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Contractile Agonists Preferentially Activate CL^- over K^+ Currents in Arterial Myocytes

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Simultaneous patch-clamp and Ca^{2+} fluorescence measurements have revealed depolarising oscillations in the membrane potential of arterial (pulmonary) myocytes in response to adenosine 5′-triphosphate (ATP) and endothelin-1 (ET-1). These oscillations (i) are due to the preferential activation of Ca^{2+}-activated Cl^-, over K^+ currents, (ii) occur through a mechanism involving Ca^{2+} release from intracellular Ca^{2+} stores and (iii) are likely to promote constriction. These results provide a novel perspective into the relative contribution and importance of Ca^{2+}activated Cl^- and K^+ channels in controlling membrane potential of arterial smooth muscle in response to contractile agonists.

Purinergic (P_{2X} or P_{2Y}) and endothelin (ET_A or ET_B) receptor stimulation is associated with an elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a variety of smooth muscle cell types (1,2). This elevation in $[Ca^{2+}]_i$ is likely to have several effects which may play an important role in promoting constriction such as activation of Ca^{2+}-activated depolarising current. Such an effect would tend to enhance Ca^{2+} entry through voltage-dependent entry pathways and thereby promote constriction. Pulmonary arterial smooth muscle, like many types of arterial smooth muscle, contains both Ca^{2+}-activated K^+ (K_{Ca}) (3) and Cl^- (Cl_{Ca}) (4) channels. Thus elevation of $[Ca^{2+}]_i$ is likely to elicit activation of both channel types. Since the membrane potential of pulmonary arterial smooth muscle is ~ -50mV (5) activation of Cl_{Ca} channels is likely to induce depolarisation while activation of K_{Ca} channels will cause hyperpolarisation. Paradoxically, this latter effect would tend to prevent constriction. In the present study we have resolved this problem by examining the effect of ATP and ET-1 on $[Ca^{2+}]_i$ and the resulting changes in the Ca^{2+}-activated Cl^- and K^+ conductance of pulmonary arterial smooth muscle.

MATERIALS AND METHODS

Male albino Wistar rats (200-250g body weight) were killed by an overdose of i.p. Euthatol (pentobarbitone sodium B. P., Rhone Merieux, Ireland). The lungs were excised and the small (diameter 200-400μm) pulmonary arterial vessels removed. Arterial smooth muscle cells were isolated using methods similar to those previously described by us (6). Membrane current was recorded from myocytes using the perforated-patch configuration (7) of the patch-clamp recording technique (8). This configuration was used in both current- and voltage-clamp modes. To record membrane current cells were voltage-clamped at -50mV unless otherwise stated. Recording pipettes (3-7MΩ) were pulled from borosilicate glass capillaries (Clark Electromedical, Pangbourne, UK) using a vertical puller (Narishige Ltd, Tokyo, Japan). Electrophysiological measurements were made with 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 2 kHz for subsequent off-line analysis. Prior to analysis currents were digitised at 100 Hz using a

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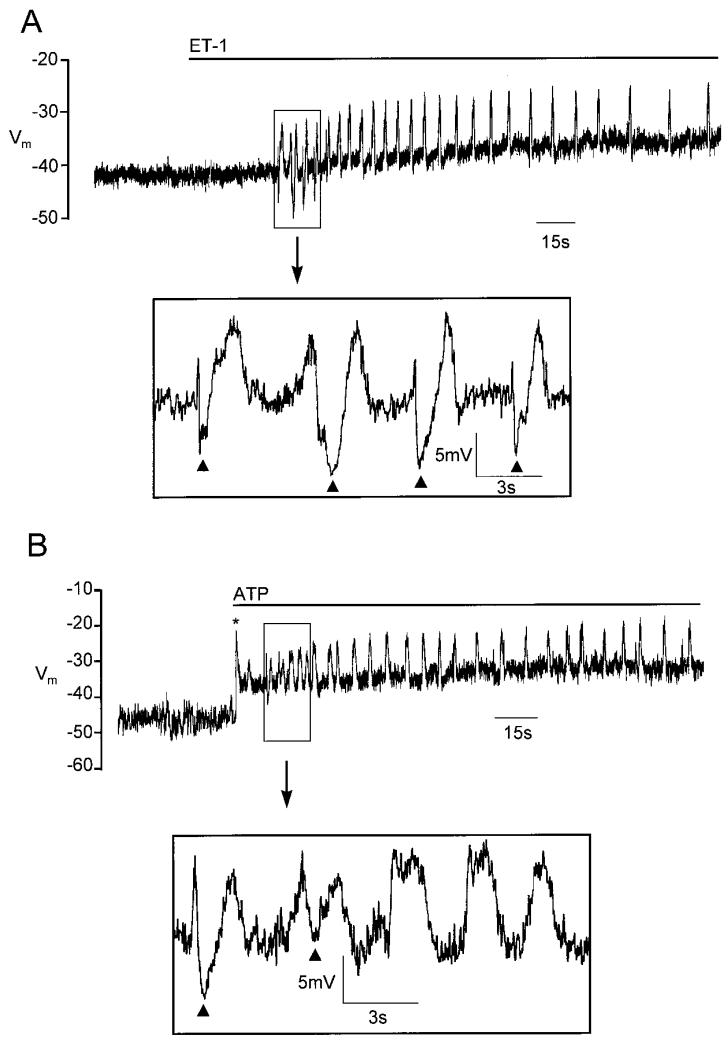
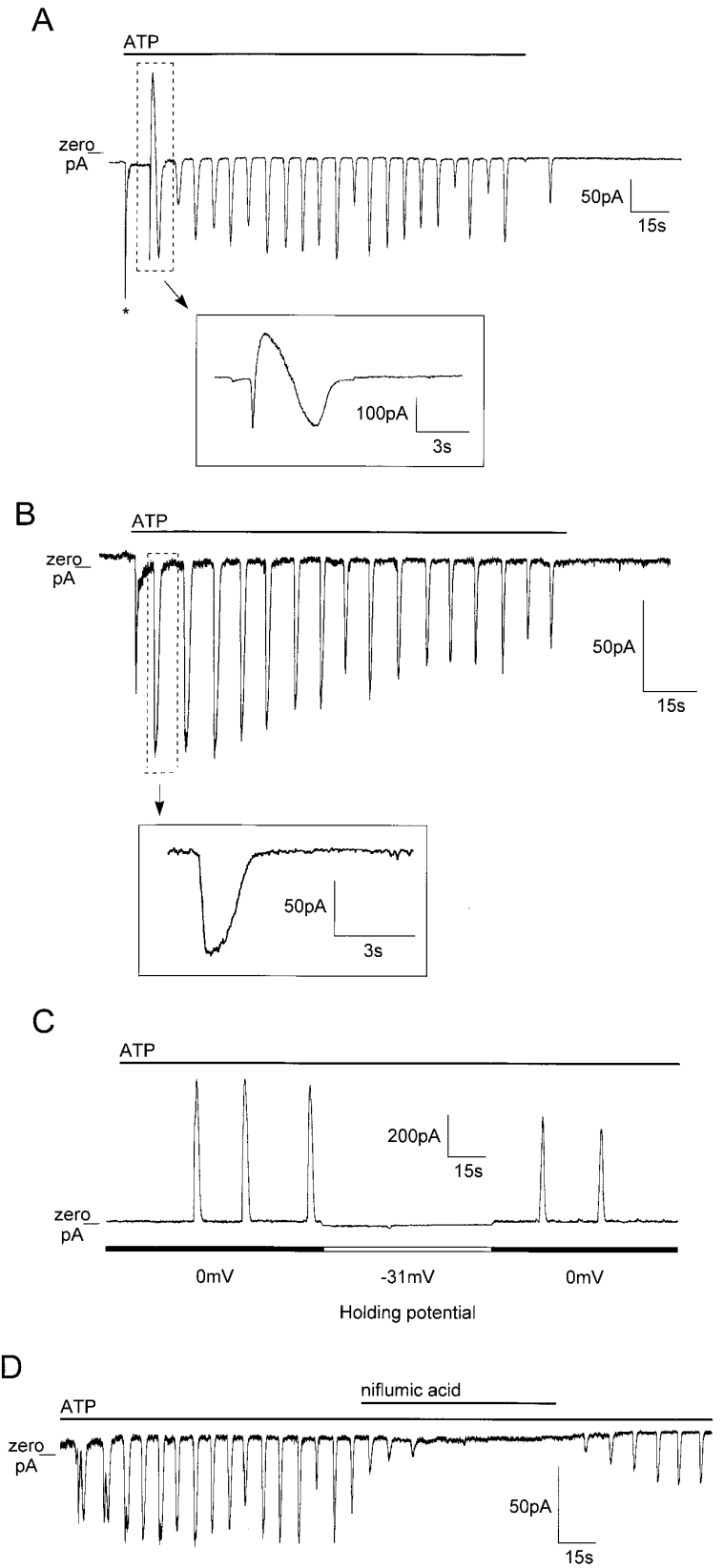


FIG. 1. Effect of extracellular application of ET-1 and ATP on membrane potential of pulmonary arterial myocytes. Cells were maintained in the perforated patch configuration (solution A in the bath and solution B in the pipette) under current-clamp. (A) Prior to the application of ET-1 (10nM) the resting membrane potential of this cell was ~ -43mV. Following application of the peptide a gradual depolarisation was observed. Superimposed upon this depolarisation were oscillations of membrane potential shown on a faster time base in the inset. Note the presence of M-shaped oscillations indicated by filled triangles. (B) Current-clamp recording of cell membrane potential. Prior to application of ATP (10μM) the resting membrane potential of this cell was ~ -46mV. Following application of ATP there was a rapid transient depolarisation (*) probably mediated through opening of a cation channel (15) followed by oscillations in the membrane potential (shown on a faster time base in the inset with W-shaped oscillations also indicated by filled triangles).

DigiData 1200 interface (Axon Instruments). Data were analysed on a 486 microcomputer using pClamp software (version 6.1; Axon Instruments).

In both voltage- and current-clamp experiments cells were bathed in solution A which consisted of (in mM): 150 NaCl, 5.4 KCl, 1.2 $CaCl_2$ 1.2 $MgCl_2$, 10 glucose, 5 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES); pH 7.4 with NaOH. On occasion 1.2mM $CaCl_2$ was replaced with 2.0mM $CaCl_2$ or omitted altogether. Pipettes contained solution B which contained (in mM, unless otherwise stated): 125 KCl, 4 $MgCl_2$, 10 HEPES, 0.02 ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′,-tetraacetic acid (EGTA) and 240μg/ml amphotericin B; pH 7.3 with KOH.


A
ATP
zero pA
50pA
15s
*
100pA
3s
B
ATP
zero pA
50pA
15s
50pA
3s
C
ATP
200pA
15s
zero pA
0mV
-31mV
0mV
Holding potential
D
niflumic acid
ATP
zero pA
50pA
15s

In anion substitution experiments cells were bathed in solution C containing (in mM): 135 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 5 HEPES and 2.5 EGTA; pH 7.4 with NaOH, while the pipettes contained solution D (in mM): 40 KCl, 1 $MgCl_2$, 10 HEPES, 85 KOH and 85 aspartic acid; pH 7.3 with KOH.

In order to estimate $[Ca^{2+}]_i$ pulmonary arterial myocytes were pre-incubated for ~10min with 3-5μM {1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid pentaacetoxymethylester} (Indo-1/AM). The $[Ca^{2+}]_i$ was estimated from the Indo-1 fluorescence using single-wavelength excitation (460nM) and dual emission (405 and 485nM) (9) detected with a spectrophotometer system (Nikon, Kingston, UK). Emission at 405nM (F_{405}) and 485nM (F_{485}) was sampled at 20 Hz after background subtraction and the F_{405}/F_{485} ratio calculated using pClamp software (version 6.1; Axon Instruments). An estimate of the $[Ca^{2+}]_i$ was made from this ratio according to equation 1 (9) where R is the flourescence ratio and R_{min} and R_{max} are the minimum and maximum fluorescence ratios, K_d (250nM) is the dissociation constant for Indo-1 (9) and β the F_{485} signal ratio in the absence and presence of a saturating concentration of Ca^{2+}(see below).

$$[Ca^{2+}]_i = K_d\ \beta\ (R-R_{min})\ /\ (R_{max}-R) \qquad \text{equation 1}$$

R_{min} was determined from cells bathed in solution A and dialysed with a solution containing (in mM): 85 KCl, 10 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA; titrated with ~42mM KOH), 4 Mg-ATP, 10 HEPES and 40μM Indo-1 (pentasodium salt); pH 7.3 with KOH. R_{max} was determined following exposure of cells to solution A containing 10μM ionomycin and 2mM Ca^{2+}. Experimentally determined values for R_{min}, R_{max} and β were 0.12±0.01, 2.36±0.39 and 4.8±1.0 (n=4), respectively.

ATP (disodium salt), ATP (magnesium salt), amphotericin B, BAPTA, caffeine, collagenase (type VIII), dithiothreitol, elastase (type II-A), EGTA, ET-1, HEPES, iberiotoxin, niflumic acid, papain (papaya latex) and protease (type I) were all purchased from Sigma (Poole, UK). Indo 1/AM, Indo 1 (pentasodium salt) and ionomycin were purchased from Calbiochem (Nottingham, UK). Experiments were performed at 20-24°C and solutions exchanged within 5s by superfusing the bath using gravity feed.

RESULTS

Throughout this study ET-1 and ATP were used at concentrations of 10-16nM and 10μM, respectively unless otherwise stated. These concentrations were chosen since they are known to induce constriction of pulmonary arterial smooth muscle (10,11). Both ET-1 and ATP when applied to cells maintained in the perforated-patch configuration under current-clamp conditions produced depolarising oscillations in membrane potential which on occasion showed a ''M-shaped'' profile (Fig. 1). Under voltage-clamp (solution A in the bath and solution B in the pipette) inward current oscillations were observed in the presence of both ATP and ET-1. ATP in 37 out of 56 cells and ET-1 in 2 out of 11 cells induced ''W-shaped'' current oscillations; mirroring the M-shaped oscillations seen under current-clamp. The occurrence of these W-shaped currents evoked in response to ATP was prevented in 7 out of 8 cells by application of iberiotoxin (20nM), a selective blocker of K_{Ca} channels (12), indicating this current profile was due to activation of these channels. Examples of these effects initiated by ATP, are shown in Fig. 2A,B. To further characterise the ionic basis of ET-1 and ATP induced oscillations of membrane current anion substitution experiments were performed. Under a Cl^- gradient of 143mM $[Cl^-]_o$ (solution C, bath) : 42mM $[Cl^-]_i$ (solution D, pipette), E_{Cl} = −31mV, and a

FIG. 2. (A) Current records obtained under voltage-clamp (solution A in the bath and solution B in the pipette) at a holding potential of −50mV. Application of ATP (10μM) activated a fast transient current (*) consistent with the rapid depolarisation seen in current clamp (see Fig. 1B) followed by sustained periodic oscillations of inward current. On occasion W-shaped currents were observed shown on a faster time base in the inset. (B) Under similar conditions to those described in (A), ATP (10μM) in the presence of iberiotoxin (20nM) did not evoke W-shaped currents (see inset). (C) Current record obtained from a cell voltage-clamped at 0mV under a Cl^- gradient of 143mM $[Cl]_o$ (solution C, bath) : 42mM $[Cl]_i$ (solution D, pipette). Under these conditions ATP (10μM) activated an oscillatory outward current which was abolished at −31mV. (D) Current record showing the effect of niflumic acid (50μM) applied extracellularly to a cell voltage-clamped at a potential of −50mV (solution A in the bath and solution B in the pipette).

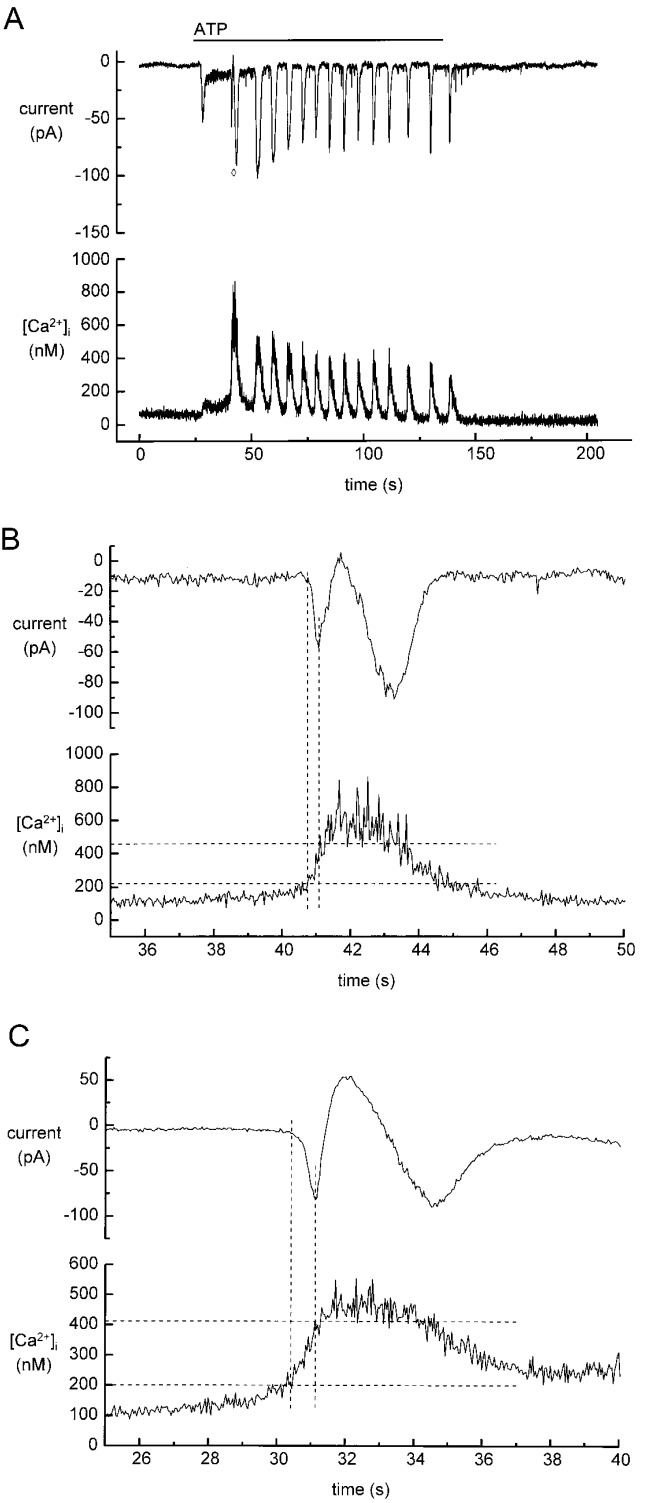


FIG. 3. Results from simultaneous patch-clamp and Indo-1 fluorescence studies showing the effect of ATP and ET-1 on membrane current and $[Ca^{2+}]_i$. Cells were maintained in the perforated-patch configuration (solution A in

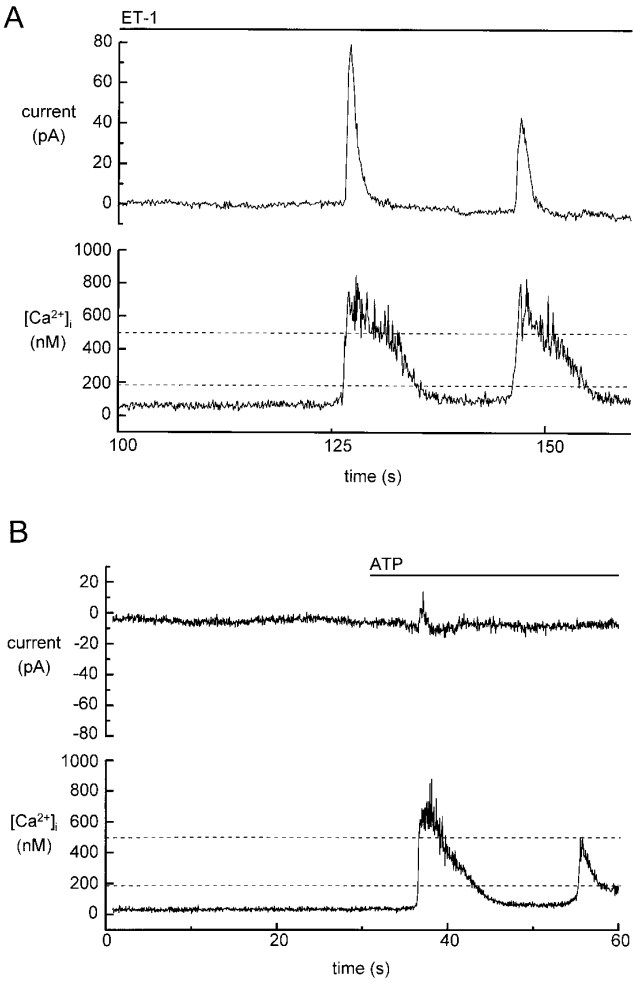


FIG. 4. Effect of ATP and ET-1 on membrane current and $[Ca^{2+}]_i$ in the presence of niflumic acid. Cells were maintained in the perforated-patch configuration (solution A in the bath and solution B in the pipette) at a holding potential of −50mV. In the presence of niflumic acid (50μM) application of (A) ET-1 (10nM) or (B) ATP (10μM) only activated an outward current even though the $[Ca^{2+}]_i$ increased to a level sufficient to activate ICl_{Ca}. The hatched lines on the lower panels of both (A) and (B) show the mean levels of $[Ca^{2+}]_i$ required to activate IK_{Ca} (upper line) and ICl_{Ca} (lower line).

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the bath and solution B in the pipette) at a holding potential of −50mV. (A) Application of ATP (10μM) caused oscillations in membrane current (upper panel) and corresponding oscillations of $[Ca^{2+}]_i$ (lower panel). Note, in this cell ATP activated a W-shaped current (◇ ; upper panel). (B) Records of the W-shaped current response and associated changes in $[Ca^{2+}]_i$ seen upon application of ATP (10μM) in (A). The level of $[Ca^{2+}]_i$ required to initiate an inward current was 210nM with 460nM $[Ca^{2+}]_i$ resulting in an apparent reduction and eventual reversal in the direction of the current (hatched lines). (C) Expanded trace showing a W-shaped current response and the associated changes in $[Ca^{2+}]_i$ in the presence of ET-1 (10nM). Similar levels of $[Ca^{2+}]_i$ (hatched lines corresponding to 200nM and 410nM, respectively) were required to initiate an inward current and induce an apparent reduction and eventual reversal in the current direction in the presence of ET-1.

holding potential of 0mV both ATP (n=5) and ET-1 (n=5) induced outward current oscillations. Upon changing the holding potential to −31mV the current oscillations were virtually abolished: an observation which indicates that these current oscillations are due to the movement of Cl^- ions. In support of this notion niflumic acid (50μM), a Cl^- channel blocker (13), reversibly inhibited inward current oscillations induced by ATP (n=10) and ET-1 (n=5). Example data using ATP is provided in Fig. 2C,D.

To verify whether oscillations in the $[Ca^{2+}]_i$ were involved in ATP and ET-1 induced current oscillations the effect of caffeine, known to deplete intracellular Ca^{2+} stores, was examined. Application of 10mM caffeine in the absence of extracellular Ca^{2+} prevented both ATP (n=6) and ET-1 (n=7) induced current oscillations (data not shown). On the basis of these, and the preceding results, it appears that both ET-1 and ATP activate a Ca^{2+}-activated Cl^- (ICl_{Ca}) and K^+ (IK_{Ca}) current as a consequence of Ca^{2+} release from intracellular stores. Consistent with this notion fluorescence measurements following loading of cells with 3-5μM Indo-1/AM revealed that both ATP and ET-1 produce oscillations of $[Ca^{2+}]_i$ which results in initiation of the oscillatory membrane currents described above (Fig. 3A). Rigorous analysis of our data revealed that the threshold for initiation of inward current oscillations due to activation of ICl_{Ca} is much lower than for activation of IK_{Ca} (as determined by an apparent, and not absolute, reversal of the current direction). Indeed, following calibration of our system (see Methods) it appears that an $[Ca^{2+}]_i$ of 184±7nM (n=25) and 498±29nM (n=17) is required for apparent activation of ICl_{Ca} and IK_{Ca}, respectively (Fig. 3B). Consistent with this finding in the presence of 50μM niflumic acid only outward current oscillations were observed at an $[Ca^{2+}]_i$ over 480nM in the presence of either ET-1 or ATP (Fig. 4A,B).

DISCUSSION

These results show for the first time simultaneous oscillations of $[Ca^{2+}]_i$ and membrane current in isolated arterial smooth muscle cells. They also clearly demonstrate the ability of ET-1 and ATP to induce oscillations of $[Ca^{2+}]_i$: an effect which leads to activation of ICl_{Ca} and IK_{Ca}. It appears that these agonists, at concentrations which induce constriction of arterial smooth muscle, mediate elevation of $[Ca^{2+}]_i$ to a level which preferentially activates ICl_{Ca} over IK_{Ca}. The concentrations required for apparent activation of IK_{Ca} (∼500nM) and ICl_{Ca} (∼185nM) are consistent with our previous studies examining the Ca^{2+} sensitivity of K_{Ca} channels (5) and ICl_{Ca} (6). The depolarising action of ICl_{Ca} is likely to promote constriction by facilitation of Ca^{2+} entry through voltage-dependent pathways. Activation of IK_{Ca} at high concentrations of $[Ca^{2+}]_i$ may represent a fine tuning mechanism that serves to repolarise the membrane thereby protecting cells from voltage-dependent Ca^{2+} overload.

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