell was challenged twice with the same concentration (100 μM) of $[ATP]_o$.

Drugs

All chemicals used in preparing Tyrode solution as well as the standard external bathing solution and internal pipette solution were purchased from Sigma Chemical Co. (U.S.A.). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and 2-methylthioATP tetrasodium (2-MeSATP) were obtained from Research Biochemicals International (U.S.A.); suramin sodium, 8-phenyltheophylline (8-PT) and tetrodotoxin (TTX) were purchased from Calbiochem-Novabiochem Corporation (U.S.A.); pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) was obtained from Tocris Cookson (U.K.); reactive blue 2, adenosine 5'-triphosphate disodium (ATP), α,β-methylene-ATP lithium (α,βMeATP), adenosine 5'-O-(3-thiotriphosphate) tetralithium (ATPγS), uridine 5'-triphosphate sodium (UTP), adenosoine 5'-diphosphate sodium (ADP), adenosine 5'-monophosphate sodium (AMP) and adenosine were purchased from Sigma Chemical Co. (U.S.A.). All drugs were dissolved in de-ionized water and stored at −30°C, except 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-phenyltheophylline which were dissolved in dimethyl sulphoxide (DMSO) and used within a month after preparation. In a small number of cells tested ($n=3$), ten times the concentration of DMSO used in the present study was found to have no effect on sinoatrial nodal cell L-type Ca^{2+} channel currents (data not shown).

Statistics

Results were calculated in which the peak amplitude of the L-type Ca^{2+} channel current recorded in the control condition was considered as 100% and any change observed in the presence of nucleotides or nucleosides as a % of control. In the antagonist experiments (Figures 3 and 4), we compared the change in current amplitude X (the change due to $[ATP]_o$ alone) with the change in current amplitude Y (the change due to $[ATP]_o$ in the presence of the particular antagonist). Data are expressed as mean ± s.e. mean. Statistical analysis was performed by using ANOVA and a P value of <0.05 was considered statistically significant.

Results

Effects of extracellular ATP ($[ATP]_o$) on L-type Ca^{2+} channel currents

Under our experimental conditions, the L-type Ca^{2+} channel current was activated at ~ -10 mV and peaked at $+20$ mV (holding potential $= -40$ mV, voltage pulses were delivered from -60 mV to $+70$ mV with a 10 mV increment for a duration of 100 ms at 1 Hz). The L-type Ca^{2+} channel current was inhibited in a concentration-dependent fashion by $[ATP]_o$ (2 μM to 1 mM) ($n = 5-8$ for each concentration tested) (Figure 1b) and the inhibitory effect of $[ATP]_o$ could be reversed after washout. In the current-voltage relationship experiments, application of $[ATP]_o$ only caused a decrease in the peak current amplitude of the L-type Ca^{2+} channel current plus a 6–9 mV hyperpolarizing shift in the reversal potential without a change in the peak potential (Figure 1a). Figure 1b shows the concentration-response relationship for inhibition of the L-type Ca^{2+} channel current by $[ATP]_o$. Application of $[ATP]_o$ (2 μM to 1 mM) caused a concentration-dependent inhibition of the Ca^{2+} channel current. The inhibitory effect of $[ATP]_o$ could be reversed after washout (Figure 1b). To avoid desensitization by $[ATP]_o$ only one concentration of $[ATP]_o$ was tested on each cell. The curve was fitted using the Hill equation: $I_{Ca(L)\,([ATP]0)}/I_{Ca(L)(control)} = 1/(1+(K/[ATP]_o)^{n_h})$ where K is the concentration at which inhibition is half maximal (IC_{50}) and n_H is the Hill coefficient. Our results showed that $[ATP]_o$ inhibited the Ca^{2+} channel current with an IC_{50} of 100 μM, and a Hill coefficient of 1.83.

Using a train protocol designed to measure the onset of block of the L-type Ca^{2+} channel currents (Kass & Arena, 1989) by $[ATP]_o$ (holding potential $= -40$ mV, pulsed to $+20$ mV for a duration of 100 ms, once every 5 s), $[ATP]_o$ was applied after a stable control condition was established. Application of 100 μM $[ATP]_o$ caused a very rapid inhibition of the L-type Ca^{2+} channel current, with over 90% of the maximum steady-state inhibition being reached within 5 s (Figure 2a). The inhibitory effect of $[ATP]_o$ could be reversed after washout. Effects of two stable (ectoATPase-resistant) ATP analogues, ATPγS and α,β-methylene-ATP, (100 μM) on Ca^{2+} channel current were also tested. Application of either ATPγS or α,β-methylene-ATP also caused an inhibition of Ca^{2+} channel current and the inhibition observed can be reversed by washing (Figure 2b and c).

Determination of the type of purinoceptors involved

The reproducibility of the $[ATP]_o$-induced inhibition of the L-type Ca^{2+} channel current was examined by challenging the same single pacemaker cell twice with the same concentration of $[ATP]_o$ ($n = 6$). Our results showed that there is no apparent change in the % inhibition caused by 100 μM$[ATP]_o$, of the L-type Ca^{2+} channel current between two challenges (35.2 ± 3.8 vs 33.2 ± 5.7% inhibition). To determine the type of purinoceptor involved in the present study, different purinoceptor antagonists were used. Following the first challenge with 100 μM $[ATP]_o$ and the subsequent washout, the cell was then challenged with a particular purinoceptor antagonist to observe the effect of the antagonist on the L-type Ca^{2+} channel current. Once the effect

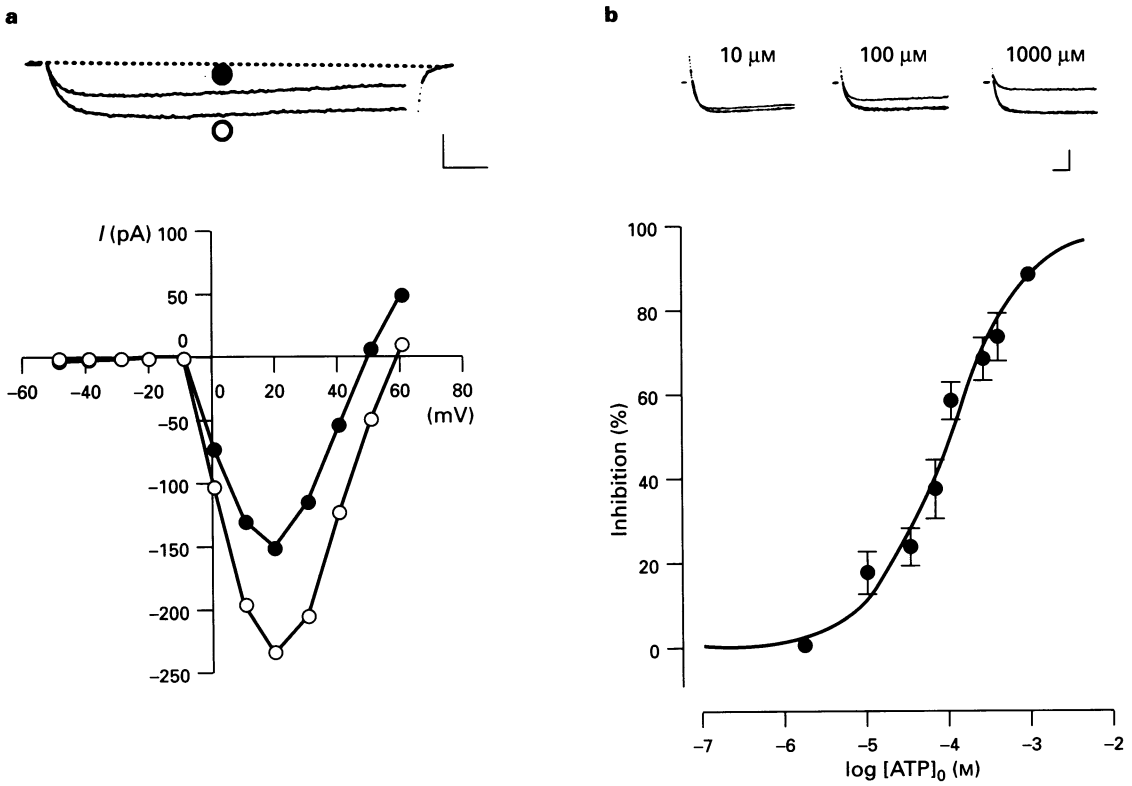

Figure 1 (a) Inhibition of Ca^{2+} channel current by $[ATP]_o$. The inset shows leaked subtracted superimposed whole-cell current traces before (○) and after (●) application of 100 μM $[ATP]_o$, in response to 100-ms voltage pulses to $+20$ mV at 1 Hz. The holding potential was -40 mV. Calibration bars: 150 pA, 10 ms. Current-voltage (I-V) relationships of the peak current amplitude of Ca^{2+} channel current in control (○) and in the presence of 100 μM $[ATP]_o$ (●) from a single sinoatrial nodal cell. (b). Concentration-response curve for the $[ATP]_o$-induced inhibition of Ca^{2+} channel current in a single sinoatrial nodal cell. Test pulses of 100 ms to $+20$ mV from the holding potential of -40 mV. The curve was fitted using the Hill equation: $I_{Ca(L)([ATP]_o)}/I_{Ca(L)\,(control)} = 1/(1+(K/[ATP]_o)^{n_H})$ where K is the concentration at which inhibition is half maximal (IC_{50}) and the n_H is the Hill coefficient. The data are presented as mean $\pm$ s.e.mean. ($n = 6-13$). The inset shows representative whole-cell current traces in the presence of 10, 100 and 1000 μM $[ATP]_o$ and the effect of $[ATP]_o$ can be reversed by washing. Calibration bars: 100 pA, 10 ms.

of the particular antagonist on the L-type Ca^{2+} channel current had been established, the cell was challenged with 100 μM$[ATP]_o$ in the continuous presence of the antagonist.

Suramin, a P_{2X} and P_{2Y} receptor antagonist, (1, 10 and 100 μM), on its own inhibited the L-type Ca^{2+} channel currents (8.4±2.6%, 20.1±3.7% and 34.2±5.0% inhibition, respectively) (Figure 3a) ($n=6-8$ for each concentration). The presence of suramin failed to affect the $[ATP]_o$-induced inhibition of the L-type Ca^{2+} channel current ($n=4-6$) (Figure 3a) and a further decrease in Ca^{2+} channel current amplitude was observed which was not significantly different from control conditions (35.2±3.8 vs. 33.6±4.7%) (Figure 3a).

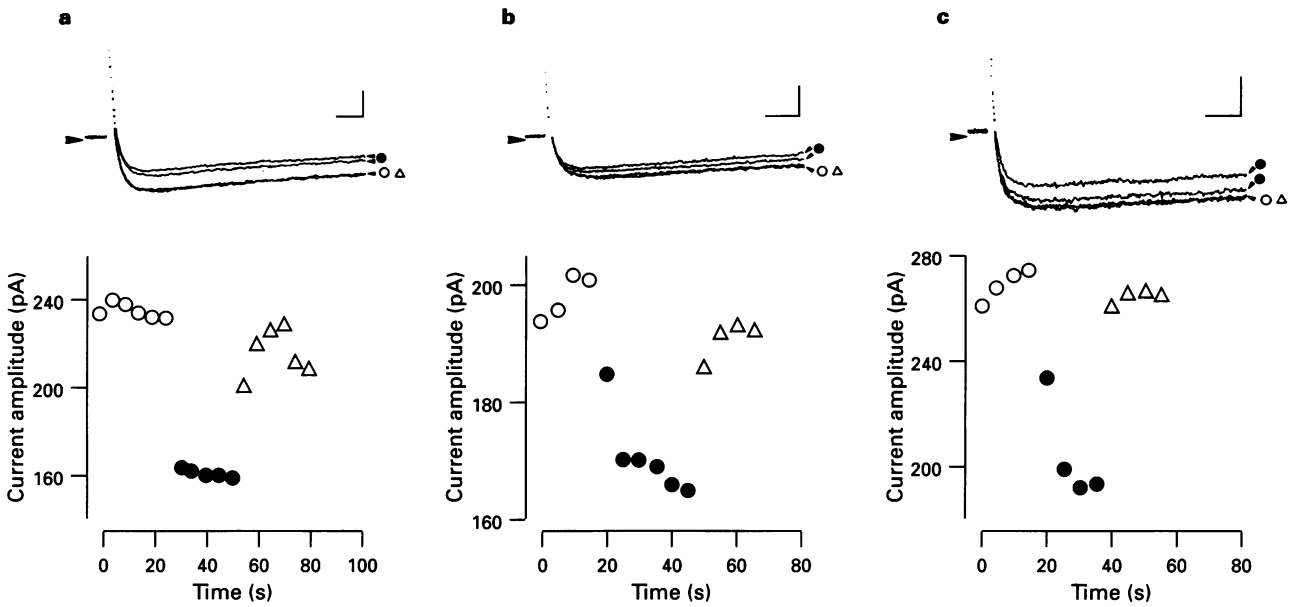

Figure 2 Inhibition of Ca^{2+} channel current by 100 μM (a) ATP, (b) ATPγS and (c) α,βMeATP. The inset shows superimposed whole-cell current traces before (○) and after (●) application of the particular nucleotide (the first and the last traces in the presence of the particular nucleotide). Effect of the nucleotide on Ca^{2+} channel current can be reversed by washing (△). Currents were evoked using a train protocol of a test pulse at +20 mV, 100 ms duration, from a holding potential of −40 mV stimulated once every 5 s. Calibration bars: (a) 120 pA and 10 ms; (b) 100 pA and 10 ms; (c) 140 pA and 10 ms.

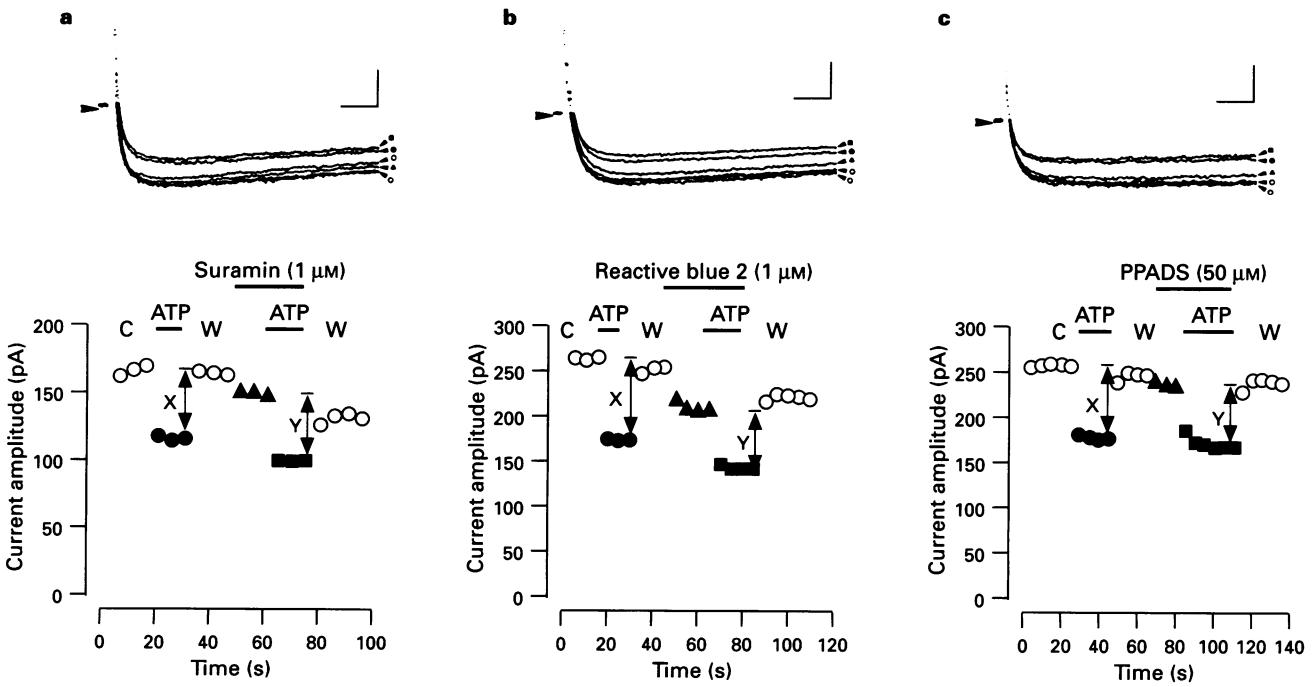

Figure 3 Inhibition of Ca^{2+} channel current by 100 μM $[ATP]_o$ in the presence of (a) suramin (1 μM), (b) reactive blue 2 (1 μM) and (c) PPADS (50 μM) in guinea-pig single sinoatrial nodal cells. The insets show the representative whole-cell current traces of Ca^{2+} channel current in control and washes (○), in the presence of $[ATP]_o$ (●), effect of individual antagonist (▲) and the effect of $[ATP]_o$ in the continuous presence of a particular antagonist (■). The cell was stimulated to +20 mV for 100 ms from a holding potential of −40 mV, once every 5 s. Effect of individual antagonist on $[ATP]_o$-induced inhibition of Ca^{2+} channel current was examined by comparing the effect of $[ATP]_o$ on its own (X) and in the presence of particular antagonist (Y). C and W indicate control and washes, respectively. Calibration bars: (a) 80 pA and 10 ms; (b) 125 pA and 10 ms; (c) 125 pA and 10 ms.

The P_{2Y} receptor antagonist, reactive blue 2, inhibited the L-type Ca^{2+} channel current (1 μM, 14.7±3.2%; 10 μM, 21.3±4.5%) ($n=5-6$ for each concentration). The presence of reactive blue 2 also failed to affect the $[ATP]_o$-induced inhibition of the L-type Ca^{2+} channel current (Figure 3b) ($n=5-6$ for each concentration) but $[ATP]_o$ still caused a further inhibition of Ca^{2+} channel current which was not significantly different from control conditions (33.8±3.9% vs. 32.4±4.1%) (Figure 3b).

Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, 50 and 100 μM), a P_{2X} receptor antagonist, was also used. It inhibited the L-type Ca^{2+} channel current (50 μM, 5.7±1.6%; 100 μM, 12.4±2.8%) ($n=6$ for each concentration), and the presence of PPADS (50 and 100 μM) also failed to affect the $[ATP]_o$-induced inhibition of the L-type Ca^{2+} channel currents ($n=5-7$ for each concentration) (Figure 3c) and a further inhibition of the Ca^{2+} channel current by $[ATP]_o$ was observed (34.3±4.3 vs 32.8±4.0%) (Figure 3c).

In view of this unsuccessful attempt to characterize the purinoceptors involved, the possible involvement of adenosine which can be formed from the enzymatic breakdown of ATP was also considered. Therefore, two adenosine receptor antagonists, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-phenyltheophylline (8-PT) were examined. DPCPX is an adenosine A_1 receptor antagonist. The presence of 0.1 μM DPCPX had no apparent effect on the $[ATP]_o$-mediated inhibition of the L-type Ca^{2+} channel current (34.1±4.1% vs 32.0±5.2%) (Figure 4a) ($n=8$); however it caused 22.3±4.2% inhibition of the L-type Ca^{2+} channel current on its own. On the other hand, adenosine (500 μM) caused 23.1±3.6% inhibition of the L-type Ca^{2+} channel currents which was abolished by 0.1 μM DPCPX ($n=8$) (data not shown).

Like all purinoceptor antagonists tested so far, the presence of 8-PT (10 μM) (A_1 and A_2 receptor antagonist) did not have any significant effect on the $[ATP]_o$ mediated inhibition of the L-type Ca^{2+} channel currents (Figure 4b) ($n=7$). $[ATP]_o$ still caused a further inhibition of the Ca^{2+} channel current which was not significantly different from control conditions (34.1±4.1 vs 32.0±5.2%) (Figure 4b).

Comparison of relative inhibitory potency of different nucleotides and nucleosides on L-type Ca^{2+} channel currents

Due to the lack of effects of all the known purinoceptor and adenosine receptor antagonists tested on the $[ATP]_o$-induced inhibition of the L-type Ca^{2+} channel currents in our study, we used another approach to determine the type of purinoceptor involved. With the same concentration of agonist (100 μM), the effect of different nucleotides and nucleosides was investigated by comparing the % inhibition of the Ca^{2+} channel current. Among all the compounds tested, adenosine 5'-triphosphate (ATP) and α,β-methylene-ATP (α,βMeATP) were found to be the most potent, whereas adenosine 5'-monophosphate (AMP) and adenosine were the least potent inhibitors of the L-type Ca^{2+} channel current in our study (Figure 5) ($n=5-7$ for each compound tested). The relative rank order of potency in inhibiting L-type Ca^{2+} channel current is: ATP = α,βMeATP >>2-MeSATP ≥ ATPγS >> UTP = ADP > AMP ≥ adenosine.

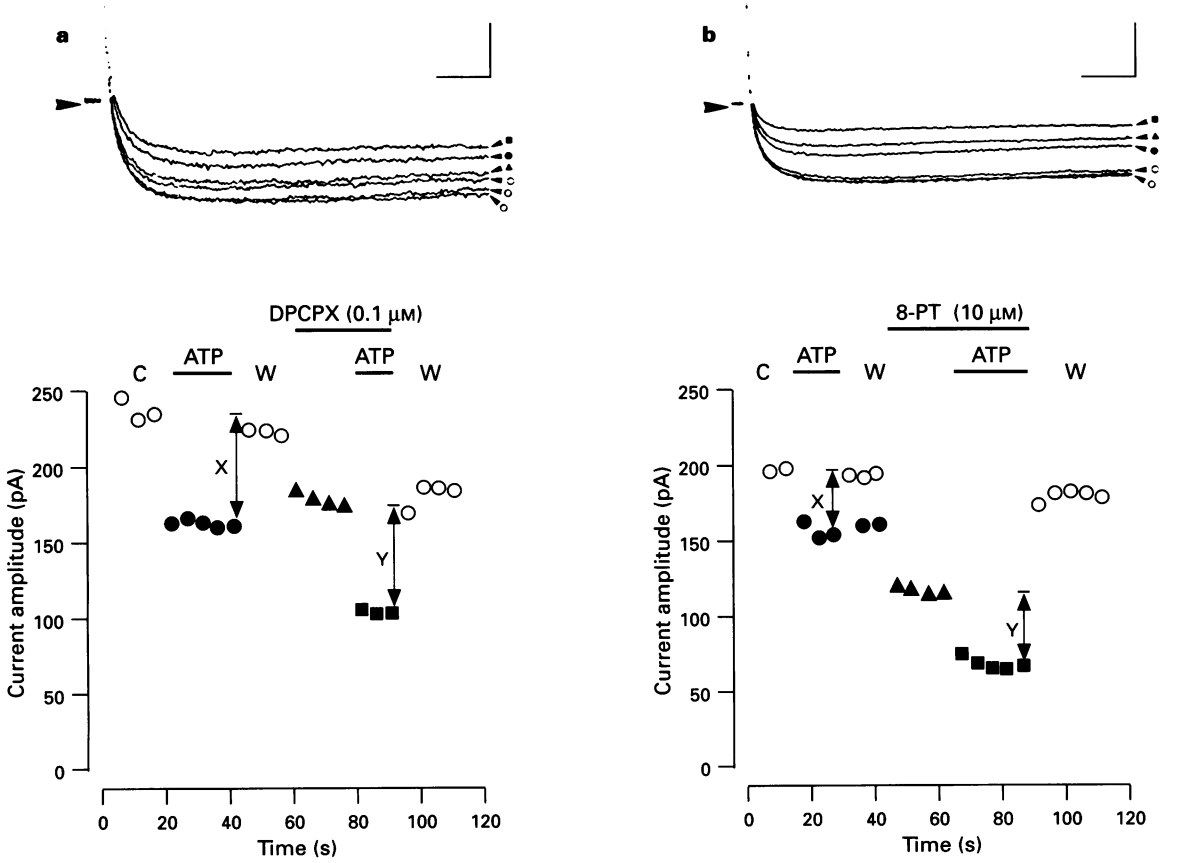

Figure 4 Inhibition of Ca^{2+} channel current by 100 μM $[ATP]_o$ in the presence of (a) DPCPX (0.1 μM) and (b) 8-PT (10 μM) in guinea-pig single sinoatrial nodal cells. The insets show the representative whole-cell current traces of Ca^{2+} channel current in control and washes (○), in the presence of $[ATP]_o$ (●), effect of an individual antagonist (▲) and the effect of $[ATP]_o$ in the continuous presence of a particular antagonist (■). The cell was stimulated to +20 mV for 100 ms from a holding potential of −40 mV, once every 5 s. Effect of individual antagonist on $[ATP]_o$-induced inhibition of Ca^{2+} channel current was examined by comparing the effect of $[ATP]_o$ on its own (X) and in the presence of particular antagonist (Y). C and W indicate control and washes, respectively. Calibration bars: (a) 125 pA and 10 ms.

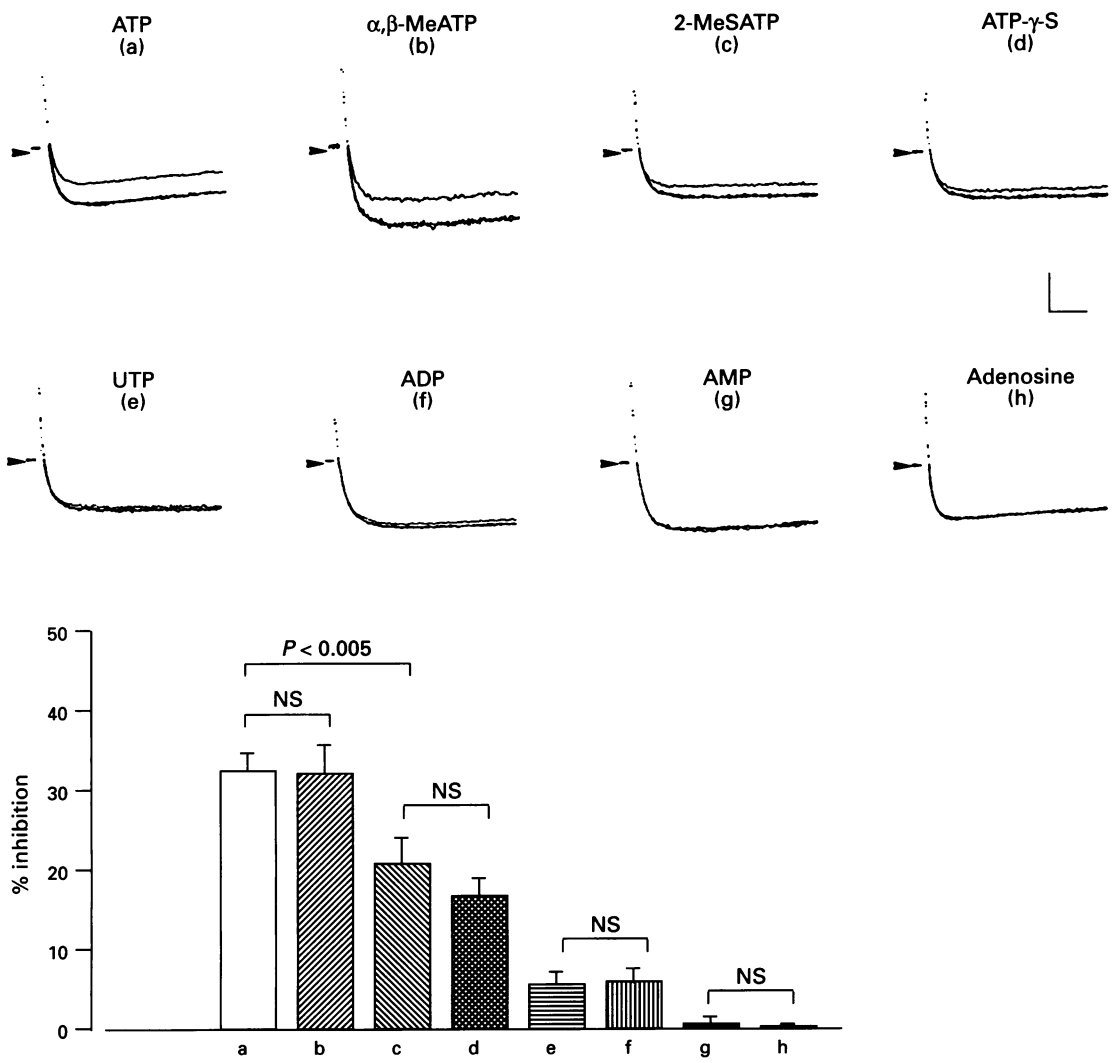

Figure 5 Effect of extracellular nucleotides and nucleosides (100 μM) on Ca^{2+} channel current of guinea-pig single sinoatrial nodal cells. The insets show representative whole-cell current traces in control, in the presence of individual nucleotide and nucleoside and washout. Effect of all nucleotides and nucleosides can be reversed by washing. (a): Adenosine 5'-triphosphate (ATP); (b): α,β-methylene-adenosine 5'-triphosphate (α,βMeATP); (c) 2-methylthio-adenosine 5'-triphosphate (2-MeSATP); (d) adenosine 5'-0-(3-thiotriphosphate) (ATPγS); (e) uridine 5'-triphosphate (UTP); (f): adenosine 5'-diphosphate (ADP); (g): adenosine 5'-monophosphate (AMP); (h): adenosine. Results are expressed as % inhibition of Ca^{2+} channel current, compared to drug-free control conditions. Values are mean ± s.e.mean ($n = 5-7$). NS: no significant difference. Calibration bars: 120 pA and 10 ms.

Discussion

It has been suggested that the L-type Ca^{2+} channel current is involved in sinoatrial pacemaking activity (Hagiwara *et al.*, 1988 and reviewed by Irisawa *et al.*, 1993). ATP is released into the extracellular space from the sympathetic nerve terminals innervating the sinoatrial nodal region of the heart in normal physiological conditions, as well as under pathophysiological conditions e.g. platelet aggregation and cardiac ischaemia (Clemens & Forrester, 1980; Gordon, 1986). Unlike atrial and ventricular myocytes, L-type Ca^{2+} channels have been suggested to be responsible for the phase 0 of the action potential of the sinoatrial node (Irisawa & Noma, 1984; Doerr *et al.*, 1989; Irisawa *et al.*, 1993) and therefore are important in regulating the heart rate. The present study provides evidence, for the first time, that extracellular adenosine 5'-triphosphate ($[ATP]_o$) may modulate the mammalian heart beat by inhibiting the L-type Ca^{2+} channel current. Inhibition of the Ca^{2+} channel current by $[ATP]_o$ observed in our study was not due to the 'speeding up' of the run-down of the Ca^{2+} channel current or the $[ATP]_o$-induced desensitization process, as the effect of $[ATP]_o$ could be removed following washout, and a second challenge with $[ATP]_o$ caused a similar % inhibition of the Ca^{2+} channel current.

In contrast to many other studies (Alvarez *et al.*, 1990; Scamps *et al.*, 1992; 1993; Scamps & Vassort, 1994) on atrial and ventricular preparations, $[ATP]_o$ consistently caused an inhibition of the L-type Ca^{2+} channel current in a concentration-dependent manner with an IC_{50} of 100 μM and a Hill coefficient of 1.83. These results suggested that 2 molecules of $[ATP]_o$ are required to bind to its receptor in order to produce its effect on the single sinoatrial nodal cells. The IC_{50} value obtained in our study is 100 times higher than in ferret isolated ventricular myocytes ($IC_{50} = 1$ μM) (Qu *et al.*, 1993) but these results may represent a genuine species difference. Complete inhibition of the L-type Ca^{2+} channel current could be achieved with high concentrations of $[ATP]_o$ (1 mM), whereas in ferret ventricular myocytes (Qu *et al.*, 1993), the maximum inhibition caused by 1 mM $[ATP]_o$ was only ~50%. On the other hand, there was a 6–9 mV shift in the reversal potential of the current-voltage relationship of Ca^{2+} channel currents, caused by 100 μM $[ATP]_o$. This represents a change in the gatings of the Ca^{2+} channels (unpublished observations).

Using a train protocol to measure the rate of block of the L-type Ca^{2+} channel current (Kass & Arena, 1989) by $[ATP]_o$, over 90% of the maximum steady-state inhibition was achieved within 5 s following exposure to $[ATP]_o$. The time required to reach steady-state inhibition was much less than any study

previously reported on atrial and ventricular preparations in the literature (which occurred within several minutes instead of seconds) (Alvarez *et al.*, 1990; Qu *et al.*, 1993). The $[ATP]_o$-induced inhibition of the Ca^{2+} channel currents in sinoatrial nodal cells could be completely reversed in $[ATP]_o$-free solution within 5–10 s. Such fast inhibitory effects of $[ATP]_o$ and rapid washout on the sinus node activity has been reported in only one study on the isolated, spontaneously beating guinea-pig heart preparation (Stark *et al.*, 1993), whereas in other reported studies the $[ATP]_o$-induced effect disappeared gradually (3–5 min) following washout (Alvarez *et al.*, 1990; Scamps *et al.*, 1992; 1993; Qu *et al.*, 1993; Scamps & Vassort, 1994). Our results suggest that in sinoatrial nodal cells, binding of $[ATP]_o$ to its receptors is not very tight and could easily be removed following washout. Taken together, our results suggest that the purinoceptors studied are located at the extracellular surface of the sinoatrial nodal cells, and that these receptors have a low affinity for $[ATP]_o$. On the other hand, the exact location of the purinoceptors was not determined as the ionized $[ATP]_o$ and/or $[Ba^{2+}]_o$ molecules can enter the cell through the channel pore and act intracellularly. Furthermore, the possibility of $[ATP]_o$ acting directly on the Ca^{2+} channels could not be ruled out. Nevertheless, our results provide evidence to suggest that $[ATP]_o$ has a physiological role in heart beat regulation, as the observed effect of $[ATP]_o$ on the Ca^{2+} channel currents occurred in the range of seconds instead of 3–5 min, as reported in other cardiac muscle preparations (Scamps *et al.*, 1992; 1993; Qu *et al.*, 1993).

In order to investigate the type(s) of purinoceptor involved in our present study, different commercially available purinoceptor antagonists were examined. Suramin, a nonselective P_2-purinoceptor antagonist (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990); (1, 10 and 100 μM) failed to affect the inhibitory activity of $[ATP]_o$ on the Ca^{2+} channel current and the inability of suramin to affect the $[ATP]_o$ response has been observed in other preparations (Matsuoka *et al.*, 1995; Knight & Burnstock, 1996). In addition to suramin, the effects of other purinoceptor antagonists, reactive blue 2 and PPADS were also investigated. Neither reactive blue 2 (1 and 10 μM), a P_{2Y} purinoceptor antagonist, nor PPADS (50 and 100 μM), a selective P_{2X}-purinoceptor antagonist (Ziganshin *et al.*, 1993; Westfall *et al.*, 1996; Knight & Burnstock, 1996), had any effect on the $[ATP]_o$-induced inhibition of sinoatrial nodal cell Ca^{2+} channel currents. Our results are therefore different from the previous reports on the effects of $[ATP]_o$ on the Ca^{2+} channel current in rat (Scamps & Vassort, 1994) and ferret (Qu *et al.*, 1993) ventricular myocytes. These authors reported that P_{2Y}-purinoceptors are involved as the ATP effects could be abolished by suramin (10 μM) and reactive blue 2 (1 μM). These observations may suggest that the typical P_2-purinoceptors (P_{2X} and P_{2Y}) are not involved in myocytes obtained from guinea-pig sinoatrial node.

It has been reported that ectoenzymes are located on the cell membrane which can efficiently breakdown the ATP to adenosine (Welford *et al.*, 1986; 1987). Some reported effects of $[ATP]_o$ have been attributed to the action of these ectoenzymes (Mantelli *et al.*, 1993) which result in the activation of the adenosine receptor (Olsson & Pearson, 1990; Ragazzi *et al.*, 1991). Activation of the adenosine receptor has been reported to cause an inhibition of the Ca^{2+} channel current (Belardinelli *et al.*, 1988; Cerbai *et al.*, 1988; Kato *et al.*, 1990; Alvarez *et al.*, 1990). In principle, therefore, the inhibitory effects of $[ATP]_o$ observed in the present study could be due to an indirect effect of $[ATP]_o$. Adenosine can cause an inhibition of the L-type Ca^{2+} channel current which then contributes to the negative chronotropism caused by $[ATP]_o$ (Pelleg *et al.*, 1986; Stark *et al.*, 1994). To explore this possibility, two adenosine receptor antagonists DPCPX (0.1 μM) and 8-phenyltheophylline (8-PT, 10 μM) were also examined. Similar to the other P_2-purinoceptor antagonists tested in our study, pre-incubation of either antagonist had no apparent effect on the $[ATP]_o$-induced inhibition of the sinoatrial nodal cell Ca^{2+} channel current. Both DPCPX and 8-PT, at the concentrations tested in the present study, have been shown to abolish the adenosine-mediated effects in various preparations (Mantelli *et al.*, 1993; Goncalves & Queiroz, 1996). These results suggested that adenosine receptors are not involved in the present study.

It is important to note that the reported adenosine-mediated inhibition of the Ca^{2+} channel currents required a prestimulation of Ca^{2+} channels (e.g. β-adrenoceptor activation of Ca^{2+} current amplitude) with the basal or unstimulated Ca^{2+} channel current unaffected by adenosine (Belardinelli *et al.*, 1983; 1988; Kato *et al.*, 1990). However in our study, application of $[ATP]_o$ caused an inhibition of the basal Ca^{2+} channel current. In contrast to $[ATP]_o$, equimolar concentrations (100 μM) of adenosine had only a minimal effect on the Ca^{2+} channel current (Figure 5). Although 0.1 μM DPCPX failed to affect the $[ATP]_o$-mediated response in our study, it abolished the 500 μM adenosine-mediated inhibition of the basal Ca^{2+} channel current. Moreover, application of stable analogues (ectoATPase-resistant) of ATP e.g. α,β-MeATP and ATPγS also caused a significant inhibition of the sinoatrial nodal cell Ca^{2+} channel current (Figure 2). All these results strongly suggest that the $[ATP]_o$-mediated inhibition of the Ca^{2+} channel current was not mediated through the formation of adenosine.

The lack of a selective antagonist, which has a minimal effect of its own on the Ca^{2+} channel current, makes it impossible to identify the purinoceptor that is involved. In view of the unsuccessful attempt to characterize the type of purinoceptors involved, we decided to used another approach by comparing the relative rank order of inhibitory potency of different ATP nucleotides and nucleosides on the L-type Ca^{2+} channel current. Using single, fixed concentrations of nucleotides and nucleosides of $[ATP]_o$ (100 μM), ATP and α,βMeATP were found to be the most potent compounds tested, causing $32.6 \pm 2.22\%$ and $32.2 \pm 3.8\%$ inhibition of the sinoatrial nodal cell Ca^{2+} channel current, respectively, whereas AMP and adenosine had a minimal effect ($0.8 \pm 0.9\%$ and $0.3 \pm 0.6\%$ inhibition, respectively). The relative rank order of potency of the different nucleotides and nucleosides on inhibition of the Ca^{2+} channel current was as follows: ATP = α,βMeATP > > 2-MeSATP $\geqslant$ ATPγS > > UTP = ADP > AMP $\geqslant$ adenosine. This potency order has not been reported in any other preparations in which the subclassification of P_2-purinoceptor receptors has been studied (for review see Harden *et al.*, 1995). Based on the results obtained from molecular biology experiments, there is a wealth of information on P_2 families of purinoceptors. In our study, the ATP analogues tested exhibited the relative potency order of ATP > ADP > adenosine, which is the same as the potency order used for the involvement of P_2 purinoceptors (Londos *et al.*, 1980; Burnstock & Kennedy, 1985). We therefore propose that P_2-purinoceptors are involved in these $[ATP]_o$ effects in SAN. This type of P_2-purinoceptor may be novel: the P_{2P}-purinoceptors (the second letter P stands for pacemaker cells) (based on the results of the antagonist experiments as well as the comparison of the relative order of potency) may be found in cardiac pacemaker cells. There are no data in the literature about the type(s) of purinoceptors in the pacemaker region of the heart except that P_{2Y} purinoceptors have been found in guinea-pig left atrium (Mantelli *et al.*, 1993). Our data do not, of course, eliminate the possibility of the involvement of P_2 subclasses. The final confirmation of the identity of the purinoceptors found in guinea-pig sinoatrial nodal cells will rely on future molecular biological experiments for working out the amino acid sequences of the purinoceptors involved.

In conclusion, application of $[ATP]_o$ caused an inhibition of basal Ca^{2+} channel currents in guinea-pig single sinoatrial nodal cells. Based on the inability of various purinoceptor antagonists tested to affect the $[ATP]_o$-induced effects and the relative rank order of different ATP analogues observed, a novel type of purinoceptor may be involved.

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