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ATP increases Ca^{2+}-activated K^+ channel activity in isolated rat arterial smooth muscle cells

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Abstract

Large conductance Ca^{2+}-activated K^+ (K_{Ca}) channels are known to be activated by phosphorylation through cAMP- and cGMP-dependent kinase activation. In pulmonary arterial smooth muscle K_{Ca} channels are directly activated by ATP (but not by non-hydrolysable analogues) independently of the presence of cyclic nucleotides or the catalytic subunits of protein kinases. This study was designed to determine whether direct activation of K_{Ca} channels by ATP is apparent in other types of arterial smooth muscle. K_{Ca} channels of similar conductance to those of rat pulmonary artery ($\approx$ 250 pS) were found in membrane patches excised from isolated smooth muscle cells from rat aorta, mesenteric and basilar arteries. In myocytes isolated from each of these arteries, intracellular application of ATP (in the absence of exogenous cyclic nucleotides or catalytic subunits) reversibly increased the open state probability of K_{Ca} channels: a response markedly reduced by a specific inhibitor of protein kinase A. Nucleotide sequence analysis of K_{Ca} channels revealed no homology with the majority of protein kinases. It is concluded that phosphorylation of K_{Ca} channels through the activity of a membrane tethered kinase related to protein kinase A (but lacking its regulatory subunits) may play an important role in controlling K^+ flux in a range of arterial smooth muscle cell types.

Keywords: Calcium-activated potassium ion channel; Potassium ion channel; Arterial smooth muscle cell; Pulmonary circulation; Protein kinase

1. Introduction

Large conductance Ca^{2+}-activated K^+ (K_{Ca}) channels have been identified in most types of arterial smooth muscle where they may operate as a negative feedback pathway responding to fluctuations in membrane potential and intracellular Ca^{2+}, and in this way may serve to control the level of myogenic tone [1]. The activity of K_{Ca} channels is regulated by both membrane potential and by the concentration of Ca^{2+} in contact with their intracellular membrane surface [2–4]. In addition, it has been shown that the activity of K_{Ca} channels in a number of tissues can also be increased by phosphorylation through the action of a number of different kinases [5–7]. In these experiments the presence of exogenous regulatory factors such as cAMP or cGMP or the catalytic subunits of their respective kinases, in addition to ATP, was a prerequisite for channel activation. It has been previously speculated that an apparently novel class of K_{Ca} channel (originally referred to as $K_{Ca,ATP}$) exists in the pulmonary circulation of the rat which can be activated directly by ATP in the absence of exogenous cAMP, cGMP or catalytic subunits [8]. It is not known, however, whether this process (i) occurs as a consequence of autophosphorylation, (ii) is due to the activity of a closely-associated, membrane-bound kinase or (iii) is a direct result of ATP binding. However, because of the sensitivity of pulmonary arterial K_{Ca} channels to intracellular ATP, it has been suggested that these channels may play a unique role in the pulmonary circulation in linking cellular metabolism to K^+ flux during hypoxia [9]: a notion based on the assumption that membrane ATP levels may decrease during hypoxia [10,11].

Direct activation of K_{Ca} channels by ATP, through the activity of closely associated kinase, has been consistently reported in neuronal tissue [12–14]. However, in the case of smooth muscle the effects of ATP are uncertain. Indeed, ATP has been reported to inhibit [15,16], activate [8] and elicit no effect [17] on these channels. In the present study, we have addressed this problem by comparing the ATP sensitivity of K_{Ca} channels in smooth muscle cells isolated from aorta, pulmonary, mesenteric and basilar arteries of the rat.

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2. Materials and methods

2.1. Cell isolation

Male albino Wistar rats (200–250 g body weight) were killed by an overdose of i.p. Euthatol (pentobarbitone sodium B.P., Rhone Merieux, Ireland) and cervically dislocated. The main pulmonary artery, aorta, mesenteric artery and basilar artery were removed and arterial smooth muscle cells isolated using a dispersion procedure (previously described by Albarwani et al. [18]) modified to include an incubation with collagenase (type VIII; 1.5 mg/ml), proteinase (type I; 0.1 mg/ml) and elastase (type II-A; 0.3 mg/ml) for 5 min at 37°C following pre-treatment with papain. Cells were stored at 4°C and remained viable for ≈ 10 h.

2.2. Electrophysiology

Single channel currents were recorded from inside-out membrane patches using standard patch-clamp recording procedures [19]. Recording pipettes were pulled from borosilicate glass capillaries (Clark Electromedical, Pangbourne, UK) using a vertical puller (Narishige, Tokyo, Japan) and when filled with electrolyte had a resistance of 10–15 MΩ. Single channel currents were measured using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) and recorded onto digital audio tape (DTC 1000ES, Sony, Japan) at a frequency of 1 kHz for subsequent off-line analysis. Prior to analysis single channel currents were digitised at 5 kHz using a DigiData 1200 interface (Axon Instruments). Data were analysed on a 486 microcomputer using pClamp software (version 6.1; Axon Instruments).

2.3. Solutions

The extracellular membrane surface of inside-out patches was bathed in a solution which consisted of (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes): pH 7.4 with KOH. The intracellular aspect of the patch was bathed in a solution containing (in mM): 140 KCl, 1.1 $MgCl_2$, 10 Hepes; pH 7.3 with KOH. To buffer the levels of free Ca^{2+} in the intracellular solution different proportions of $CaCl_2$ and ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) or 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) were used (in mM): 4.8 $CaCl_2$ and 5 EGTA for 2.6 μM Ca^{2+}; 5.35 $CaCl_2$ and 6 EGTA or 4.38 $CaCl_2$ and 5 BAPTA for 0.9 μM Ca^{2+}; no added $CaCl_2$ and 5 EGTA for ≈ 1 nM Ca^{2+}. Levels of free 'intracellular Ca^{2+}' ($[Ca^{2+}]_i$) were determined using a programme for calculating metal ion to ligand binding (Peter Griffiths, University Laboratory of Physiology, Oxford). On addition of ATP the concentration of $MgCl_2$ in the intracellular solution was raised to 1.5 mM to compensate for chelation of Mg^{2+} by ATP. All experiments were performed at room temperature 21–24°C. ATP dipotassium salt, EGTA, BAPTA, Hepes, collagenase (type VIII), elastase (type II-A), proteinase (type I), papain, dithiothreitol, H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride), PKI (peptide inhibitor of protein kinase A) were all purchased from Sigma (Poole, UK). Okadaic acid and Rp-cAMPS (adenosine-3′,5′-cyclic monophosphothioate-Rp-isomer triethylammonium salt) were purchased from Calbiochem (Nottingham, UK). Solutions were exchanged during recordings by superfusing the whole bath using a gravity feed system. The time for complete solution exchange was ≈ 7 s.

2.4. Statistics and data analysis

Data are presented as means ± S.E.M. When presented graphically, the S.E.M. is represented by the associated error bars. Statistical significance of raw data was assessed using Student's *t*-test. *P*-values ≤ 0.05 were considered significant. Changes in channel activity are expressed as changes in open state probability (P_{open}). The P_{open} of K_{Ca} channels was calculated once any changes in channel activity following application of Ca^{2+}, ATP or any other exogenous agent had stabilised. Representative stretches of data never shorter than 30 s in duration were used for data analysis.

3. Results

Upon excising inside-out membrane patches from acutely dissociated arterial smooth muscle cells all patches tested contained between 1 and 6 K_{Ca} channels ($n = 196$ patches). In symmetrical 140 mM KCl, K_{Ca} channels displayed a linear current–voltage relationship with mean single channel conductances (over the range of −50 to 50 mV) of 250 ± 3 pS (main pulmonary artery; $n = 4$), 240 ± 7 pS (aorta; $n = 4$), 254 ± 3 pS (mesenteric artery; $n = 4$) and 260 ± 2 pS (basilar artery; $n = 3$). In addition to a high unitary conductance, and in agreement with others [20], the activity of K_{Ca} channels was dependent upon both membrane voltage and the concentration of Ca^{2+} in contact with the intracellular membrane surface of the channel: the P_{open} of the channel increasing with both membrane depolarisation and an elevation of $[Ca^{2+}]_i$. At a membrane potential of +40 mV and a $[Ca^{2+}]_i$ of 0.9 μM K_{Ca} channel activity was clearly apparent ($P_{open} = 0.01$–0.07; $n = 163$) as shown in Fig. 1A. This degree of activity represented a clear 'base-line' from which subsequent changes in response to ATP or modulatory factors could be measured.

In myocytes isolated from basilar arteries at a membrane potential of +40 mV ($[Ca^{2+}]_i = 0.9$ μM) intracellular application of 405 μM ATP (340 μM Mg-ATP,

throughout; the species of ATP responsible for activating K_{Ca} channels [8]) induced a significant ($P \leq 0.01$) increase in the P_{open} of K_{Ca} channels in 20 out of 30 patches (Fig. 1A). Under the same experimental conditions, this concentration of ATP also caused a similar significant increase in K_{Ca} channel activity in patches excised from mesenteric (11/15), aortic (11/18) or pulmonary (40/58) arterial myocytes (Fig. 1B). Note, changes in K_{Ca} channel activity were expressed as P_{open} since the total number of channels contained in each patch was determined following application of 2.6 μM Ca^{2+} to its intracellular surface at a membrane potential of +40 mV. This $[Ca^{2+}]_i$ is known to maximally activate K_{Ca} channels at this membrane potential [20,29], indeed the P_{open} under these conditions was close to unity. In cells isolated from all four arteries the

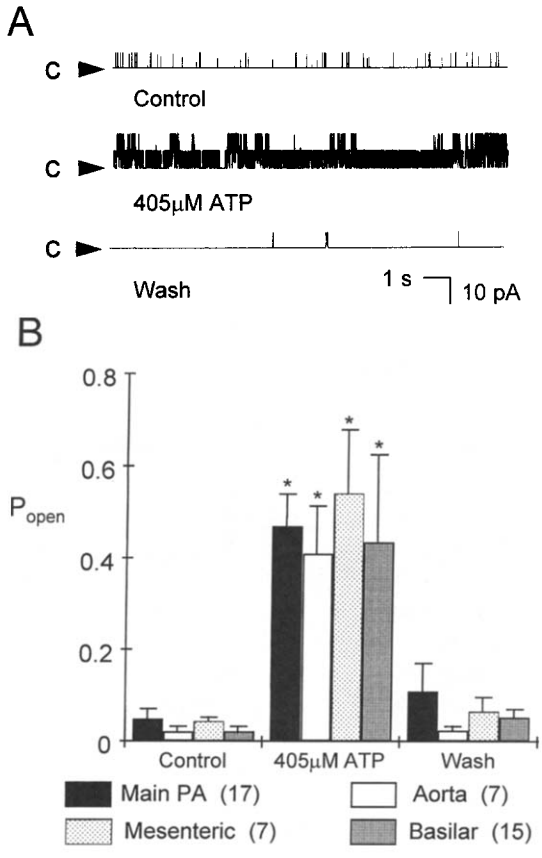

Fig. 1. Intracellular application of ATP and its effect on K_{Ca} channel activity. (A) Single K_{Ca} channel currents recorded from an inside-out membrane patch (excised from a basilar myocyte) held at a membrane potential of +40 mV in symmetrical 140 mM KCl (see Section 2 for details). The intracellular aspect of the patch was superfused initially with a solution containing 0.9 μM Ca^{2+} (control). Addition of 405 μM ATP (340 μM Mg-ATP; throughout) caused an increase in channel activity (P_{open}) which was reversible upon washout. For all traces, channel openings are shown as upward deflections from the closed-level current denoted by C. P_{open} values are as follows: control, 0.015; 405 μM ATP, 0.314; and wash, 0.003. (B) Histogram showing the activating action of ATP (405 μM) on K_{Ca} channels in four types of arterial myocyte at a membrane potential of +40 mV. Bars represent S.E.M, * $P \leq 0.01$ when compared to control. The number of patches is shown in parentheses in this and all subsequent figures.

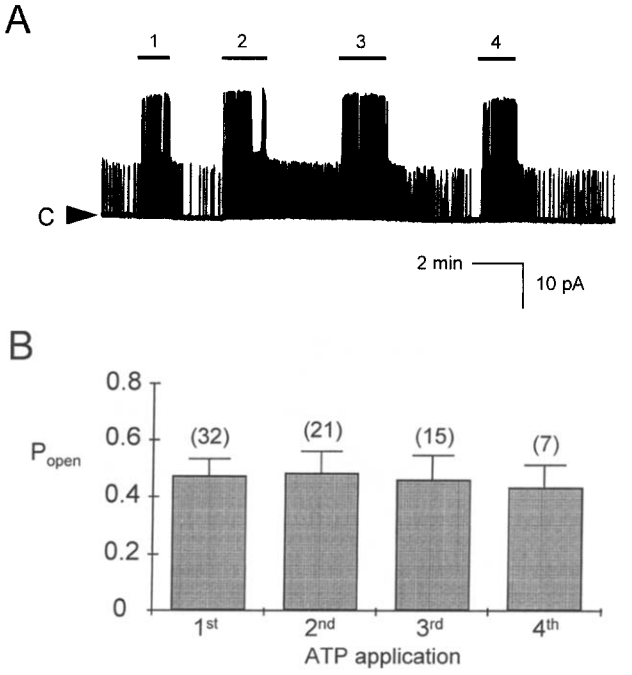

Fig. 2. Reversible activation of K_{Ca} channels by ATP. (A) Single K_{Ca} channel currents recorded from an inside-out membrane patch (excised from a basilar myocyte) held at a membrane potential of +40 mV in symmetrical 140 mM KCl. The intracellular aspect of the patch was superfused with a solution containing 0.9 μM Ca^{2+}. The filled bars indicate when the ATP (405 μM) was present in the bath, the application number being indicated above each bar. Note, ATP causes a reversible increase in K_{Ca} channel activity which can be elicited several times on a single patch without any apparent decrease in activity. P_{open} values are as follows: application 1, 0.314; application 2, 0.331; application 3, 0.367; and application 4, 0.309. (B) Histogram showing the activating action of subsequent applications of 405 μM ATP (see abscissa). Data from all four types of arterial myocyte have been pooled.

effect of ATP was found to be reversible following washout and could be elicited several times on a single patch without any apparent decrease in K_{Ca} channel activity (Fig. 2A,B). In these experiments ATP was applied up to four times for 3–4 min over a patch life time of up to 1 h. In the presence of ATP no apparent K_{Ca} channel run-down was observed. To obviate the possibility that K_{Ca} channel activation by ATP was due to fluctuation in $[Ca^{2+}]_i$ (resulting from a disruption of the ionic equilibrium by the nucleotide) some experiments were repeated using the pH-insensitive Ca^{2+} chelator BAPTA. Similar results to those illustrated in Fig. 1 were obtained: activation of K_{Ca} channels by 405 μM ATP occurred in 15 out of 22 patches from pulmonary, and in 25 out of 37 patches from basilar myocytes (data not shown). Analysis of K_{Ca} channel activity revealed similar mean open and closed times for K_{Ca} channels irrespective of the source myocyte. Mean open and closed times for K_{Ca} channels in pulmonary myocytes obtained upon raising the $[Ca^{2+}]_i$ from 0.9 μM to 2.6 μM and following activation by 405 μM ATP, in the presence of 0.9 μM $[Ca^{2+}]_i$, are shown in Table 1. It appears that the mean open time is similar following K_{Ca} channel

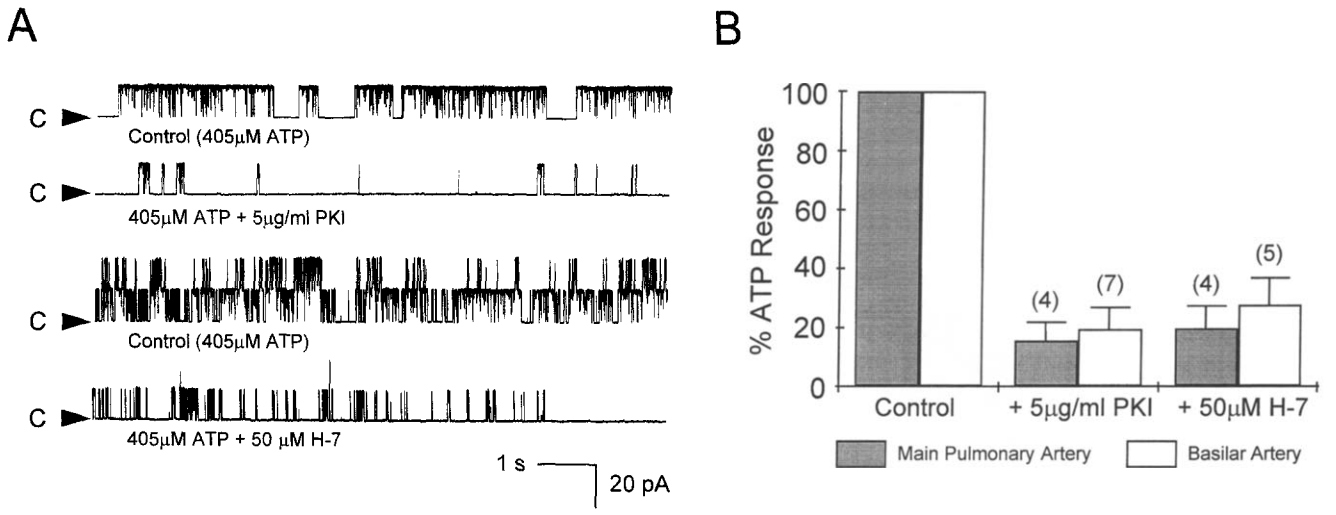

Fig. 3. Effects of protein kinase inhibitors PKI and H-7 on ATP-mediated K_{Ca} channel activation. (A) Single channel currents recorded from an inside-out membrane patch (excised from a basilar myocyte) held at a membrane potential of +40 mV in symmetrical 140 mM KCl. The intracellular aspect of the patch was initially superfused with a solution containing 0.9 μM Ca^{2+} and 405 μM ATP. Following co-application of PKI (5 μg/ml) or H-7 (50 μM) activation of K_{Ca} channels by ATP (405 μM) was markedly reduced. P_{open} values are as follows (upper two records) control, 0.712; +PKI, 0.091; (lower two records) control, 0.343; +H-7, 0.063. (B) Histogram showing the inhibition of ATP-mediated K_{Ca} channel activation by PKI (5 μg/ml) and H-7 (50 μM) on both basilar and pulmonary myocytes. Activation of K_{Ca} channels is expressed as a percentage of K_{Ca} channel activity in the presence of 405 μM ATP (control). Analysis of raw data revealed that both inhibitors caused a significant ($P \leq 0.05$) reduction in K_{Ca} channel activity when compared to control.

activation by ATP (405 μM) or high (2.6 μM) $[Ca^{2+}]_i$. However, the mean closed time is shorter in the presence of 2.6 μM $[Ca^{2+}]_i$, reflecting the higher P_{open} under these conditions.

In pulmonary arterial smooth muscle direct activation of K_{Ca} channels by ATP is not mimicked by non-hydrolysable analogues of ATP, suggesting that phosphorylation may be involved in this effect [8]. In order to test whether activation was due to phosphorylation or was the consequence of ATP binding, we examined the effects of kinase inhibitors on K_{Ca} channel activation by ATP. Activation of K_{Ca} channels by 405 μM ATP in all four types of arterial myocyte was markedly reduced by the non-specific kinase inhibitor H-7 [21] and the peptide inhibitor of protein

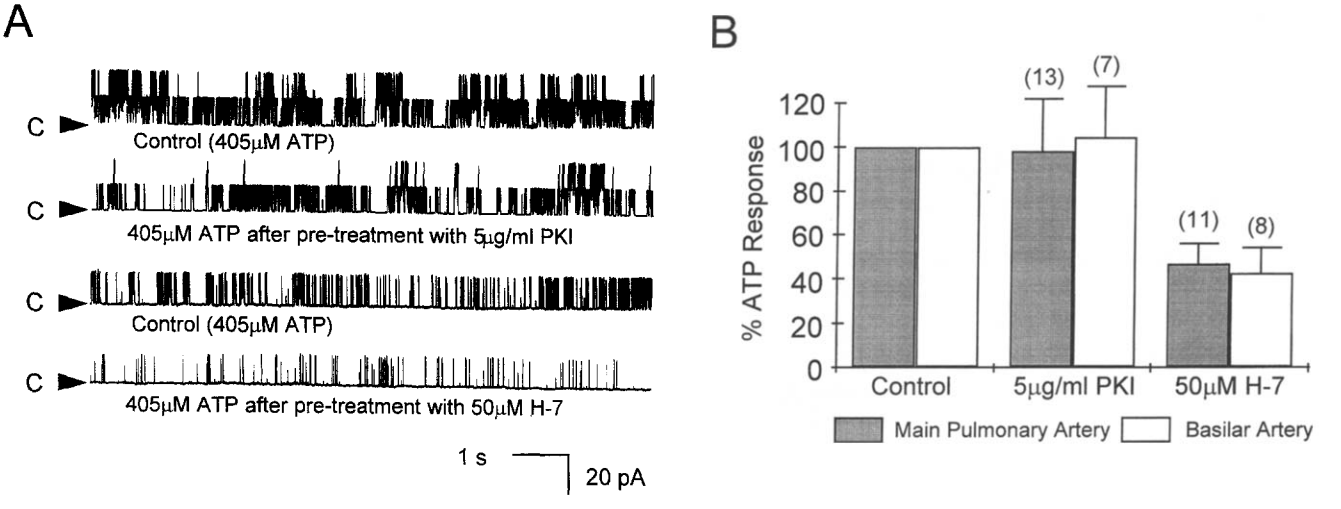

Fig. 4. Effect of pre-treatment of patches with protein kinase inhibitors PKI and H-7 on ATP-mediated K_{Ca} channel activation. (A) Single channel currents recorded from an inside-out membrane patch (excised from a basilar myocyte) held at a membrane potential of +40 mV in symmetrical 140 mM KCl. The intracellular aspect of the patch was initially superfused with a solution containing 0.9 μM Ca^{2+} and 405 μM ATP. Pre-treatment of patches with H-7 (50 μM) for between 3 and 4 min inhibited ATP-mediated K_{Ca} channel activation whereas pre-treatment of patches with PKI (5 μg/ml) was ineffective. P_{open} values are as follows (upper two records) control, 0.367; +PKI, 0.331; (lower two records) control, 0.239; +H-7, 0.054. (B) Histogram showing the effect of pre-treatment of patches excised from basilar and pulmonary myocytes with the inhibitors PKI (5 μg/ml) and H-7 (50 μM). Activation of K_{Ca} channels is expressed as a percentage of K_{Ca} channel activity in the presence of 405 μM ATP (control). Analysis of raw data revealed that H-7 but not PKI caused a significant ($P \leq 0.05$) reduction in K_{Ca} channel activity when compared to control.

Table 1
Effect of 2.6 μM $[Ca^{2+}]_i$ and 405 μM ATP on P_{open}, mean open and mean closed times for K_{Ca} channels

	0.9 μM $[Ca^{2+}]_i$	2.6 μM $[Ca^{2+}]_i$	405 μM ATP +0.9 μM $[Ca^{2+}]_i$
P_{open}	0.06 ± 0.02	0.92 ± 0.02	0.52 ± 0.07
Mean open time (ms)	12.49 ± 3.80	32.20 ± 3.71	28.96 ± 2.79
Mean closed time (ms)	127.05 ± 28.05	1.85 ± 0.24	3.75 ± 0.76
Number of patches	10	9	9

Note, data for pulmonary arterial K_{Ca} channels.

kinase A, PKI [22]. Typical results showing significant ($P \leq 0.05$) inhibition of the activating action of ATP in pulmonary and basilar myocytes are illustrated in Fig. 3. The effect of H-7 appeared poorly reversible in contrast to that of PKI: an effect which may be due to its lipophilic nature. Indeed others have found this compound to be a poorly reversible kinase inhibitor of K_{Ca} channel activation by ATP when applied to cell free patches [14]. Consistent with this observation pre-treatment of patches (Fig. 4) with H-7 for 3 to 4 min prevented subsequent activation of K_{Ca} channels by ATP applied approx. 1 min following H-7 removal, although similar pre-treatment with PKI failed to prevent activation of K_{Ca} channels by ATP. In contrast to the activation induced by ATP, neither H-7 (50 μM) nor PKI (5 μg/ml) had any significant effect upon increases in K_{Ca} channel activity induced by elevating $[Ca^{2+}]_i$ (data taken from both basilar and pulmonary arterial myocytes). The P_{open} in the presence of 2.6 μM Ca^{2+} (0.829 ± 0.03; $n = 6$) was not significantly different to that observed (0.811 ± 0.06; $n = 6$) when 2.6 μM Ca^{2+} and 50 μM H-7 were applied concomitantly. Likewise, the P_{open} in the combined presence of 2.6 μM Ca^{2+} and 5 μg/ml PKI (0.864 ± 0.04; n = 6) was not significantly different to that observed in the presence of 2.6 μM Ca^{2+} alone (0.871 ± 0.04; $n = 6$). In order to further discriminate between the activating action of ATP and Ca^{2+}, the time for K_{Ca} channel activation by the two agents was examined. In the presence of 0.9 μM $[Ca^{2+}]_i$, the onset time for activation of K_{Ca} channels by application of 405 μM ATP and 2.6 μM Ca^{2+} was similar. However, K_{Ca} channels remained significantly ($P \leq 0.01$) more active upon returning to control following the washout of ATP than following a reduction in $[Ca^{2+}]_i$ from 2.6 μM to 0.9 μM. In the presence of the phosphatase inhibitor okadaic acid (100 nM), the activating action of 405 μM ATP was enhanced by 87 ± 42% ($n = 7$) and the time required for its reversal upon washout significantly ($P \leq 0.01$) increased when compared to ATP alone (Fig. 5A,B). Taken together the results shown in Figs. 3–5 obviate the possibility of artefactual increases in K_{Ca} channel activity due to changes in $[Ca^{2+}]_i$ in the presence of ATP, H-7 and PKI and reinforce the notion that activation of K_{Ca} channels by ATP requires phosphorylation, perhaps by a kinase related to PKA. This raised the possibility that exogenously applied ATP could be converted locally to cAMP which could subsequently activate membrane associated PKA. However, the activating action of 405 μM ATP (P_{open} = 0.49 ± 0.12; $n = 3$) was unaffected (P_{open} = 0.44 ± 0.08; $n = 3$) by concomitant application of the cAMP antagonist [23–25] Rp-cAMPS (10 μM).

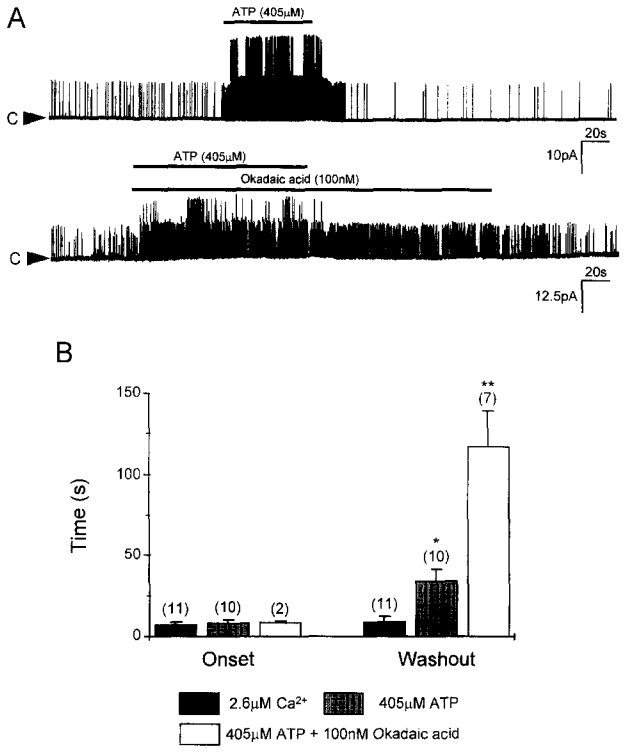

Fig. 5. (A) Single channel currents recorded from two different inside-out membrane patches (excised from pulmonary arterial myocytes) held at a membrane potential of +40 mV in symmetrical 140 mM KCl. In both cases the intracellular aspect of the patch was initially superfused with a solution containing 0.9 μM Ca^{2+}. Then either 405 μM ATP (upper record) or 0.405 μM ATP and 100 nM okadaic acid (lower record) was added. Note, the markedly reduced reversibility of the ATP effect in the presence of okadaic acid. (B) Onset and washout times for K_{Ca} channel activation by Ca^{2+} and ATP in the absence and presence of the phosphatase inhibitor okadaic acid. The histogram shows the times required for increases and subsequent decreases in K_{Ca} channel activity in 0.9 μM $[Ca^{2+}]_i$ following addition and removal of 2.6 μM Ca^{2+} or the application and washout of 405 μM ATP in the absence and presence of okadaic acid (100 nM). The onset times for activation of K_{Ca} channels under these three different conditions were similar. However, K_{Ca} channels remained significantly more active (*; $P \leq 0.01$) following washout of ATP when compared to washout of Ca^{2+}. In the presence of okadaic acid K_{Ca} channel activity was further, and significantly (**, $P \leq 0.01$), prolonged following the washout of ATP. Note, the onset and washout times given are a measure of the time required for activation, and reversal of activation, of K_{Ca} channels by Ca^{2+} or ATP in our experiments using a perfusion system which required ≈ 7 s for complete equilibration. These values are not indicative of the binding kinetics for Ca^{2+} to K_{Ca} channels or actual rates of phosphorylation. Onset and washout times were determined by measuring the time interval between the point at which solution exhange commenced and the point at which the ensuing response stabilised.

4. Discussion

K_{Ca} channels are recognised as playing a vital role in controlling the membrane potential of a variety of cell types [1]. Indeed, changes in their activity are associated with the control of myogenic tone in arterial tissue [1] and in the reduction of tone through direct activation by nitric oxide [26] and indirect activation following cAMP- [5,6] or cGMP-dependent phosphorylation [7]. However, only a few reports [12,14,27,28] exist which suggest that these channels possess the capacity for autophosphorylation, as a consequence of endogenous kinase activity, or through a kinase firmly associated with this channel at the membrane level. Our initial findings [8] revealed that K_{Ca} channels in pulmonary arterial myocytes could be activated by ATP in the absence of an intact intracellular milieu in contrast to K_{Ca} channels in other tissues (which were reported to be either inhibited [15,16] or unaffected [17] by ATP). As a consequence it was speculated that K_{Ca} channels in the pulmonary circulation possess a unique role for linking cellular metabolism to K^+ flux during hypoxia [29]. Our present findings clearly indicate that this phenomenon is likely to be of more generalised importance, since K_{Ca} channels in a range of arteries are activated by ATP. Furthermore, on the basis of our observations using H-7 and PKI, it is likely that this activation is mediated by a kinase related to protein kinase A (PKA). Using the GenBank data base comparison of the amino acid sequence of mSlo, a gene which encodes a large number of K_{Ca} channel variants [30], with sequences for the catalytic domain found in the majority of known protein kinases, including PKA, showed no sequence homology. Thus it is unlikely that K_{Ca} channels exhibit endogenous kinase activity but are more likely to be associated at the membrane level with a PKA-related kinase which lacks its regulatory subunits. Such a putative kinase is thought to regulate neuronal K_{Ca} channels [14]. Consistent with this notion the cAMP antagonist Rp-cAMPS which binds to the regulatory subunits of PKA [23–25] had no effect on ATP-induced activation of K_{Ca} channels. In further support for the existence of an associated kinase is the finding that activation of K_{Ca} channels by ATP is not observed in all membrane patches, indicating that patch excision may result in its loss. The diversity associated with K_{Ca} channels [30] could, however, result in a differential sensitivity to phosphorylation. Indeed, ATP has been reported to activate K_{Ca} channels in rabbit gastric antrum myocytes [31] and rat cortical neurones [14] by a similar process to the one described here, albeit utilising higher concentrations of ATP (> 1 mM). Kinetic analysis of K_{Ca} channels revealed that phosphorylation of K_{Ca} channels by ATP had a similar effect on mean open and closed times to $[Ca^{2+}]$: a finding consistent with our previous suggestion (based on a biophysical analysis of multichannel patch data) that phosphorylation enhances the sensitivity of K_{Ca} channels to $[Ca^{2+}]_i$ [29]. In view of the reversible activation of K_{Ca} channels by phosphorylation it is likely that changes in K_{Ca} channel activity are likely to play an important role in controlling the membrane potential of arterial smooth muscle cells under conditions where membrane levels of ATP may fluctuate; for example during hypoxic episodes such as those encountered physiologically in the lungs or pathologically during the ischaemia which follows stroke.

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