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Mechanism of muscarinic control of the high-threshold calcium current in rabbit sino-atrial node myocytes

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Abstract. The mechanism of the action of acetylcholine (ACh) on the L-type calcium current ($I_{Ca,L}$) was examined using a whole-cell voltage-clamp technique in single sino-atrial myocytes from the rabbit heart. ACh depressed basal $I_{Ca,L}$ at concentrations in the range 0.05–10 μM, without previous β-adrenergic stimulation. The ACh-induced reduction of $I_{Ca,L}$ was reversed by addition of atropine, indicating that muscarinic receptors mediate it. Incubation of cells with a solution containing pertussis toxin led to abolition of the ACh effect, suggesting that this effect is mediated by G proteins activated by muscarinic receptors. Dialysis of cells with protein kinase inhibitor or 5′-adenylyl imidodiphosphate, inhibitors of the cAMP-dependent protein kinase, decreased basal $I_{Ca,L}$ by about 85% and suppressed the effect of ACh. The ACh effect was also absent in cells dialysed with a non-hydrolysable analogue of cAMP, 8-bromo-cAMP. The results suggest that, in basal conditions, a large part of the L-type calcium channels should be phosphorylated by protein kinase A stimulated by a high cAMP level correlated with a high adenylate cyclase activity. The depressing effect of ACh on $I_{Ca,L}$ may occur via inhibition of the high basal adenylate cyclase activity leading to a decrease of cAMP-dependent protein kinase stimulation and thus to a dephosphorylation of calcium channels.

Key words: Sino-atrial node – Ca^{2+} current – Acetylcholine – cAMP-dependent phosphorylation

Introduction

In the heart the calcium current (I_{Ca}), which plays an essential role in generating the action potential and controlling contractility, is regulated by autonomic transmitters. β-Adrenergic agonists increase I_{Ca} in both amphibian and mammalian myocardium. This effect is mediated by stimulation of adenylate cyclase [12, 24, 25]. Inhibition of I_{Ca} by acetylcholine takes place through an opposite mechanism of β-adrenergic stimulation. However, in ventricular cells of the heart, acetylcholine by itself lacks an inhibitory effect on I_{Ca}; significant action of the drug occurs only in the presence of β-adrenergic stimulation [12, 21]. Such a result has been also reported in bullfrog atrial cells [31]. A plausible mechanism for the muscarinic inhibition of I_{Ca} is that acetylcholine can inhibit adenylate cyclase activity via stimulation of inhibitory GTP-binding proteins [11, 20, 21]. An alternative mechanism by which acetylcholine can lower the intracellular cyclic AMP concentration involves a cyclic-GMP-stimulated cyclic AMP phosphodiesterase, which acts by increasing cAMP hydrolysis [18]. Muscarinic stimulation also increases phosphoinositide turnover with a process involving inositol trisphosphate and diacylglycerol as second messengers [4, 30].

In chick ventricle, frog myocardium and some mammalian atrial cells, it has been reported that acetylcholine can inhibit calcium-dependent action potentials or I_{Ca} directly, even in the absence of a β-adrenergic agonist [1, 14, 23, 35]. Brown and Denyer [5] and Di Francesco and Tromba [9] have observed a similar effect in the sino-atrial node cells of the mammalian heart. Moreover, Di Francesco and Tromba [9], analysing the inhibitory action of acetylcholine on the hyperpolarization-activated current (I_f), have concluded that the effect of drug also occurs in the absence of any previous β-adrenergic stimulation via inhibition of a high basal adenylate cyclase activity.

The aim of the present study was to clarify the mechanism by which acetylcholine inhibits the high-threshold calcium current ($I_{Ca,L}$) in rabbit sino-atrial node myocytes. Given that the inhibitory action of acetylcholine on $I_{Ca,L}$ in these cells seems to occur in the absence of any previous β-adrenergic stimulation, it was of interest to verify whether the action of the drug can be mediated by a cyclic-AMP-down-regulating process.

Materials and methods

Cell isolation procedure. Sino-atrial node cells were isolated according to the methods of Di Francesco et al. [10] and Denyer and

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Table 1. Solutions used for cell isolation, cell external perfusion and pipette filling

Constituent[a]	Concentration (mM) in solution						
	1 Tyrode	2 Ca-free EGTA	3 Ca-free	4 Enzyme	5 KB	6 External	7 Pipette
NaCl	140	140	140	140		125	
KCl	5.4	5.4	5.4	5.4	70		
$MgCl_2$	1					1	1
$MgSO_4$		3.5	3.5	3.5	5		
$CaCl_2$	1.8	0.02	0.02	0.08	0.08	1.8	
TEACl						20	
CsCl							30
HEPES/NaOH	5	5	5	5		5	
HEPES/KOH					10		
HEPES/CsOH							10
CsAsp							90
KGlu					5		
K_2ATP					5		
KH_2PO_4					20		
MgATP							3
Na_2GTP							0.3
EGTA		0.05			0.5		5
Glucose		10	10	10	10		
Creatine					5		
Taurine		50	50	50	20		
Collagenase (U/ml)				190			
Elastase (U/ml)				24			
pH	7.4	7.4	7.4	7.4	6.9	7.4	7.2

[a] TEACl, tetraethylammonium chloride; CsAsp, caesium aspartate; KGlu, potassium glutamate

Brown [7]. In brief, albino rabbits weighing 500−1200 g were killed by a blow on the neck. The heart was quickly removed and put in Tyrode solution pre-warmed at 37° C and pre-equilibrated with O_2. The sino-atrial node region was dissected out from the other parts of the cardiac tissues. The sino-atrial node was cut in strips perpendicularly to the crista terminalis. After a recovery period in Tyrode bubbled with O_2 at 37° C, strips were put in solution 2 (see Table 1) for 5 min and rinsed with solution 3. They were then transferred to the enzyme solution (solution 4). After 7−10 min digestion the strips were rinsed once in solution 3, twice in KB medium (solution 5) and then transferred to a KB solution containing 50 g/l polyvinylpyrrolidone (PVP 40). After a resting period at 4° C (2 h) in KB/PVP, cells were mechanically dispersed in KB medium using a glass pipette with a small tip diameter. Aliquots of filtered cell suspension were put in petri dishes to allow cells to settle down (20 min). Tyrode solution was then reintroduced. After equilibration, cells were rinsed with Tyrode before experimental use. Atrial cells were prepared by the same procedure.

Solutions and drugs. The solutions used for cell isolation, cell external perfusion and pipette filling are described in Table 1. When the calcium currents were being recorded, all K^+ currents were blocked with intracellular Cs^+ and extracellular tetraethylammonium ions. Na^+ current was blocked with 10 μM tetrodotoxin. External solutions were delivered to the external membrane of the cell with a specific perfusion device placed less than 100 μm from the cell and allowing fast solution exchange. Collagenase (CLS 2) was purchased from Worthington, N. J., USA; Elastase was from Serva, FRG. All other chemicals were purchased from Sigma. Isoprenaline was prepared as a 1 mM stock solution with 1% ascorbic acid in distilled water and kept at 4° C. Acetylcholine (ACh) was prepared as 10 mM and 0.1 mM stock solutions in distilled water and kept at 4° C. 5′-Adenylyl imidodiphosphate (p[NH]ppA, lithium salt) was dissolved in standard internal solution to make a final 5 mM concentrated solution. Protein kinase inhibitor (PKI, rabbit sequence) was dissolved in standard internal solution to make a final 5 μM concentrated solution. 8-bromo cyclic AMP (8-Br-cAMP) was dissolved in standard internal solution to make a 200 μM concentrated solution. Pertussis toxin (PTX) was dissolved in Tyrode solution to make a 0.5 μg/ml final solution in which cells were incubated at room temperature for 5−7 h before recording was commenced.

Voltage-clamp procedures and data analysis. Whole-cell currents were recorded under conditions similar to those described by Hamill et al. [16]. Patch electrodes (2−5 MΩ when filled with standard internal solution) were prepared from glass microtubes (Assistent, Bardram, Denmark) on a two-stage pipette puller (Narishige PP-83, Tokyo, Japan). Experiments were performed using a patch-clamp amplifier (RK 300, Biologic, Grenoble, France) driven by an IBM-PC-AT-compatible microcomputer (IPC, Essex Electric PTE Ltd., Singapore) equipped with an A/D, D/A conversion board (Labmaster TM 40, Scientific Solutions, Solon, USA). Experiments were programmed and analysed with specific software (pClamp V5-5, Axon Instruments, Foster City, USA).

Cells were previously superfused with Tyrode solution to obtain the whole-cell configuration. In Tyrode solution, a large quantity of isolated cells were rod-shaped and had a central bulge where a single nucleus was located [7]. They showed spontaneous contractions and were approximately 7−10 μm in width and 80−100 μm in length. Cells were then stimulated with hyperpolarizing voltage pulses (HP = −40 mV) between −50 mV and −110 mV during 2.4 s in order to verify the presence of hyperpolarization-activated current I_f. Only cells exhibiting I_f current and lacking inwardly rectifying background current, I_{K1}, were considered as sino-atrial node cells and were kept for experiments on $I_{Ca,L}$ current.

Cells were stimulated with a double voltage pulse: they were depolarized every 5 s from −70 mV to −40 mV during 300 ms and subsequently to 0 mV during 280 ms. This procedure was carried out to avoid voltage-dependent run-down of $I_{Ca,L}$ current. Calcium currents were sampled at 2.85 kHz and filtered at 1 kHz. Peak I_{Ca} current was measured as the difference from the zero

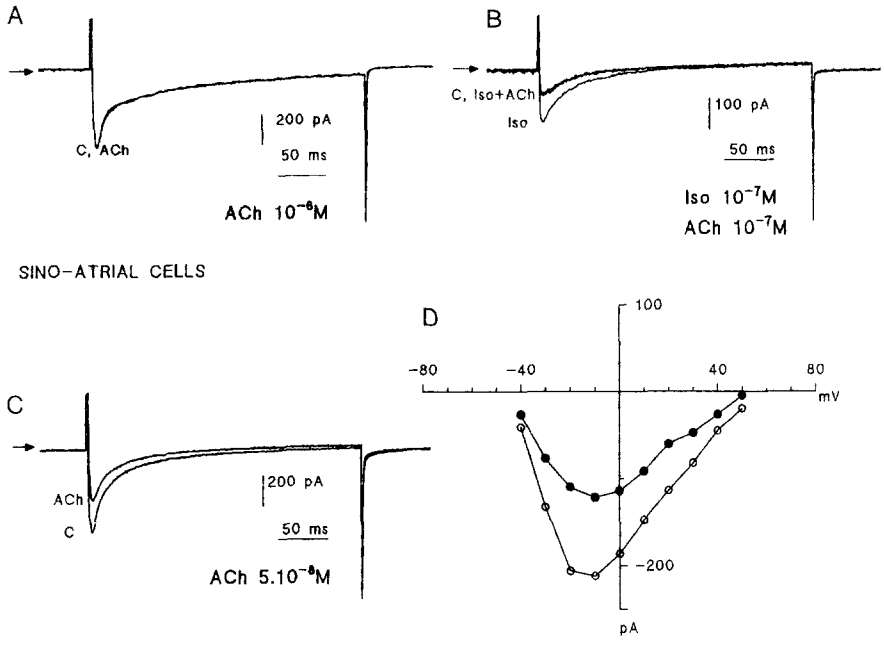

Fig. 1 A–D. Effect of acetylcholine (*ACh*) on L-type calcium current ($I_{Ca,L}$) in atrial and sino-atrial cells of the rabbit heart. $I_{Ca,L}$ was elicited by a voltage step from −40 mV to 0 mV. **A** ACh (1 μM) had no effect on the basal $I_{Ca,L}$ in an atrial cell. **B** In another atrial cell, ACh (0.1 μM) suppressed the increase of calcium current induced by 0.1 μM isoprenaline. **C** ACh at low concentration (50 nM) reduced basal $I_{Ca,L}$ in a sino-atrial cell. **D** The current/voltage relationships were plotted for the L-type calcium current of a sino-atrial cell in control conditions (○) and in the presence of 50 nM ACh (●). Note the reduction of the current values at any voltage. The holding potential was −50 mV

current level. In some experiments, membrane capacitance was recorded to allow measurements of total calcium current density (sampling: 41.6 kHz; filtering: 3 kHz). The average membrane capacitance was 27 ± 2 pF (n = 14). The series resistance was 9 ± 0.54 MΩ (n = 8) and it was not compensated. Statistical data were expressed as the means ± SEM. All experiments were done at room temperature (20–23° C).

Results

Effect of ACh on $I_{Ca,L}$ in atrial and sino-atrial cells

The L-type calcium current was elicited by a clamp pulse from −40 mV to 0 mV in atrial (Fig. 1 A, B) and in sino-atrial cells (Fig. 1 C). In atrial cells, ACh at concentrations as high as 1 μM had no significant effect on the basal $I_{Ca,L}$ (Fig. 1 A). However, ACh had potent effects on $I_{Ca,L}$ stimulated by isoprenaline. $I_{Ca,L}$ was reduced to control levels by the addition of 0.1 μM ACh to the solution containing 0.1 μM isoprenaline (Fig. 1 B). Such a result is similar to those obtained in frog ventricular and atrial cells [12, 19] and in guinea-pig ventricular cells [21].

In sino-atrial cells, ACh at low concentration (50 nM) had a marked inhibitory effect on the amplitude of basal $I_{Ca,L}$ without causing a change in the time to peak (Fig. 1 C). The effect of ACh was fully reversible on washout. Figure 1 D shows the current/voltage relationships for $I_{Ca,L}$ in a sino-atrial cell.

The L-type calcium current was elicited by various depolarizations from −50 mV and the amplitude of the peak current was plotted as a function of the voltage. The *I/V* relation shows a threshold potential at about −45 mV, a maximum at −10 mV and an apparent reversal potential at about + 60 mV. Application of 50 nM ACh depressed $I_{Ca,L}$ at every potential. The shape of the *I/V* relation was unaffected by ACh. This result is in accordance with that reported by Brown and Denyer [5] in the same preparation.

Effect of various doses of ACh on $I_{Ca,L}$ in sino-atrial cells

$I_{Ca,L}$ was activated by impulses from a holding potential of −40 mV to 0 mV. Figure 2 A shows the percentage of $I_{Ca,L}$ reduction as a function of cumulative doses of acetylcholine. The depression of $I_{Ca,L}$ was 18.4 ± 3.3% (mean ± SEM, n = 8) by 50 nM ACh, 30.7 ± 3% (n = 8) by 0.1 μM ACh, 44.3 ± 6.1% (n = 6) by 1 μM ACh and 55.5 ± 6.5% (n = 4) by 10 μM ACh. The maximal effect of ACh was obtained at 10 μM.

Sino-atrial node cells show a relative variability in the $I_{Ca,L}$ amplitude and thus the reducing effect of ACh could be correlated with the current amplitude. Figure 2 B illustrates the effect of 0.1 μM ACh in eight cells where different values of control $I_{Ca,L}$ density were calculated with an impulse induced from −40 mV to 0 mV. The same percentage of $I_{Ca,L}$ reduction was obtained in nearly every cell, indicating that it was not correlated with the control current density. A similar result was found on application of other doses of ACh.

Muscarinic action of ACh on $I_{Ca,L}$ in sino-atrial myocytes

The L-type calcium current was elicited by clamp pulses from −40 mV to 0 mV. Figure 3 B shows that when the cell was superfused with 0.1 μM ACh, a significant re-

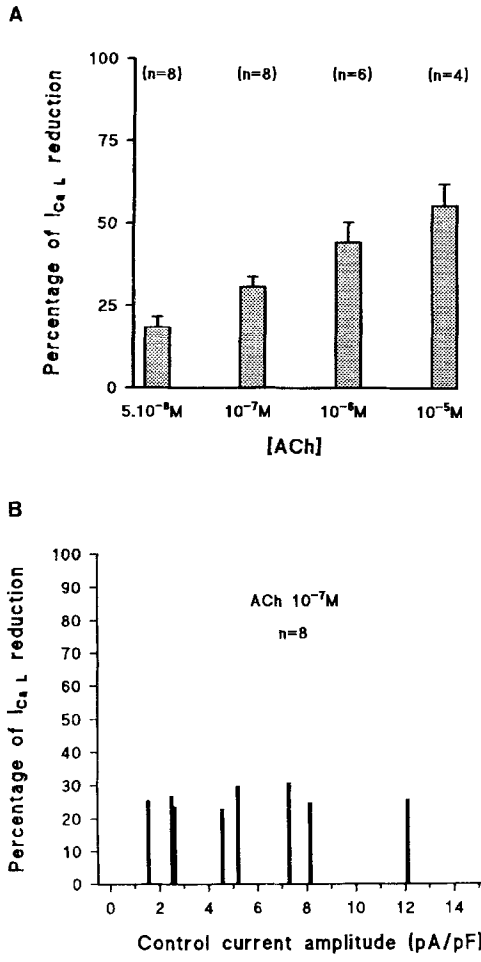

Fig. 2 A, B. Effect of various doses of ACh on $I_{Ca,L}$ in sino-atrial cells. The L-type calcium current was activated by a depolarizing pulse from −40 mV to 0 mV. **A** *Columns* represent the average calcium current reduction on application of cumulative doses of ACh. The percentage of current depression was calculated as the ratio of $I_{Ca,L}$ amplitudes with and without ACh. *Vertical bars* are means ± SEM. The number of cells (*n*) is indicated above the columns. The mean value of $I_{Ca,L}$ amplitude was 200 ± 37 pA (n = 8) in control solution. **B** *Columns* represent the percentage of $I_{Ca,L}$ reduction on application of 0.1 μM ACh as a function of control current density in eight cells. The mean value of $I_{Ca,L}$ depression was 26.3 ± 0.9% (mean ± SEM). Note the absence of correlation between $I_{Ca,L}$ reduction by ACh and the control current density

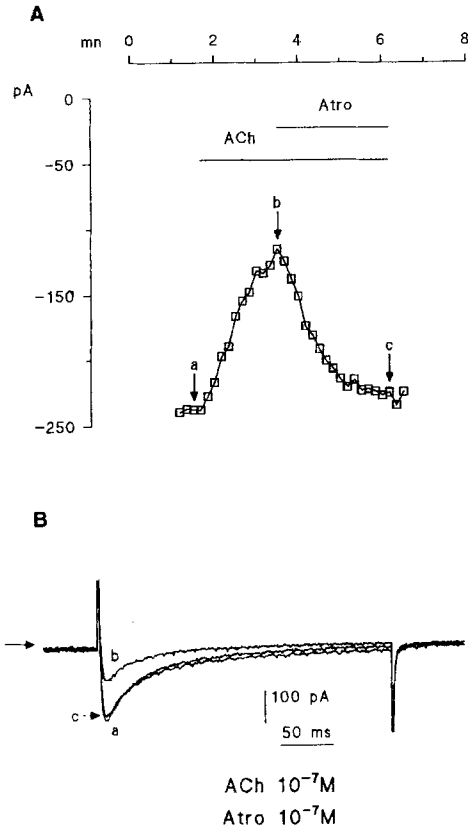

Fig. 3 A, B. Removal of ACh action on $I_{Ca,L}$ by atropine in sino-atrial cell. **A** Time course of the calcium current elicited by a voltage step from −40 mV to 0 mV, during perfusion with 0.1 μM ACh and with ACh and 0.1 μM atropine (*bars*). *Symbols* mark the times where the current traces were recorded. **B** Current traces recorded in the control solution (*a*), during perfusion with ACh (*b*) and during perfusion with ACh and atropine (*Atro*; *c*)

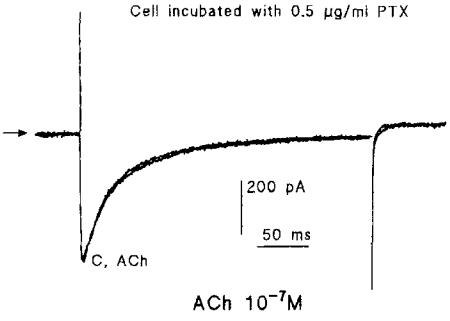

Fig. 4. Effect of treatment with pertussis toxin (*PTX*) on the acetylcholine action on $I_{Ca,L}$ in a sino-atrial cell. The current traces were recorded at a voltage pulse induced from −40 mV to 0 mV, in a cell incubated with 0.5 μg/ml PTX before (*C*) and after 4 min of superfusion with 0.1 μM ACh. Note the lack of acetylcholine action in the presence of PTX

duction of $I_{Ca,L}$ was obtained in about 3 min. Addition of 0.1 μM atropine, a muscarinic antagonist, fully abolished the ACh effect, as shown by the time course of $I_{Ca,L}$ amplitude during drug perfusion (Fig. 3 A). Similar results were obtained in two further experiments.

Involvement of GTP-binding proteins in ACh effects

Muscarinic agonists are known to decrease cyclic AMP levels by inhibition of adenylate cyclase activity [28, 33, 38]. Moreover, the involvement of GTP-binding proteins in the muscarinic-agonist-mediated action of ACh on calcium currents has been demonstrated in atrial and ventricular cells [3, 21, 31]. It is therefore of interest to see if the $I_{Ca,L}$ reduction by ACh is also mediated by G proteins in sino-atrial cells. Figure 4 shows the effect of treatment with pertussis toxin on the ACh action on $I_{Ca,L}$. PTX is a protein known to prevent G-protein-mediated responses to muscarinic stimulation [22, 26]. Figure 4 illustrates $I_{Ca,L}$ activated by a clamp pulse from −40 mV

to 0 mV in a cell incubated for 5 h with 0.5 μg/ml PTX at room temperature. The addition of 0.1 μM ACh failed to reduce the calcium current ($n = 5$).

Effects of inhibitors of cAMP-dependent protein kinase

The enhancement of I_{Ca} by catecholamines is generally attributed to β-adrenergic-receptor-stimulated cAMP-dependent phosphorylation of calcium channels [17, 37]. The reduction of adenylate cyclase activity by ACh, which depresses cAMP production, can lead to a significant lowering of the cAMP-dependent protein kinase A activity and thus to a decrease of the calcium channel phosphorylation. If this is the case, blocking the phosphorylation by protein kinase A inhibitors should suppress the effect of ACh.

5′-Adenylyl imidodiphosphate (p[NH]ppA) is a non-hydrolysable ATP analogue that binds to the active site of protein kinase A and produces dead-end inhibition by formation of an unproductive enzyme-p[NH]ppA complex [32]. The inhibitor protein of the cAMP-dependent protein kinase (PKI) acts by interaction with the catalytic subunit of the protein kinase [39].

Figure 5 A shows the time course of changes in $I_{Ca,L}$ peak amplitude elicited by clamp pulses from −40 mV to 0 mV and expressed as current density, in a cell dialysed with 5 μM PKI. The current density declined by about 85% in 4 min. There were no significant changes when the cell was superfused with 0.1 μM ACh after 4 min of cell dialysis with PKI. In Fig. 5 B the time course of the mean current density of three cells in control conditions was compared with that of five cells dialysed with 5 mM p[NH]ppA. In control conditions, the run-down was illustrated by a mean current density decline of 37%. In the cells dialysed with the protein kinase inhibitor, the time course of change was faster and stronger. The mean current density was decreased by about 85%.

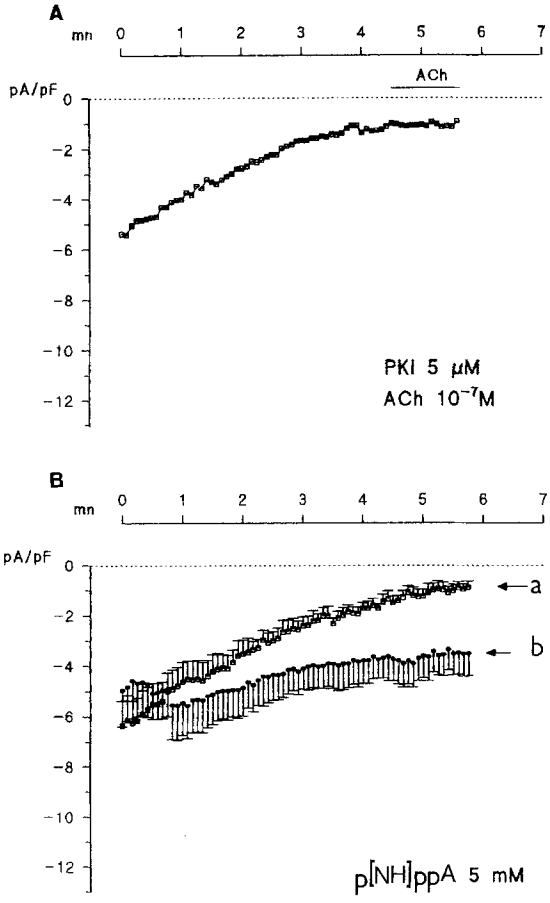

Fig. 5 A, B. Effect of protein kinase A inhibitors PKI and p[NH]ppA on $I_{Ca,L}$. **A** Time course of changes in $I_{Ca,L}$ peak amplitude, elicited by clamp pulses from −40 mV to 0 mV and expressed as current density, in a cell dialysed with 5 μM PKI and after addition of 0.1 μM ACh in the bath solution. Note the lack of ACh action on the remaining calcium current density. **B** Time course of the mean current density illustrating the "run-down" of the calcium current of three cells in control conditions (*b*) and of five cells dialysed with 5 mM p[NH]ppA (*a*). *Vertical bars* are means ± SEM. Note the marked enhancement of run-down slope in cells dialysed with p[NH]ppA

Involvement of cAMP in the ACh effects on $I_{Ca,L}$

In order to study the involvement of cAMP in the ACh reduction of $I_{Ca,L}$, ACh was applied under conditions where the intracellular cAMP concentration was clamped. To do this, cells were loaded with 8-Br-cAMP, a non-hydrolysable analogue of cAMP. Figure 6 shows that the presence of 200 μM 8-Br-cAMP in the pipette solution led to a progressive increase of the $I_{Ca,L}$ amplitude from 10 s (trace 1) to 6 min of whole-cell recording (trace 2) where the steady-state effect of 8-Br-cAMP was achieved. In this case, the superfusion with 0.1 μM ACh for 4 min failed to change the amplitude of $I_{Ca,L}$ (trace 3). Similar results were obtained in four further cells.

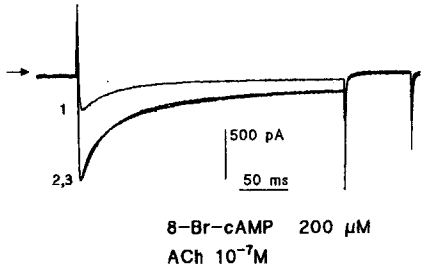

Fig. 6. Lack of ACh action on $I_{Ca,L}$ in cell loaded with 8-Br-cAMP. The whole-cell pipette contained 200 μM 8-Br-cAMP. *Traces 1 and 2:* $I_{Ca,L}$ activated by a depolarizing pulse from −40 mV to 0 mV at 10 s (*1*) and 6 min (*2*) of whole-cell recording in a cell superfused with the standard external solution. *Trace 3:* $I_{Ca,L}$ recorded after 4 min of 0.1 μM ACh application

Discussion

The present study shows that the L-type calcium current is reduced by acetylcholine in sino-atrial cells of the rabbit heart and that this effect is mediated by stimulation of muscarinic receptors. Such a result has been reported by Di Francesco and Tromba [9] and by Brown and Den-

yer [5] in the same preparation. Association of acetylcholine to the muscarinic receptor in the heart activates guanine-nucleotide-binding protein, G_i, which causes the inhibition of adenylate cyclase activity [2, 15, 33], inducing the reduction of the intracellular cyclic AMP level. This effect is blocked by pertussis toxin [11]. The present results with PTX and with cells loaded with 8-Br-cAMP are in accordance with the view that cAMP acts as a second messenger in the ACh-dependent modulation of $I_{Ca,L}$.

It is known that β-adrenergic agonists increase I_{Ca} in both amphibian and mammalian myocardium. The effect is mediated by stimulation of adenylate cyclase. The resulting increase in intracellular cAMP level activates a cAMP-dependent protein kinase, inducing an enhancement of I_{Ca} by phosphorylation [12, 24, 25, 31, 37]. On the other hand, a reduction of cAMP level by acetylcholine [9, 21, 28, 31] should lead to a smaller number of phosphorylated calcium channels and thus to a decrease of the calcium current. In the present experiments, the lack of any effect of ACh on $I_{Ca,L}$ in the presence of protein kinase inhibitors, which block the phosphorylation of calcium channels, is in agreement with such a pathway.

The inhibitory effects of ACh on the calcium current have been hypothesized to be caused by other, different mechanisms: activation of a cyclic-GMP-dependent phosphodiesterase [13], stimulation of a protein phosphatase [38] or activation of protein kinase C [27]. Although a detailed study of these mechanisms has not been performed in the present work, it appears that the activation of a phosphodiesterase or a phosphatase by ACh may lead to a further reduction of I_{Ca} when the PTX-treated cells are superfused with ACh. In fact, Fig. 4 shows that in the presence of PTX the addition of ACh failed to reduce the calcium current. It may be assumed that ACh can activate phosphodiesterase or phosphatase via a PTX-sensitive G protein. However, the lack of an ACh effect on $I_{Ca,L}$ in cells loaded with 8-Br-cAMP rules out the possibility of the stimulation of a protein phosphatase by ACh via this pathway. Activation of protein kinase C via muscarinic-receptor-mediated phosphoinositide hydrolysis has been reported to be insensitive to PTX [29]. Thus, the fact that $I_{Ca,L}$ inhibition by ACh was abolished in PTX-treated cells, rules against phospholipase C being a second messenger in the present case. Such results suggest that the site of ACh action is mainly proximal to cAMP production.

It has been reported that in neuronal cells, ACh inhibits I_{Ca} via a direct coupling of inhibitory G protein with the calcium channels [36]. The same pathway has been proposed in the inhibitory effect of ACh on the I_f current of sino-atrial cells in mammalian heart [40]. The present study seems to rule out such a mechanism since ACh had no further effect when the phosphorylation cycle was blocked by protein kinase inhibitors or when the cells were loaded with 8-Br-cAMP.

In amphibian and mammalian ventricular cells acetylcholine by itself lacks an inhibitory effect on basal I_{Ca}. A significant effect of ACh occurs only in the presence of β-adrenergic stimulation [21, 31]. In the sino-atrial myocytes, ACh reduces $I_{Ca,L}$ without previous β-receptor-mediated I_{Ca} stimulation. The result is similar to those reported by Di Francesco and Tromba [9] and Brown and Denyer [5] in the same preparation. Analysing the effect of ACh on I_f, Di Francesco and Tromba [9] have assumed that, in sino-atrial node cells, basal adenylate cyclase activity is particularly high at rest and implies a high cyclic AMP level. A large basal cAMP level, which has been observed in sino-atrial tissue [34], may lead to a marked increase of the calcium channel phosphorylation. However the preceding authors [34] have measured cAMP levels in a tissue with nerve endings. In these conditions, a part of the cAMP content may be attributable to adrenergic transmitter release. Direct measurements of cAMP concentration are needed in isolated cells of the sino-atrial node to shed more light on the basal involvement of cAMP. Nevertheless, the present results show that, in cells dialysed with protein kinase inhibitors (PKI or p[NH]ppA), basal $I_{Ca,L}$ was reduced by about 85%. In single ventricular myocytes, PKI suppresses the basal $I_{Ca,L}$ only by about 20%, indicating that phosphorylation by cAMP-dependent protein kinase is not a prerequisite for maintaining a large part of the basal $I_{Ca,L}$ in these cells [25].

Finally, as shown previously on I_f [8, 10], the results reported here suggest that in the sino-atrial node cells of the rabbit heart, $I_{Ca,L}$ is controlled by cyclic AMP via adenylate cyclase and support the view that a reduction of cAMP is a common step in the ACh action on basal I_f and basal $I_{Ca,L}$ [9]. Moreover, a large part of the L-type calcium channels seems to be phosphorylated in basal conditions, in contrast with the Purkinje fibres [6], atrial [19, 31] and ventricular [12, 21] cells where ACh is ineffective on basal calcium current.

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