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Modulation of ionic currents in smooth muscle balls of the rabbit intestine by intracellularly perfused ATP and cyclic AMP

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Abstract. The effects of intracellularly perfused ATP and cyclic-AMP (c-AMP) on ionic currents recorded from fragmented smooth muscle cells (smooth muscle ball; SMB) were investigated, using the single electrode whole cell voltage clamp method. The Ca^{2+} current was distinguished from K^+ currents, using pipette solution containing Cs^+, TEA^+ and 4 mM EGTA. ATP enhanced the Ca^{2+} current dose-dependently between 0.3 and 10 mM, and slightly slowed the slow component of the decay of the Ca^{2+} current, while the steady-state inactivation curve remained unaffected. Intracellular application of 5′-adenylyl-imidodiphosphate (AMP-PNP; 1 mM) inhibited the Ca^{2+} current by competition with ATP, but c-AMP (up to 300 μM) had no effect. With a high-K^+ solution containing 0.3 mM EGTA and ATP in the pipette and physiological salt solution in the bath, a net inward current with transient (Ca^{2+} dependent) and delayed (Ca^{2+} independent) K^+ outwart currents were evoked. Increased concentrations of ATP (above 1 mM) but not c-AMP (up to 100 μM) in the pipette enhanced the transient K^+ outward current. Neither agent had any effect on the delayed outward current.When repetitive stimulations of intervals shorter than 5 s were applied, the amplitude of the transient outward current was markedly reduced, and 100 μM c-AMP partially prevented this attenuation. ATP may act on the Ca^{2+} channel either by phosphorylating the channel protein or by other ATP requiring mechanisms, independently from those induced by the action of c-AMP. Thus, the different responses of cardiac and visceral smooth muscles induced by β-adrenoceptor stimulation may be explained in part by the different natures of the Ca^{2+} channel in response to c-AMP.

Key words: Smooth muscle cell – Whole cell voltage clamp – Intracellular perfusion – ATP – c-AMP – Ionic currents – Ca^{2+} channel

Introduction

Cyclic nucleotides and Ca^{2+} are important cellular signal transductors (Rasmussen et al. 1985). In guinea-pig ventricular cells, intracellularly applied ATP increased and stabilized the Ca^{2+} current (Irisawa and Kokubun 1983; Taniguchi et al. 1983). An increase in the Ca^{2+} current was also observed by activation of the cyclic-AMP (c-AMP) dependent phosphorylation of the Ca^{2+} channel (Trautwein et al. 1982; Irisawa and Kokubun 1983; Osterrieder et al. 1982; Kameyama et al. 1985). Thus, the role of β-adrenoceptor stimulants as promoters of positive inotropic action can be explained at least in part. Excitatory actions of ATP and c-AMP (c-AMP-dependent protein kinase) have also been noted in other excitable cells (Fedulova et al. 1981; Byerly and Moody 1984; Doroshenko et al. 1984). However, it is not certain whether ATP and c-AMP act on the same site by c-AMP dependent phosphorylation of the channel.

In visceral smooth muscle tissues, β-adrenoceptor stimulants which increased the cellular content of c-AMP, relaxed the tissues, hyperpolarized the membrane and suppressed the spontaneous Ca^{2+} spike activity (Bülbring and Tomita 1969; Kroeger and Marshall 1973). Such phenomena were also observed in the main pulmonary artery on an application of dibutyryl c-AMP (Somlyo et al. 1970). Thus, the actions of c-AMP on smooth muscles seem to differ from those observed on cardiac muscle. Further, Klöckner and Isenberg (1985b) observed that c-AMP did not prevent the run-down phenomena of the Ca^{2+} current in single smooth muscle cells of guinea-pig urinary bladder. However, detailed information on the action of c-AMP on ionic currents in smooth muscle cells, using the voltage clamp technique, have not been reported.

In the present study, we attempted to clarify the actions of ATP and c-AMP, and their interactions on ionic currents recorded from intestinal smooth muscle cells, using intracellular perfusion techniques.

Materials and methods

Single cell dispersion. Part of the longitudinal muscle layer was peeled from the ileum of male albino rabbits (1.8–2.2 kg) which had been anesthetized with sodium pentobarbital (i.v. 40 mg/kg). The procedures used to disperse the muscle tissue were similar to those described by Ohya et al. (1986b). In brief, small segments of the ablated longitudinal muscle tissue were incubated in Ca^{2+}-free physiological salt solution (incubating solution) which contained 0.1% collagenase, 0.1% trypsin inhibitor and 0.2% bovine serum albumin, at 35 ± 1°C for 20–30 min. After completion of digestion, single cells including smooth muscle balls (SMBs) were dispersed by gentle agitation, using a glass pipette. The cell suspension was filtered through a fine nylon mesh, then centrifuged at 1,000 rpm for 1 min. The cell sediment was re-suspended in fresh physiological

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Table 1

Bath solution (mM)						
	NaCl	KCl	TEACl	$CaCl_2$	$MnCl_2$	Glucose
PSS	137	6	–	2.5	–	12
Mn^{2+} solution	137	6	–	–	2.5	12
TEA^+ solution	103	–	40	2.5	–	12
Pipette solution (mM)						
	KCl	CsCl	TEACl	EGTA		
High-K^+ solution	125	–	–	0.3 or 4		
Cs^+-TEA^+ solution	–	105	20	4		

salt solution and stored at 10°C. All experiments were carried out within 2 h after cell harvest.

Each smooth muscle cell (SMC) was 200–300 μm in length and 5–10 μm in diameter. Together with these spindle-shaped cell, we obtained a spheroidal fragment 10–30 × 20–60 μm, smooth muscle ball (SMB) (Ohya et al. 1986a, b). Both SMCs and SMBs could be also contracted by electrical stimulation or by application of agonists. The electrical characteristics of SMCs and SMBs were similar in our experiments. The values of specific membrane capacitance were much the same (SMC, 1.2 ± 0.2 μF/cm^2, $n = 5$; SMB, 1.5 ± 0.3 μF/cm^2, $n = 6$).

Electrical recordings. All of the data presented were obtained from fragments of the smooth muscle cells (smooth muscle ball; SMB). The SMB containing cell suspensions were taken into a small chamber (0.2 ml) and placed on the stage of the microscope (TMD-Diaphoto, Nihon Kogaku Co., Tokyo, Japan). The electrodes were approached towards the SMB, by using three dimensional oil driven micromanipulators (MO-102, Narishige Sci. Inst. Lab., Tokyo, Japan). Recording electrodes, made of Pyrex glass capillary tubes, were prepared by using a double stepped electrode puller and the tips of the electrodes were polished using a microforge (PP-83 and MF-83. Narishige Sci. Inst. Lab., Japan).

Whole cell voltage clamp of SMB was performed with a patch electrode (1–4 MΩ) through a single electrode voltage clamp amplifier (switching type) (Brennecke and Lindemann 1974). The switching frequency was fixed at 10 KHz and the closed time of the switch was set at 5% of the cycling time. A 100 MΩ resistor was used in the I–V converter. The output of the maximal current was 2.5 nA, since the voltage source of the passing current was 5 V (Ohya et al. 1986b). After formating a membrane-pipette seal with a high resistance (over 5 GΩ), the patch membrane was disrupted by a negative pressure (10–30 mm H_2O). The capacitive surge was completed within 5 ms. Electrical responses were monitored on a storage oscilloscope and on a thermo-writing pen-recorder (VC-10 and RJG-4124, Nihon Kohden Co., Tokyo, Japan). For measurement of the peak amplitude of the Ca^{2+} current, the leak current was subtracted, as estimated from the current evoked by a hyperpolarizing step. With Cs^+-TEA^+ solution in the pipette, the hyperpolarizing voltage step did not generate an active current (Ohya et al. 1986b). A command pulse was applied every 30 s.

Extracellular and intracellular perfusion. The solution in the chamber could be completely exchanged within 1 min by flushing with 5–10 ml of the test solutions. In some experiments, the constituents of the cytosole were modified by means of an "intracellular perfusion" technique. The apparatus and procedures were similar to those described (Irisawa and Kokubun 1983; Kameyama et al. 1985), i.e. briefly, a heat re-shaped fine polyethylene tube connected to a reservoir containing exchange solution was inserted into the suction electrode at a distance of 100–200 μm behind the tip. Intracellular perfusion was carried out by applying a negative pressure (30–50 cm H_2O) to the suction pipette and a hydrostatic positive pressure to inserted tube was created by lifting the reservoir (10–20 cm). In all experiments using intracellular perfusion, exchange of pipette solution was confirmed by checking the outlet volume of the solution from the pipette holder (0.03–0.05 ml/min).

Solutions. The ionic compositions of the physiological salt solution (PSS), Mn^{2+} solution and TEA^+ solution for the bath and high-K^+-solution and Cs^+-TEA^+ solution for the pipette are summarized in Table 1. Na_2-ATP concentrations in the pipette solution were 0, 0.3, 1, 3, 5 and 10 mM, and the concentrations of Na^+ and free Mg^{2+} were kept at 20 mM and 1 ± 0.1 mM, respectively, by adding appropriate amounts of NaCl (0–10 mM) and $MgCl_2$ (1–11 mM) to the pipette solution (Saida and Nonomura 1978; Itoh et al. 1982). The pH values of bath and pipette solutions were adjusted to 7.3 ± 0.1 and 7.2 ± 0.1, respectively, by 10 mM HEPES (N-2-hydroxyethylpiperazine-2-ethanesulfonic acid), titrated with Tris [Tris-(hydroxymethyl)-aminomethane]. All experiments were performed at room temperature.

Drugs. The drugs used were as follows; adenosine 5′-triphosphate (disodium salt; Na_2ATP), adenosine-3′,5′-cyclic monophosphate (c-AMP), dibutyryl cyclic AMP (dibutylyl c-AMP), 5′-adenylyl-imidodiphosphate (AMP-PNP) and theophylline (Sigma Chem. Co., St. Louis, MO, USA), and for preparation of isolated cells; collagenase (Wako Chem. Ind. Ltd., Osaka, Japan), trypsin inhibitor (type II-S) and bovine serum albumin (essentially fatty acid free); (Sigma Chem. Co., St. Louis, MO, USA).

Statistics. The values measured were expressed as the mean ± SD (number of SMBs or stimulations), and statistical significance was determined using unpaired Student's *t*-test. *P* values of less than 0.05 were considered to be significant.

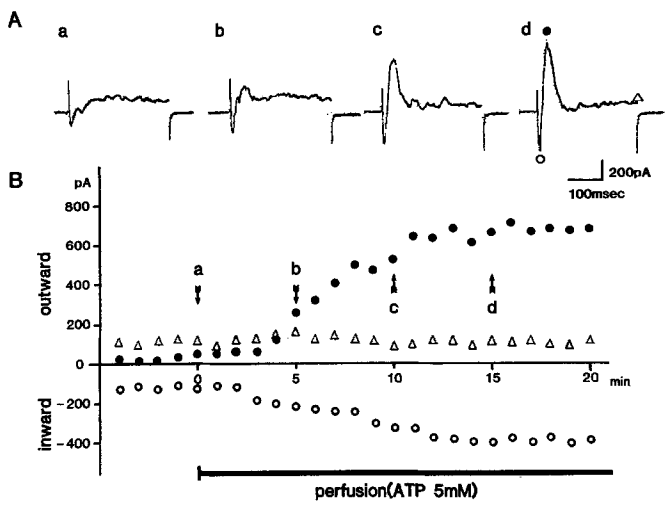

Fig. 1A,B. Effects of intracellular application of ATP (5 mM) on net membrane currents recorded from fragmented single smooth muscle cell (smooth muscle ball). **A** Actual traces (*a* before; *b* 5 min after; *c* 10 min after; *d* 15 min after the application of ATP). ○ Net inward current; ●, the transient outward current; △, the delayed outward current. **B** Changes in the amplitudes of ionic currents during perfusion of 5 mM ATP. The amplitudes of the net inward current (○) and the transient outward current (●) were measured at their peaks, and those of the delayed outward current (△) were measured at the termination of the voltage steps. The pipette was filled with high-K^+ solution containing 0.3 mM EGTA. The bath solution was PSS. The membrane potential was displaced from −60 mV to +20 mV every 30 s. *a*−*d* indicated in **B** correspond with *a*−*d* in **A**, respectively

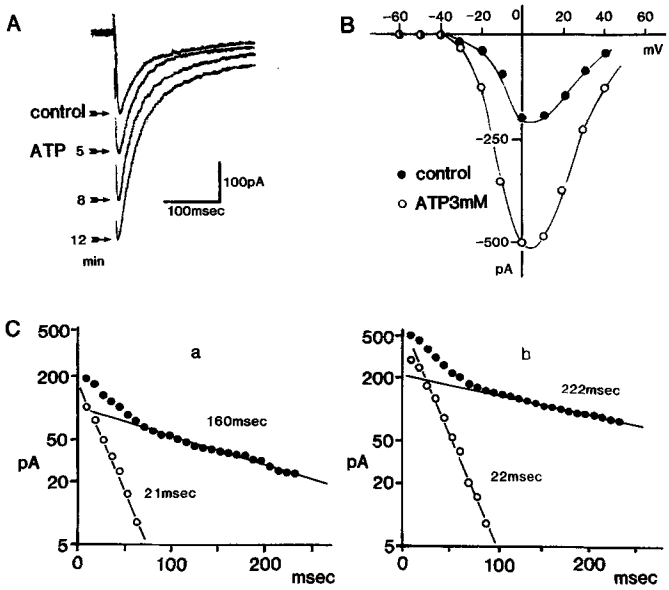

Fig. 2A–C. Effects of intracellular application of ATP (3 mM) on the Ca^{2+} current. The Ca^{2+} current was differentiated from net currents by application of Cs^+-TEA^+ solution with 4 mM EGTA in the pipette and salt solution containing 2.5 mM Ca^{2+} and 40 mM TEA-Cl in the bath. **A** Actual traces of the Ca^{2+} current recorded before (control), 5 min, 8 min and 12 min after perfusion of 3 mM ATP. Control was obtained 3 min after rupture of the patch membrane. **B** The I–V relationships of the Ca^{2+} current before ● and 12 min after the application of 3 mM ATP ○. **C** Decay of the Ca^{2+} current recorded before *a* and 12 min after *b* perfusion of ATP were plotted in semi-logarithmic scales, against time. (300 ms long pulse stepped from the holding potential of −60 mV to 0 mV.) **A–C** were recorded from the same SMB

Results

Under conditions of high-K^+ solution (0.3 mM EGTA) with no ATP in the pipette and PSS in the bath, application of a depolarizing pulse to the SMB evoked a net inward current followed by a small transient outward current (appearing as a outward notch just after generation of inward current) and delayed outward current (Fig. 1Aa). Just after rupture of the patch membrane, only a small transient outward current was observed. Perfusion without ATP in the pipette first diminished and then abolished the transient outward current. When 5 mM ATP was added to the pipette solution, the net inward and transient outward currents were enhanced (Fig. 1Ab–d). However the delayed outward current following the transient outward current was not modified by ATP (Fig. 1Ab–d). Figure 1B shows changes in amplitudes of these currents during the intracellular perfusion of ATP, plotted against time. Perfusion of the pipette solution was begun after the net inward current had stabilized (0 min in Fig. 1B). Three minutes after the perfusion, the amplitudes of the net inward and transient outward currents were gradually enhanced but the amplitude of delayed outward current remained stationary. The amplitudes of these inward and outward currents reached steady levels within 15 min.

Effects of ATP and ATP related compounds on the Ca^{2+} current

To evaluate the effects of intracellularly-applied ATP on the Ca^{2+} current, the outward currents were blocked with Cs^+-TEA^+ solution in the pipette and TEA^+ solution in the bath (Ohya et al. 1986b). Figure 2A shows actual traces of the Ca^{2+} currents recorded before (control), and 5 min, 8 min and 12 min after intracellular perfusion of 3 mM ATP (voltage step-up from −60 to 0 mV). The I–V relationship of the Ca^{2+} current measured before application of ATP revealed that the threshold potential required for the generation of the Ca^{2+} current and the membrane potential generating the maximum amplitude of the current were about −30 mV and 0 mV, respectively. Twelve minutes after application of 3 mM ATP, the amplitude of the Ca^{2+} current was enhanced at any given step of the command pulse, with no change in the threshold or the potential required to produce the maximal amplitude of the Ca^{2+} current (Fig. 2B). When the decay of the Ca^{2+} currents was replotted on a semi-logarithmic scale, it was composed of two (fast and slow) components (Fig. 2C). ATP prolonged the time constant of the slow component to 125–162% ($n = 5$) of the control values, however, that of fast component remained much the same value (105–127%, $n = 5$). Figure 2C shows a typical illustration of the action of ATP on the decay of the Ca^{2+} current. Twelve minutes after the intracellular perfusion of 3 mM ATP, the slow component increased from 160 to 222 ms and the fast component remained much the same (21 to 22 ms).

The actions of ATP on the steady-state inactivation curve of the Ca^{2+} current were examined using a double pulse procedure (2 s or 10 s of conditioning pulse) (Fig. 3). As a long conditioning pulse might damage the SMB, we used

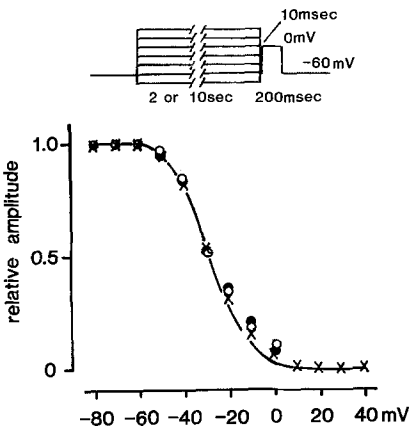

Fig. 3. Effect of ATP (3 mM) on the steady-state inactivation curve of the Ca^{2+} current. A double pulse protocol was used. Starting from a holding potential of −60 mV, the conditioning pulse (prepulse) was applied for 2 s or 10 s and then the test pulse (0 mV) was applied. The amplitude of the Ca^{2+} current evoked by the test pulse without a conditioning pulse was registered as a relative amplitude of 1.0. ● Before (control) and ○ after perfusion of 3 mM ATP using 2 s in prepulse. The results were obtained from the same SMB. The relationship observed by application of 10 s in prepulse (×) was obtained in the absence of ATP from a different SMB. Cs^+-TEA^+ solution (pipette) and TEA^+ solution (bath) were used. The continuous line was fitted by a Boltzmann distribution with parameters chosen for the best visual fit ($V_{1/2} = -29$ mV, $k = 7.2$)

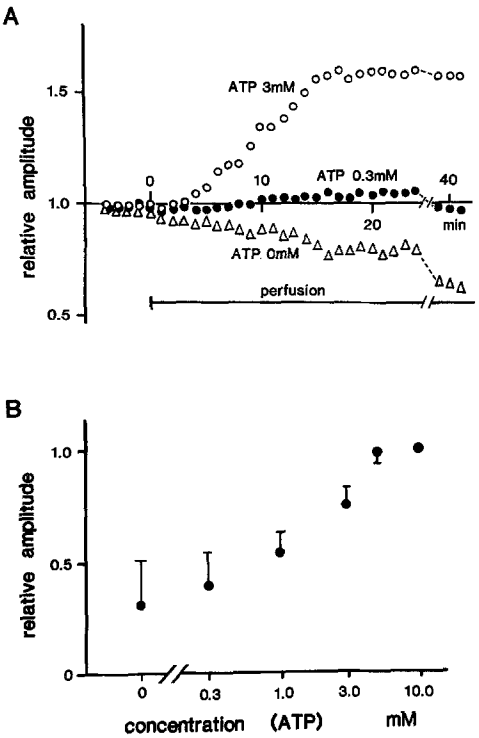

Fig. 4. A Time courses of alterations in amplitude of the Ca^{2+} current induced by the intracellularly applied ATP. ATP concentrations perfused into the SMB are shown in the figure. The amplitude of the Ca^{2+} current before perfusion of ATP was normalized as 1.0. These results were obtained from three different SMBs. A 300 ms voltage step from −60 to 0 mV was applied every 30 ms. **B** Dose-response relationship of ATP on the amplitude of the Ca^{2+} current. In this protocol, initially a certain concentration of ATP was perfused and the Ca^{2+} current was recorded. After the current had stabilized, the pipette solution was replaced with that containing 10mM ATP. The amplitude of the Ca^{2+} current measured following perfusion of 10 mM ATP in each experiment was normalized as a relative amplitude of 1.0. Cs^+-TEA^+ solution was present in the pipette and TEA^+ solution was present in the bath. The value was expressed as the mean with SD ($n = 4-6$)

2 s conditioning pulses, and voltage dependency almost the same as that obtained by application of the 10 s conditioning pulses was observed. With 2 s conditioning pulses, no significant effect of ATP (3 mM) was observed on the steady-state inactivation curve ($V_{1/2} = -29$ mV, $k = 7.2$). Thus, 3 mM ATP enhanced the amplitude of the Ca^{2+} current with no effect on the steady-state inactivation curve.

The effects of various concentrations of ATP on the time courses and amplitude of the Ca^{2+} current were then observed (Fig. 4A). With intracellular perfusions of 0, 0.3 or 3 mM ATP, the amplitude of the inward current was gradually decreased (run-down phenomenon), little changed, and enhanced, respectively. When the amplitude reached a steady level 15 min after the perfusion of ATP (over 1 mM), the shape of the Ca^{2+} current remainded unchanged during a further 20 min of perfusion.

Figure 4B shows the effects of various concentrations of ATP on the amplitude of the Ca^{2+} current. ATP (above 0.3 mM) enhanced the Ca^{2+} current, in a dose dependent manner, and almost reached the maximum value after perfusion with 5 mM ATP.

To further investigate the action of ATP on the Ca^{2+} current, we examined the effects of AMP-PNP, a non-hydrolyzable analogue of ATP, on the Ca^{2+} current. In Fig. 5, changes in the amplitude of the Ca^{2+} current following perfusion of AMP-PNP (1 mM) were plotted against time, and the amplitudes of the Ca^{2+} current before perfusion of 1 mM AMP-PNP in the presence of 0, 1.0 or 10 mM ATP were normalized as 1.0, respectively (voltage step from −60 to 0 mV with 250 ms in pulse duration). Intracellular perfusion of AMP-PNP (1 mM) inhibited the Ca^{2+} current in the presence of 0 mM and 1.0 mM ATP (0 mM ATP, to 30%; 1 mM ATP, to 60%), but only slightly so in the case of 10 mM ATP perfusion (to 90%). Inhibition of the Ca^{2+} current induced by AMP-PNP depended on the concentrations of ATP, i.e. when the concentrations of ATP were increased to 10 mM, the inhibition induced by AMP-PNP diminished (A). Figure 5B shows actual traces of the Ca^{2+} current recorded before and 15 min after application of 1 mM AMP-PNP, during perfusion with 1 mM ATP. Figure 5C shows the I−V relationship observed before and 15 min after application of 1 mM AMP-PNP. With 1 mM ATP, the subsequent application of 1 mM AMP-PNP inhibited the Ca^{2+} current, in a voltage-independent manner.

Effects of c-AMP on the Ca^{2+} current

To investigate the action of c-AMP on the Ca^{2+} current, we examined the effects on the Ca^{2+} current of extracellularly applied 1 mM dibutyryl c-AMP, a permeable derivative of cyclic nucleotide, plus additionally applied 3 mM theophylline, a phosphodiesterase inhibitor (Fig. 6). When ATP was not included in the pipette solution, the amplitude of the Ca^{2+} current decreased gradually, and neither extracellularly applied dibutyryl c-AMP nor additively applied theophylline prevented the run-down phenomenon.

For Further investigation of the action of c-AMP, c-AMP was perfused into the SMB after the Ca^{2+} current

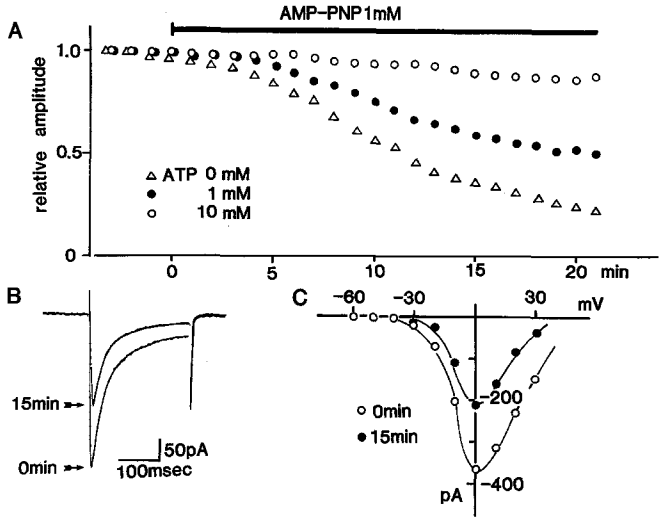

Fig. 5A–C. Effect of AMP-PNP (1 mM) on the Ca^{2+} current. **A** Effects of 1 mM AMP-PNP on the Ca^{2+} current against the perfusion time. Amplitudes of the Ca^{2+} current stabilized under perfusion of 0, 1.0 or 10 mM ATP were normalized as a relative amplitude of 1.0, respectively. Three different SMBs were used. **B** Traces of Ca^{2+} inward current measured before (0 min) and 15 min after perfusion of 1 mM AMP-PNP, in the presence of 1 mM ATP. **C** The I–V relationship observed before and 15 min after perfusion of 1 mM AMP-PNP, in the continuous presence of 1 mM ATP. **B** and **C** were obtained from same SMB. Cs^+-TEA^+ pipette solution and TEA^+ bath solution were used

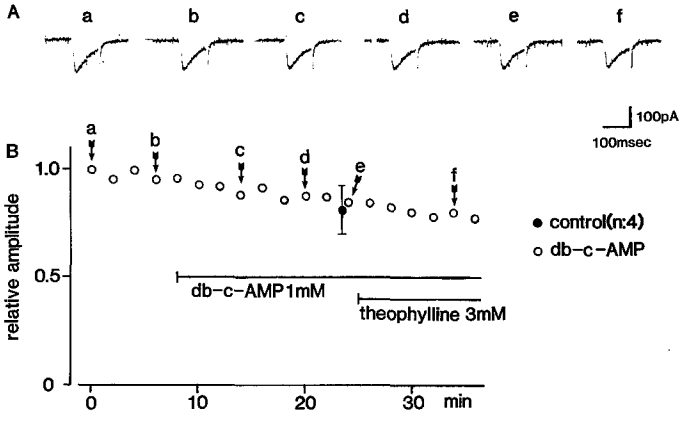

Fig. 6A,B. Effects of extracellularly applied dibutyryl c-AMP (1 mM) and additively applied theophylline (3 mM) on the Ca^{2+} current. **A** Examples of the Ca^{2+} current recorded during application of dibutyryl c-AMP *c–d* and during additively applied theophylline *f* (condition: 100 ms pulse to 0 mV every 30 s). **B** Time course of the alteration in the amplitude of Ca^{2+} current measured during application of dibutyryl c-AMP with theophylline. Dibutyryl c-AMP and theophylline were added to the bath 8 min after and 25 min after initiation of current recording, respectively. The amplitude of the Ca^{2+} current, obtained just after initiation of the recording of the current was normalized as 1.0. *a–f* in **B** correspond with actual records in **A**, respectively. *Closed circle in* **B** indicates the amplitude of Ca^{2+} current recorded in the absence of dibutyryl c-AMP as control ($n = 4$, *bar* indicates SD × 2). Cs^+-TEA^+ solution without ATP was present in the pipette in this experiment (TEA^+ solution was present in the bath)

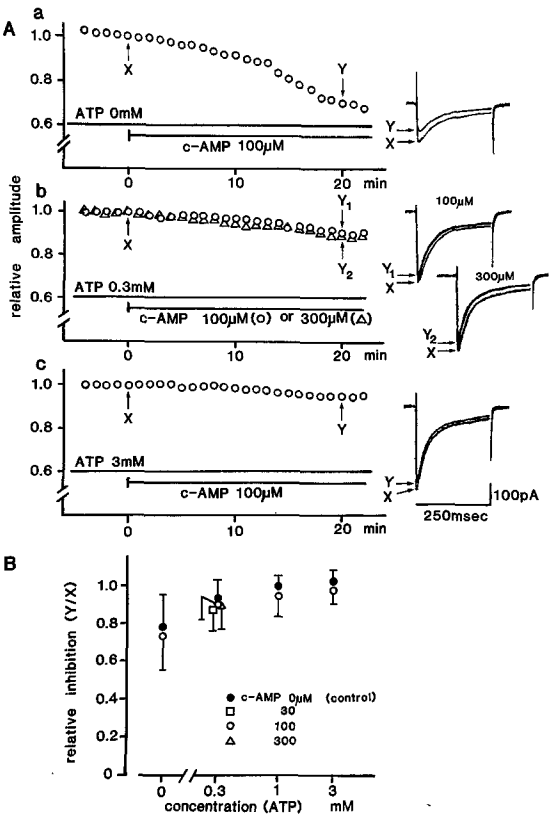

Fig. 7A,B. Effects of c-AMP on the Ca^{2+} current. **A** Changes in the amplitude of the Ca^{2+} current during perfusion of c-AMP. Amplitudes of the Ca^{2+} current stabilized with various concentrations of ATP (just before perfusion of c-AMP) were normalized as 1.0, and labeled as X. The Ca^{2+} current observed 20 min after the perfusion of c-AMP is marked by Y. (250 ms steps from −60 to 0 mV). Actual traces of the inward current before and after application of c-AMP are shown in the *right column*. **B** Absence of an effect of c-AMP (□ 30 μM, ○ 100 μM and △ 300 μM) on the Ca^{2+} current. Varied concentration of ATP (*abscissa*) and amplitudes of the Ca^{2+} current ($n = 4–6$, *bar* indicates SD) as ratio of Y/X (*ordinate*). In this figure, amplitudes of the Ca^{2+} current recorded in the presence or absence (control) of c-AMP under perfusion of ATP were measured using different SMBs. Control (●) was obtained under conditions of perfusion of various concentrations of ATP alone. Cs^+-TEA^+ pipette solution and TEA^+ bath solution were used

had stabilized with perfusion of various concentrations of ATP-containing pipette solutions (Fig. 7A). The amplitude of the Ca^{2+} current before the perfusion was normalized as 1.0 (indicated as X in the figure). Application of 100 μM c-AMP (open circles) had no effect on the amplitude of the Ca^{2+} current, at any given concentration of the pre-perfused ATP. Figure 7A b also shows the effects of 300 μM c-AMP (triangles) on the Ca^{2+} current following perfusion with 0.3 mM ATP. The right hand columns in Fig. 7A also show actual traces of the inward currents recorded before (X) and 20 min after (Y; 100 μM, Y_1; 100 μM, Y_2; 300 μM) application of c-AMP, in the presence or absence of ATP. When the amplitudes of inward current were compared before and after application of c-AMP, the amplitude was slightly decreased. To clarify whether reduction in the amplitude was due to the run-down phenomenon or to the inhibitory action of c-AMP, amplitudes of the inward

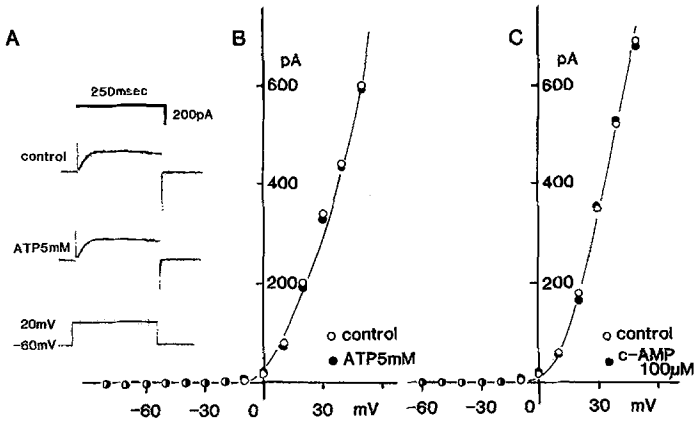

Fig. 8A–C. Effects of ATP (5 mM) and c-AMP (100 μM) on the delayed outward current (Ca^{2+}-independent K^+-current). The delayed outward current was recorded with high-K^+ pipette solution (4 mM EGTA) and Mn^{2+} bath solution. **A** Examples of the delayed outward current recorded before (*upper*) and after (*middle*) intracellular perfusion of 5 mM ATP (250 ms pulse from −60 to +20 mV). **B** The I–V relationship of the delayed outward current (amplitude measured at the termination of 250 ms voltage steps) before (○) and 20 min after (●) the perfusion of 5 mM ATP. **C** The I–V relationship of the delayed outward current before (○) and 20 min after (●) the perfusion of 100 μM c-AMP. The membrane potential was held at −60 mV and appropriate voltage step was applied to measured the current amplitude in **B** and **C**. **A** and **B** were obtained from the same SMB

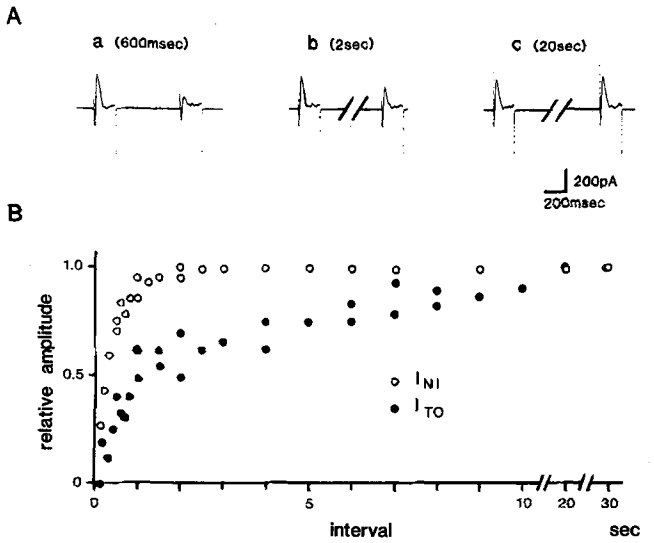

Fig. 9A,B. Interval-dependent suppressions of the net inward and transient outward currents. **A** Actual records of the evoked currents obtained by a double pulse protocol, at a interval of 600 ms (*a*), 2 s (*b*) and 20 s (*c*). (200 ms long pulses from −60 to 0 mV). **B** The amplitude of the net inward (I_{NI}) and the transient outward (I_{TO}) currents evoked by the test pulse (*second pulse*) are plotted against the interval. The amplitudes of I_{NI} and I_{TO} evoked by the first pulse were normalized to 1.0. *Abscissa* interval between two pulses (s). The electrode was filled with a high-K^+-solution containing 0.3 mM EGTA and 5 mM ATP; PSS was in the bath. **A** and **B** were obtained from the same SMB

current recorded in the presence or absence of c-AMP were compared. Figure 7B shows the effects of various concentrations of c-AMP on the Ca^{2+} current in case of perfusion of ATP. As a control, the amplitude of the Ca^{2+} current was measured at Y (20 min after start of the perfusion) under conditions of perfusion of various concentrations of ATP, without c-AMP. There were no significant differences between the values obtained from control and c-AMP (30, 100, 300 μM) perfusions, with any concentrations of ATP used. These observations indicate that reduction in the amplitude after perfusion of c-AMP was not due to the inhibitory actions of c-AMP, but rather to the run-down phenomenon. Thus, c-AMP probably has no effect on the Ca^{2+} current.

Effects of ATP and c-AMP on the outward currents

Superfusion of Mn^{2+} solution (Ca^{2+}-omitted) in the bath suppressed the Ca^{2+}-inward current and the Ca^{2+} dependent outward current (high-K^+ solution in the pipette) (Ohya et al. 1986b). The residual outward current (delayed outward current) was considered to be the Ca^{2+}-independent component. Figure 8 shows the actual traces (A) and I–V relationship (B) of the Ca^{2+}-independent outward current before and 15 min after perfusion of 5 mM ATP, and the I–V relationship (C) before and 15 min after the perfusion of 100 μM c-AMP. In the control, the outward current had a threshold of just below 0 mV and increased in a voltage dependent manner. Both the time dependence of the current trace and the shape of the I–V relations remained the same after intracellular perfusion of 5 mM ATP (15 min) or 100 μM c-AMP (15 min).

When the pipette contained high-K^+ solution (0.3 mM EGTA) and PSS was present in the bath, the depolarizing command pulse evoked the transient outward current. This current was regarded as the Ca^{2+}-dependent outward current, because a reduction in the Ca^{2+} from either side of the cell membrane (application of Ca^{2+}-free solution in the bath and/or 4 mM EGTA in the pipette) suppressed generation of this current (Ohya et al. 1986a). As the Ca^{2+}-independent outward current appeared at a command potential of 0 mV, values of the amplitude of the transient outward current were slightly overestimated. However, the percentage of the Ca^{2+} independent outward current in the entire outward current was considered to be small (only a few %). In the double pulse study, a shorter interval decreased the amplitudes of the transient outward current and the net inward current evoked by the test pulse (Fig. 9). As shown in Fig. 9B, pulsing at intervals shorter than 3 s reduced the amplitude of the net inward current evoked by the test pulse. In the case of the transient outward current, the current evoked by the test pulse was reduced at internal of less than 20 s.

Effects of 100 μM c-AMP on the amplitude of the transient outward current evoked by repetitively applied test pulses were also examined. Figure 10A shows actual records of the net inward and the transient outward currents during a train stimulation (15 pulses) with the interval of 3 s. Before perfusion of 100 μM c-AMP into the SMB, the amplitude of the transient outward current was markedly decreased (a). This reduction was partially prevented by intracellularly-applied c-AMP (b). Figure 10B shows the average amplitude of the transient outward current measured from the last ten pulses of fifteen train stimulations at various intervals. Application of c-AMP produced no significant changes in amplitudes of the transient outward current when the interval of train stimulation exceeded 15 s. However, repetitive

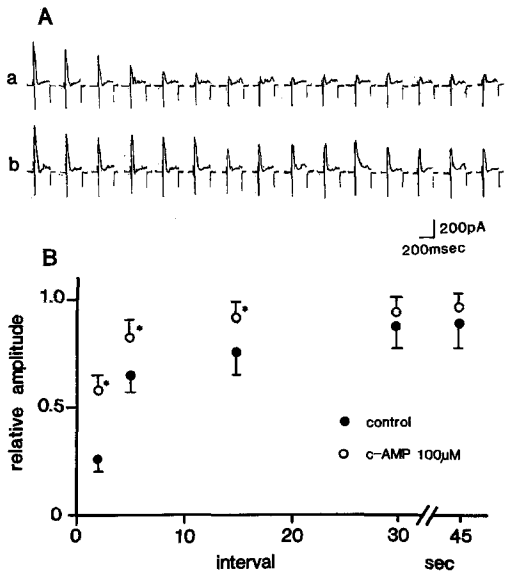

Fig. 10A, B. Effect of c-AMP on the transient outward current evoked by repetitive stimulation. **A** Typical records of currents obtained by fifteen train stimulations (200 ms long voltage steps from −60 to 0 mV; interval of 3 s). *a* Before and *b* 15 min after intracellular perfusion of 100 μM c-AMP. The compositions of the pipette and bath solutions were the same as for Fig. 9. **B** Amplitudes of the transient outward current before ● and 10 min after ○ intracellular perfusion of 100 μM c-AMP, were plotted against the interval of train pulses. The amplitude of the last ten of fifteen train pulses was averaged (pulse condition and solutions were same as in **A**). In each record, the amplitude of the transient outward current evoked by the first pulse was recorded as 1.0, respectively. Bar indicates SD. * Statistically significant between the values of before and after the perfusion of c-AMP

stimulation with intervals shorter than 15 s reduced the amplitude of the transient outward current and this reduction was partially prevented by intracellular application of 100 μM c-AMP, in a stimulus frequency-dependent manner. Using this procedure, a similar tendency was observed in five other examined SMBs. These results indicate that c-AMP partially prevents the reduction in the amplitude of the transient outward current (Ca^{2+}-dependent outward current) induced by repetitive stimulation, at short intervals.

Discussion

In ventricular muscle cells and sensory neurons, intracellularly-applied ATP enhanced and stabilized the Ca^{2+} current (Fedulova et al. 1981; Irisawa and Kokubun 1983; Taniguchi et al. 1983; Byerly and Moody 1984). The mechanisms underlying these effects of ATP are as follows; 1. ATP regulates phosphorylation of the Ca^{2+} channel protein and this phosphorylation is c-AMP dependent (Reuter 1983). 2. ATP as well as c-AMP activates the Ca^{2+} pump in the plasma membrane and in the intracellular Ca^{2+} storage sites (Byerly and Yazejian 1986). This Ca^{2+} extrusion mechanism would be linked to amplitude of the Ca^{2+} current, because the Ca^{2+} current is modified by intracellular Ca^{2+} concentrations (Ca^{2+}-dependent Ca^{2+}-current inactivation) (Eckert and Tillotson 1981; Brown et al. 1981; Kokubun and Irisawa 1984).

The present experiments revealed that in the smooth muscle membrane, the intracellularly applied ATP enhanced the Ca^{2+} current, as has been observed in other tissues. However, c-AMP had no effect on the Ca^{2+} current in SMB, thereby indicating that in the smooth muscle membrane, control of the Ca^{2+} current by ATP is not mediated by c-AMP-dependent phosphorylation. Since 4 mM EGTA in the pipette could control the Ca^{2+} in the cytosol at low concentration, it is rather unlikely that acceletation of the Ca^{2+} pump by ATP and c-AMP account for the enhancement of the Ca^{2+} current (release from the Ca^{2+}-dependent Ca^{2+}-current inactivation) in the present study. In the guinea-pig ventricular muscle cell, AMP-PNP, reduced but adenosine-5′-(γ-thio)-triphosphate (ATP-γ-S) markedly enhanced the effects of β-stimuli (enhancement of the Ca^{2+} current). Therefore, in cardiac muscles, ATP and c-AMP seem to phosphorylate the same site in the Ca^{2+} channel (Kameyama et al. 1985). If activation of Ca^{2+} channels in smooth muscles reguires phosphorylation of the channel protein, ATP but not c-AMP may not phosphorylate the Ca^{2+} channel.

AMP-PNP inhibited the Ca^{2+} current in competition with actions of ATP, therefore, it is plausible that AMP-PNP and ATP may act on the same site. When there was no ATP in the pipette, the run-down phenomenon was accelerated by intracellular application of AMP-PNP. We considered that even without ATP supplement, some ATP remained in the SMBs and was utilized in the activation of the Ca^{2+} channel. If so, AMP-PNP could compete with this ATP and the decrease in the Ca^{2+} current may be enhanced.

ATP prolonged the slow component of the decay of the Ca^{2+} current; a similar tendency was observed in cardiac muscles (Taniguchi et al. 1983; Irisawa and Kokubun 1983). Several types of Ca^{2+} channels have been demonstrated in various tissues (Bean 1985; Fedulova et al. 1985; Nowycky et al. 1985; Nilius et al. 1985; Friedman et al. 1986). These channels have different features such as voltage dependence, inactivation kinetics, sensitivity to Ca^{2+} antagonist and agonist, ion permeability etc. In dorsal root ganglion neurons, intracellular application of c-AMP together with ATP and Mg^{2+} prevented the run-down phenomenon of the second component but not the first component (low-threshold Ca^{2+} current) of Ca^{2+} current (Fedulova et al. 1985). If several types of Ca^{2+} channels also exist in ileal smooth muscle cells, then intracellular application of ATP might increase some types of Ca^{2+} channel, which have slow inactivation kinetics. However, the present study did not clarify the underlying mechanism for prolongation of the decay of the Ca^{2+} current.

The only obvious action of c-AMP in the present study was preservation of the amplitude of the transient outward current during repetitive stimulation. One possible mechanism is that c-AMP may directly phosphorylate the Ca^{2+}-dependent K^+ channel, as observed in Helix neuron (Ewald et al. 1985). They presented evidence that c-AMP and c-AMP-dependent protein kinase augmented opening of the Ca^{2+}-dependent K^+ channel. Another possible mechanism is that the amount of Ca^{2+} in the storage site is preserved by acceleration of the Ca^{2+} pump induced by c-AMP, during repetitive stimulation. In various smooth muscle tissues, the Ca^{2+} pump was found to be accelerated by c-AMP and c-AMP-dependent protein kinase and this process was also ATP-dependent (Hurwitz et al. 1973; Casteels and Raeymaekers 1979; Bhalla et al. 1978; Suematsu et al. 1984).

Therefore, a reduction in the amplitude of the transient outward current, which is regarded as being closely related to Ca^{2+} released from the storage site (Klöckner and Isenberg 1985a; Ohya et al. 1986a), may be partly prevented. Acceleration of c-AMP-dependent Ca^{2+} accumulation into the storage site has also been demonstrated in skinned muscle strips of vascular tissues (Itoh et al. 1982). In various visceral smooth muscles, isoprenaline hyperpolarized the membrane presumably due to increase in the Ca^{2+}-dependent K^+ channel (Bülbring et al. 1981). It is not known whether the channels activated during the hyperpolarization of the membrane and the channels which form the transient outward current are the same. More detailed experiments may be required to understand the mutual relationship between the c-AMP induced reduction in the free-Ca^{2+} and the c-AMP induced enhancement of the activation of the Ca^{2+}-dependent K^+ channels.

In conclusion, the c-AMP-mediated regulation of the Ca^{2+} channel is probably absent in smooth muscle of rabbit ileum, thus the nature of the membrane differs from that observed in cardiac muscles. As a consequence, together with the c-AMP-dependent accelerations of the Ca^{2+} pump at both the sarcolemma and sarcoplasmic reticulum, and with the c-AMP-dependent phosphorylation of myosin light chain kinase (Conti and Adelstein 1980), the increased amount of c-AMP synthesized by β-adrenoceptor stimulation may cause the relaxation of the smooth muscle.

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