Evidence for myosin light chain kinase mediating noradrenaline-evoked cation current in rabbit portal vein myocytes

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1. The role of myosin light chain kinase (MLCK) in the activation of the noradrenaline-evoked non-selective cation current (I_{cat}) was examined with the whole-cell recording technique in single rabbit portal vein smooth muscle cells.

2. Intracellular dialysis with 5 μM MLCK$_{(11-19)}$amide, a substrate-specific peptide inhibitor of MLCK, markedly reduced the amplitude and rate of activation of noradrenaline-evoked I_{cat}. A similar result was obtained when the cells were dialysed with 10 μM AV25, which also inhibits MLCK by an action at the auto-inhibitory domain of MLCK.

3. Inhibitors of binding of ATP to MLCK, wortmannin and synthetic naphthalenesulphonyl derivatives (ML-7 and ML-9), at micromolar concentrations, also reduced the amplitude of noradrenaline-evoked I_{cat}.

4. ML-7 and ML-9 (both at 5 μM) reduced the amplitude of I_{cat} induced by both guanosine 5′-O-(3-thiotriphosphate) (GTPγS) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG).

5. MLCK$_{(11-19)}$amide, AV25 and ML-9 did not inhibit the noradrenaline-evoked Ca^{2+}-activated potassium current at a holding potential of 0 mV. In addition, MLCK$_{(11-19)}$amide and AV25 did not reduce the non-selective cation current induced by ATP in rabbit ear artery cells.

6. Intracellular dialysis with 2 μM Ca^{2+} and 9 μM calmodulin activated I_{cat}, which developed over a period of about 5 min.

7. Intracellular dialysis with the non-hydrolysable analogue of ATP, 5′-adenylylimido-diphosphate (AMP-PNP), reduced the amplitude and rate of activation of noradrenaline-evoked I_{cat}.

8. The results indicate that MLCK mediates noradrenaline-activated I_{cat} in rabbit portal vein smooth muscle cells.

In rabbit portal vein smooth muscle cells stimulation of α_1-adrenoceptors by noradrenaline evokes calcium-activated chloride and potassium currents ($I_{Cl(Ca)}$ and $I_{K(Ca)}$) and a non-selective cation current (I_{cat}) which does not appear to be mediated by an increase in intracellular calcium concentration (Byrne & Large, 1988; Wang & Large, 1991). It has been proposed that I_{cat} may contribute to the noradrenaline-induced depolarization and may also provide a direct influx of Ca^{2+} ions in vascular smooth muscle cells (Byrne & Large, 1988; Wang & Large, 1991). There is convincing evidence which suggests that the pathway that links the α_1-adrenoceptor to the Ca^{2+}-activated conductances involves G-protein activation with inositol trisphosphate (IP$_3$) production and subsequent release of Ca^{2+} from an internal store (see Large & Wang, 1996). However, considerably less is known about the transduction mechanism which mediates I_{cat}. Previously we provided

evidence to suggest that noradrenaline activates I_{cat} via a G-protein coupled to phospholipase C (PLC) and that the resulting 1,2-diacyl-*sn*-glycerol (DAG), produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), plays a pivotal role in the opening of the cation channels (Helliwell & Large, 1997). However, it was found that phorbol esters, which activate protein kinase C (PKC), did not activate I_{cat}. Moreover chelerythrine, an inhibitor of PKC, did not inhibit I_{cat} evoked by noradrenaline or the DAG analogue 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). Therefore it was concluded that DAG activates I_{cat} by a PKC-independent mechanism (Helliwell & Large, 1997). This finding provokes the question: how does DAG activate I_{cat}? It is possible that DAG may open the channels directly or may involve another mediator.

In the present work we have explored the possibility that myosin light chain kinase (MLCK) may be involved in the

activation of I_{cat}. MLCK is a Ca^{2+}–calmodulin-dependent kinase which phosphorylates the regulatory light chain of smooth muscle myosin to produce smooth muscle contraction. Although it is often stated that myosin is the only known substrate for MLCK, it has been shown that agents which affect MLCK activity modulate ionic currents in some cell types. In bullfrog sympathetic neurons it has been shown that inhibitors of MLCK activity reduced the amplitude of the M-current but not the A-type or delayed rectifier potassium currents (Akasu et al. 1993). In guinea-pig gastric myocytes it was shown that inhibitors of MLCK reduced the amplitude of the carbachol-activated non-selective cation current (Kim et al. 1997). In this latter study, the MLCK inhibitors had little effect on the same cationic current induced by guanosine 5′-O-(3-thiotriphosphate) (GTPγS) and it was concluded that the target of MLCK is the muscarinic receptor or a receptor-related protein. Therefore the above data suggest that MLCK may modulate ionic conductances.

In the present experiments we have examined the effects on noradrenaline-evoked I_{cat} of substances that inhibit MLCK activity by three distinct mechanisms and the data suggest a central role for MLCK in the activation of I_{cat} in rabbit portal vein smooth muscle cells.

METHODS

Cell isolation

New Zealand White male rabbits (2–2·5 kg) were killed by intra-venous overdose of sodium pentobarbitone. The portal vein was removed and dissected free of connective tissue and fat in normal physiological salt solution (PSS) which contained (mM): NaCl, 126; KCl, 6; $CaCl_2$, 1·5; $MgCl_2$, 1·2; glucose, 10; and Hepes, 11; the pH was adjusted to 7·2 with 10 M NaOH. The tissue was then cut into strips and incubated in low-Ca^{2+} PSS (PSS with no added $CaCl_2$) for 5 min at 37 °C. After preincubation the solution was replaced with low-Ca^{2+} PSS containing 0·2–0·3 mg ml^{-1} protease Type XIV (Sigma) for 5 min, after which the tissue was washed with low-Ca^{2+} PSS. The low-Ca^{2+} PSS solution was then replaced with 100 μM Ca^{2+} PSS (PSS containing 100 μM rather than 1·5 mM $CaCl_2$) containing 0·5–1 mg ml^{-1} collagenase Type IV (Sigma) and the tissue was then incubated for a further 10 min before a final wash in 100 μM Ca^{2+} PSS. Cells were released by trituration of the tissue through a wide-bore Pasteur pipette in 100 μM Ca^{2+} PSS. The solution containing dissociated cells was then centrifuged at 100 g for 2 min to form a loose pellet which was resuspended in 0·75 mM Ca^{2+} PSS. In some experiments single smooth muscle cells were prepared from the rabbit ear artery. The ear artery was dissected free of connective tissue before being cut into strips. The strips were placed in 100 μM Ca^{2+} PSS containing 5 mg ml^{-1} bovine serum albumin, 1 mg ml^{-1} papain (Sigma), 0·5–1·5 mg ml^{-1} collagenase Type IV (Sigma) and 2·5 mM DL-dithiothreitol, and stored overnight at 4 °C. The following day the strips were incubated for 30–50 min at 37 °C and then washed in 100 μM Ca^{2+} PSS. The cells were then released into solution by mechanical agitation of the strips of tissue using a wide-bore Pasteur pipette. The cell suspension was then centrifuged (100 g) to form a loose pellet which was resuspended in 0·75 mM Ca^{2+} PSS. Cells were stored on coverslips at 4 °C and were used within 8 h.

Electrophysiology

Whole-cell membrane currents were measured at room temperature (20–25 °C) with a List L/M EPC-7 patch clamp amplifier. Patch pipettes were made from borosilicate glass and when filled with normal pipette solution had resistances of about 4 MΩ.

Solutions and drugs

The standard extracellular K$^+$-free PSS used in most experiments contained (mM): NaCl, 126; $CaCl_2$, 1·5; glucose, 10; and Hepes, 11; the pH was adjusted to 7·2 with NaOH. In the present experiments noradrenaline-evoked I_{cat} was recorded in 50 μM external Ca^{2+} (Ca^{2+}_o) because under these conditions the amplitude of I_{cat} is increased by about eightfold (Helliwell & Large, 1996) thus facilitating analysis of the currents. The increase in amplitude of I_{cat} in reduced $[Ca^{2+}]_o$ is thought to be due to relief from channel blockade by Ca^{2+} ions without change in the current–voltage characteristics (see Helliwell & Large, 1996). Therefore the extracellular PSS $[Ca^{2+}]$ was changed from 1·5 mM to 50 μM about 1 min before noradrenaline was applied. In a few experiments we demonstrated that MLCK inhibitors reduced noradrenaline-evoked I_{cat} when the bathing solution contained 1·5 mM Ca^{2+}. The standard pipette solution contained (mM): CsCl, 18; caesium aspartate, 108; $MgCl_2$, 1·2; glucose, 10; Hepes, 11; BAPTA, 10; and $CaCl_2$, 1 (free $[Ca^{2+}]_i$ predicted by EQCAL software (Biosoft, Ferguson, MO, USA) was approximately 14 nM); the pH was adjusted to 7·2 with Trizma base. In some experiments in which noradrenaline-evoked Ca^{2+}-activated potassium currents ($I_{K(Ca)}$) were measured the external solution contained (mM): NaCl, 131; KCl, 6; $MgCl_2$, 1·2; $CaCl_2$, 0·2; glucose, 11; and Hepes, 10. The pipette solution contained (mM): NaCl, 5; KCl, 126; glucose, 11; Hepes, 10; BAPTA, 0·1; and $MgCl_2$, 1·2. The pH of these solutions was also adjusted to 7·2 with NaOH.

In experiments in which the influence of high intracellular $[Ca^{2+}]$ (i.e. 400 nM and 2 μM Ca^{2+}_i) was studied, 400 nM and 2 μM Ca^{2+}_i were achieved by adding, respectively, 8 and 9 mM $CaCl_2$, as estimated with EQCAL software, to the standard pipette solution. GTPγS, OAG (both from Sigma) and calmodulin (from bovine brain; Sigma) were made up as 10 mM stocks in DMSO and diluted to the final concentrations with pipette solution. 5′-Adenylylimido-diphosphate (AMP-PNP) was purchased from Sigma and was made up as a 10 mM stock in distilled water (stored as frozen aliquots) and diluted to final concentrations with the pipette solution on the day of experimentation. 1-(5-Chloronaphthalene-1-sulphonyl) homo-piperazine, HCl (ML-9), 1-(5-iodonaphthalene-1-sulphonyl) homo-piperazine, HCl (ML-7) (both from Calbiochem) and wortmannin (Sigma) were also made up as 10 mM stocks in DMSO (stored as frozen aliquots) and diluted to final concentrations on the day of experimentation. The concentration of DMSO (0·1 %) had no effect on the amplitude of noradrenaline-evoked I_{cat}.

Synthetic peptide inhibitors of myosin light chain kinase

The myosin light chain kinase inhibitor peptide MLCK$_{(11-19)}$amide was purchased from Alexis Corporation Ltd (Nottingham, UK). This compound is a substrate-specific inhibitor of MLCK and corresponds to the region around the phosphorylation site (i.e. serine^{19}) of myosin light chain (MLC) from chicken gizzard with the sequence: lysine^{11}-lysine^{12}-arginine^{13}-**alanine^{14}-alanine^{15}**-arginine^{16}-alanine^{17}-threonine^{18}-serine^{19}. Two substitutions (in bold) were made where proline and glutamine at positions 14 and 15, respectively, were replaced with alanine (Kemp et al. 1983). This was done in order to improve the inhibitor potency of the synthetic peptide (see Kemp et al. 1983; Pearson et al. 1986). Similarly, AV25 (which was a gift from Dr M. P. Walsh, University of Calgary, Canada) is a synthetic peptide which inhibits the

removal of the auto-inhibitory particle of MLCK. AV25 is patterned after the auto-inhibitory domain of myosin light chain kinase (Weber *et al.* 1999). AV25 denotes the first and the last amino acid residues, and the length of the peptide. AV25 corresponds to amino acid residues 783–807 of chicken gizzard (i.e. AKKL*A*KDRMKKYMARRK*L*QK*A*GHAV), with three substitutions (in bold italics). Tryptophan at position 800 was replaced with leucine to remove any influence from interaction of the peptide with calmodulin. Serine at position 787 and threonine at position 803 were replaced by alanine to avoid peptide phosphorylation by other kinases such as Ca^{2+}–calmodulin kinase II (Weber *et al.* 1999).

$MLCK_{(11-19)}$amide and AV25 were made up as 1 mM stock solutions in distilled water (stored as frozen aliquots) and diluted to the final concentrations with the pipette solution on the day of experimentation. After the whole-cell configuration was achieved, 5–10 min was allowed for sufficient dialysis of the peptides and AMP-PNP before any recordings were made. Control currents were also obtained using a pipette solution which contained the equivalent amount of distilled water.

The values in the text are means ± S.E.M. (*n*, number of cells examined) and the test for statistical significance was Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Effect of specific peptide inhibitors of MLCK on noradrenaline-evoked I_{cat}

Figure 1*A* shows the non-selective cation current produced by bath-applied noradrenaline (100 μM) in an external solution containing 50 μM Ca^{2+}. In this cell the peak amplitude of I_{cat} was about 175 pA; the current then decayed spontaneously in the continued presence of noradrenaline due to an unknown inactivation/desensitization process. Figure 1*B* illustrates the response to 100 μM noradrenaline in a cell where the patch pipette contained the MLCK substrate-specific inhibitor $MLCK_{(11-19)}$amide (5 μM). It can be clearly seen that the current was markedly reduced compared with the control response shown in Fig. 1*A*. The mean data from three populations of cells are shown in Fig. 1*C*, where it can be seen that intracellular dialysis with 5 μM $MLCK_{(11-19)}$amide reduced the amplitude of I_{cat} by about 80%.

Another feature of the inhibitory effect of $MLCK_{(11-19)}$amide was that the rate of activation of I_{cat}, as estimated from 10–90% of the rise time, was also markedly decreased from
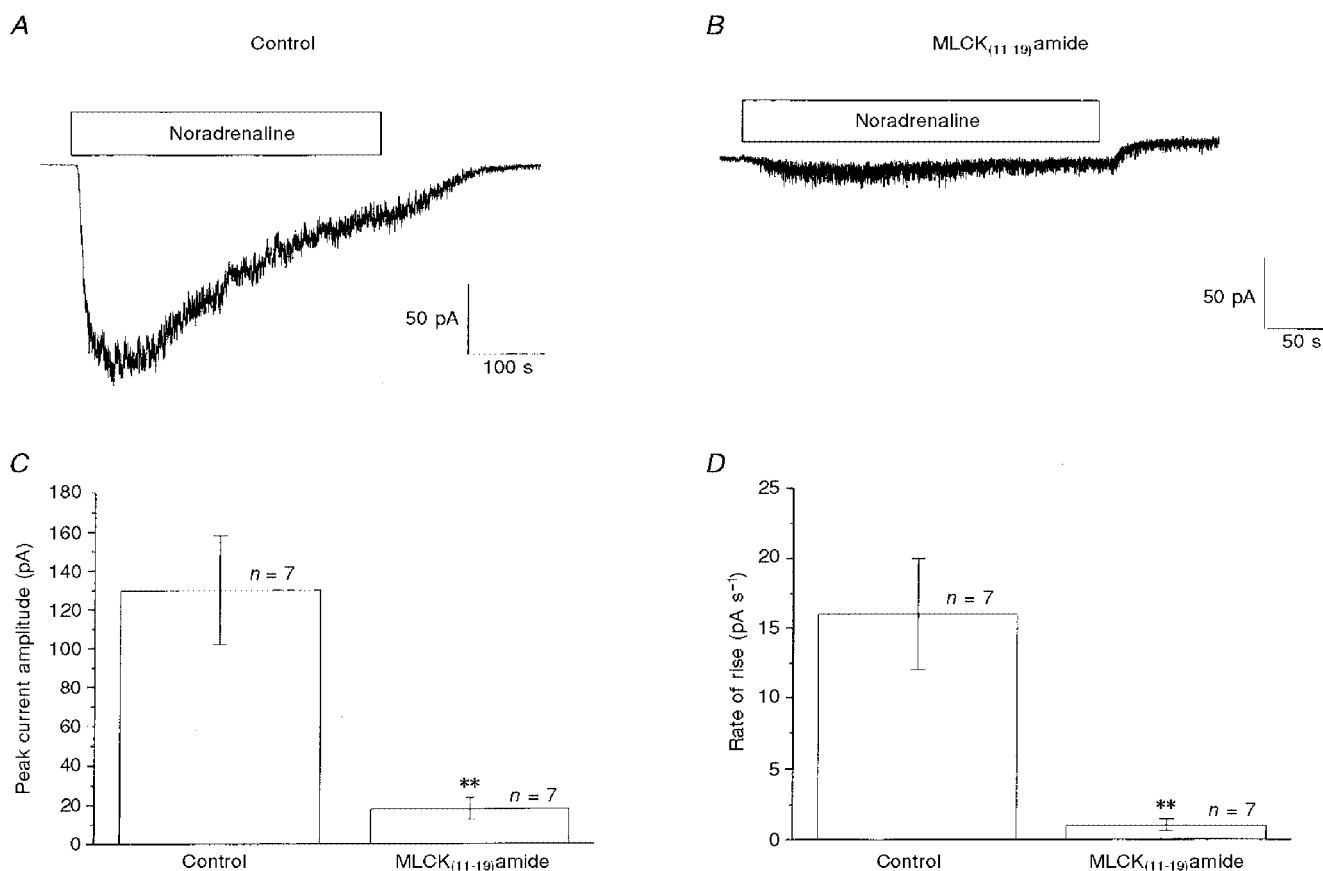

Figure 1. The effect of $MLCK_{(11-19)}$amide on noradrenaline-activated I_{cat}

A, an example of a control I_{cat} activated by bath-applied 100 μM noradrenaline, applied during the period denoted by the horizontal bar. *B*, I_{cat} activated by bath-applied noradrenaline 5 min after achieving whole-cell configuration with a pipette solution containing 5 μM $MLCK_{(11-19)}$amide. *C* and *D* show quantitatively the effects of $MLCK_{(11-19)}$amide on the peak amplitude (pA; *C*) and the activation rate (pA s^{-1}; *D*) of I_{cat}. In this and subsequent figures error bars represent the S.E.M. and the numbers (*n*) above the columns indicate the number of cells examined. Records are from different cells. **$P < 0.01$.

a control value of 16 pA s^{-1} to 1 pA s^{-1} (Fig. 1*D*). It should be noted that in these experiments control currents were obtained using a pipette solution which contained the equivalent amount of distilled water required to dissolve the peptides MLCK$_{(11-19)}$amide and AV25.

In another series of experiments we investigated the effects of intracellular dialysis with AV25, which also inhibits MLCK activity but by binding to a different site on the MLCK molecule (see Methods). Figure 2 shows noradrenaline-induced I_{cat} under control conditions (Fig. 2*A*) and in another cell after 5 min dialysis with 10 μM AV25 in the patch pipette solution, where the amplitude of I_{cat} was greatly decreased (Fig. 2*B*). In three populations of cells intracellular dialysis with 10 μM AV25 reduced the amplitude of I_{cat} by about 72% (Fig. 2*C*). Also, as with MLCK$_{(11-19)}$amide, the rate of activation of I_{cat} was markedly depressed by AV25 (Fig. 2*D*).

Effects of agents that inhibit binding of ATP to MLCK on noradrenaline-evoked I_{cat}

Experiments were then carried out to ascertain whether agents that inhibit the binding of ATP to MLCK, and hence prevent its action, affect I_{cat}. First, we investigated the

effects of wortmannin, which is also known to inhibit phosphoinositide 3-kinase (PI 3-kinase) at nanomolar concentrations (Shepherd *et al.* 1996). Figure 3*A* illustrates a control current to noradrenaline and Fig. 3*B* shows that the current activated by noradrenaline was not reduced in the presence of 50 nM wortmannin. In three populations of cells the control amplitude of noradrenaline-evoked I_{cat} was 309 ± 70 pA ($n = 7$) compared with 310 ± 56 pA ($n = 7$) in the presence of 50 nM wortmannin. These results indicate that PI 3-kinase is not involved in the activation of I_{cat} and therefore we investigated whether micromolar concentrations of wortmannin, at which it inhibits MLCK, affect I_{cat}.

A higher concentration of wortmannin (20 μM), when applied on its own, activated in some cells a 'noisy' inward current similar to noradrenaline-evoked I_{cat} (Fig. 3*C*). In five cells the mean amplitude of the wortmannin-induced current was 60 ± 17 pA, compared with a value of 158 ± 25 pA ($n = 5$) produced by noradrenaline (100 μM) in the same population of cells. The rate of activation of the wortmannin-evoked current (5 ± 1 pA s^{-1}) was slower than that of the current produced by noradrenaline (18 ± 1 pA s^{-1}). Addition of noradrenaline in the presence of
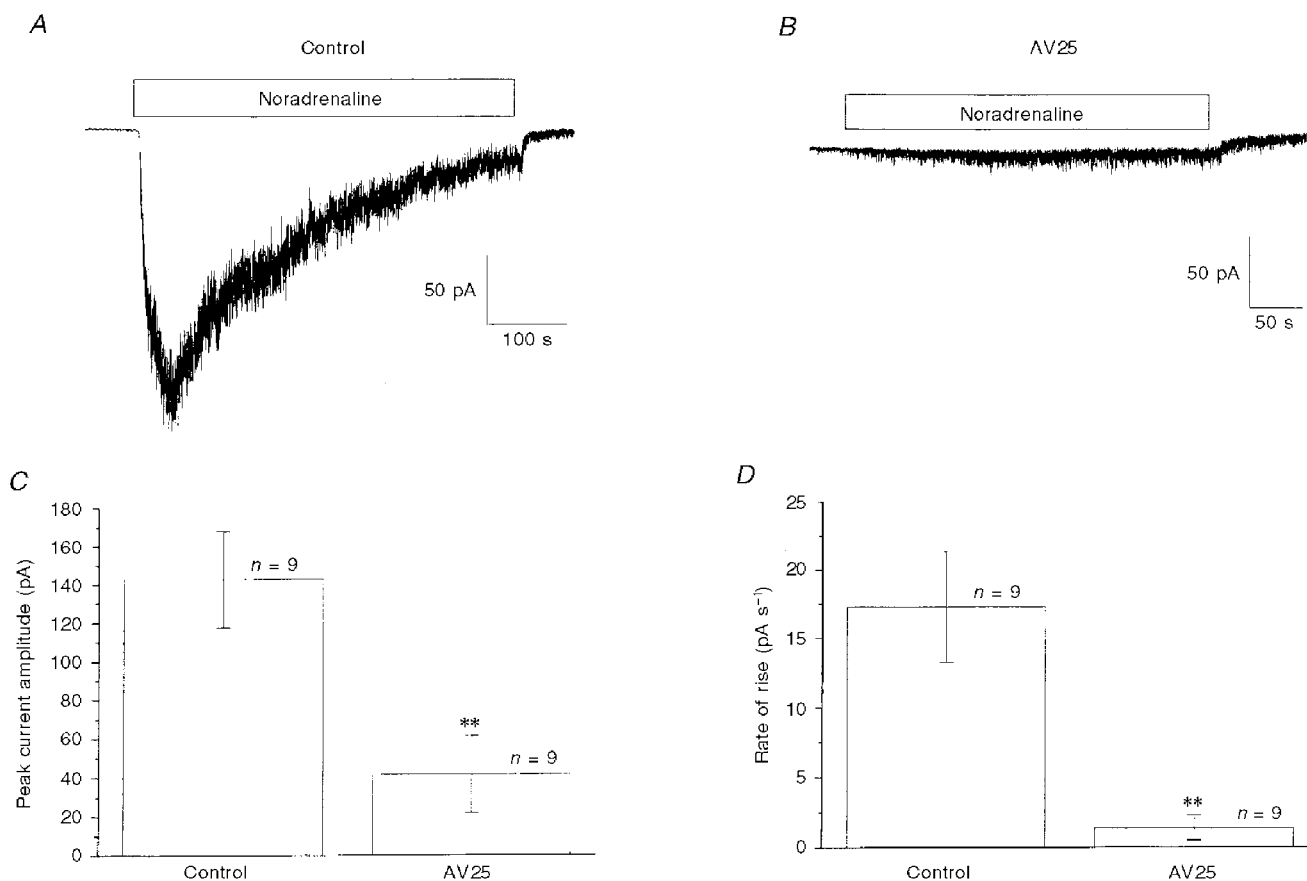

Figure 2. The effect of AV25 on noradrenaline-activated I_{cat}

A, control I_{cat} evoked by bath-applied noradrenaline (100 μM, horizontal bar). *B* illustrates I_{cat} recorded 5 min after achieving whole-cell configuration with a pipette solution containing 10 μM AV25. *C* and *D* show the effect of 10 μM AV25 on peak amplitude (*C*) and the activation rate (*D*) of I_{cat}. Records are from different cells. **$P < 0.01$.

20 μM wortmannin did not activate a current, although sometimes there was a small increase in 'noise' (e.g. Fig. 3C).

In five other cells, 20 μM wortmannin did not evoke a current but almost totally blocked the response to noradrenaline applied subsequently (Fig. 3D). Thus, the control response to noradrenaline was 148 $\pm$ 27 pA ($n = 5$) and in the presence of wortmannin (20 μM) the amplitude of noradrenaline-evoked I_{cat} was 6 $\pm$ 2 pA ($n = 5$). Overall these data show that wortmannin at micromolar concentrations inhibits noradrenaline-evoked I_{cat} but that in some cells I_{cat} is activated by wortmannin.

Subsequently, the effects of the synthetic napthalene-sulphonyl compounds ML-7 and ML-9, which also inhibit the binding of ATP to MLCK, were investigated. Figure 4A–C shows that application of ML-9 and ML-7 (both at 5 μM) during activation of I_{cat} by noradrenaline almost completely abolished the current. The relationship between the inhibitory effect on I_{cat} and the concentration of ML-7 and ML-9 is illustrated in Fig. 4D. The IC$_{50}$ values (concentration required to produce 50% inhibition of I_{cat}) were 0·8 and 2 μM for ML-7 and ML-9, respectively. It is apparent from Fig. 4D that the slope of the curves for ML-7 (0·9) and ML-9

(2·5) were different and also that in some cells these compounds produced greater than 100% inhibition, i.e. they also reduced the holding current (see Discussion). Interestingly, in seven out of 25 cells, after the initial inhibition produced by ML-7 and ML-9 there was partial recovery of the noradrenaline-evoked I_{cat}, although there was still marked inhibition of the current. There is no obvious explanation for this observation.

Effect of ML-9 on I_{cat} evoked by GTPγS and OAG

Previously we have demonstrated that intracellular dialysis with GTPγS and bath-applied OAG evoked I_{cat} (Helliwell & Large, 1997), and we carried out experiments to assess whether ML-9 inhibits these currents. Figure 5A and C shows control I_{cat} activated by, respectively, intracellular dialysis with 500 μM GTPγS and bath application of 10 μM OAG, as described previously (Helliwell & Large, 1997). Application of 5 μM ML-9 produced a marked decrease in the amplitude of I_{cat} evoked by GTPγS (Fig. 5B) and OAG (Fig. 5D). ML-9, at a concentration of 5 μM, inhibited the GTPγS- and the OAG-induced I_{cat} by, respectively, 100 $\pm$ 4% ($n = 5$) and 92 $\pm$ 5% ($n = 6$). Also, 5 μM ML-7 reduced the GTPγS-evoked current by 93 $\pm$ 7% ($n = 5$; not shown).
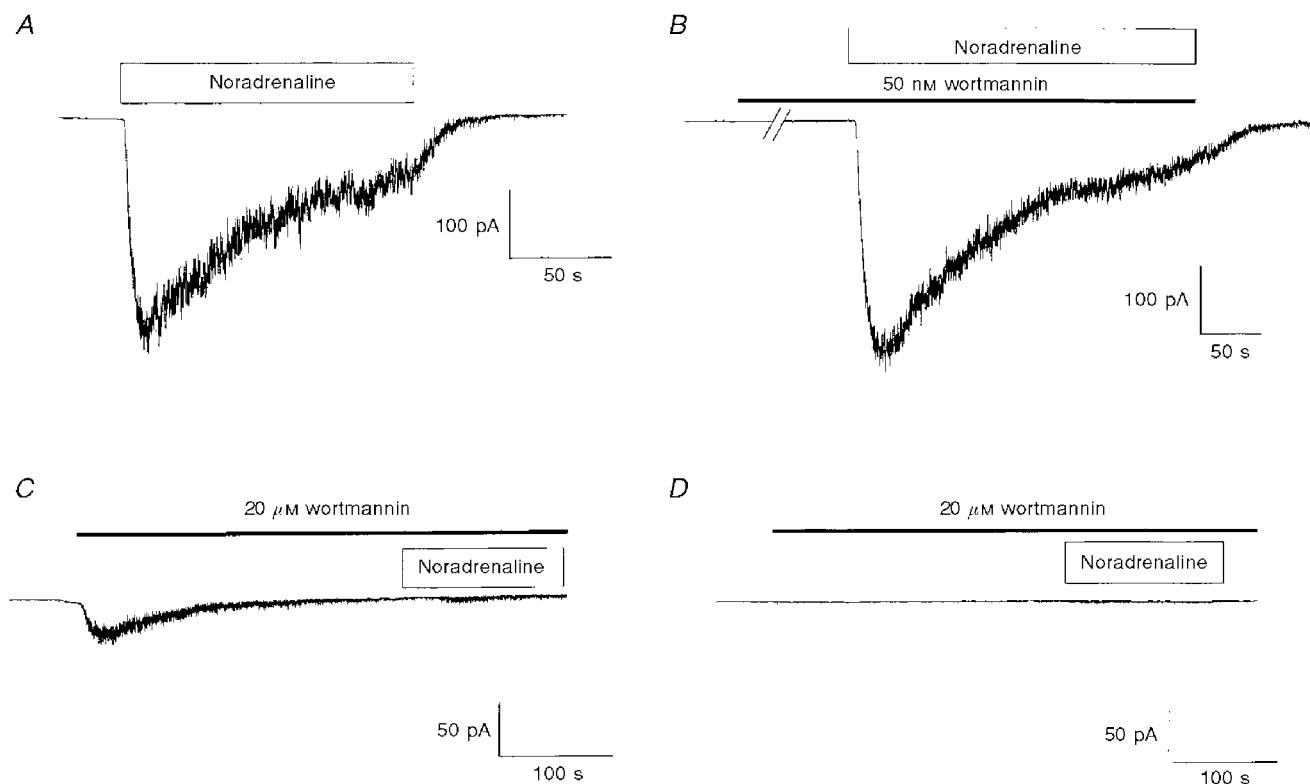

Figure 3. Effects of wortmannin on I_{cat}

A, example of I_{cat} activated by bath application of 100 μM noradrenaline in the absence of wortmannin. B, I_{cat} activated by bath-applied noradrenaline 5 min after preincubation with 50 nM wortmannin. The break in the record represents about 4 min. C, in another cell bath application of 20 μM wortmannin evoked a 'noisy' inward current similar to the noradrenaline-activated I_{cat}. However, subsequent application of noradrenaline in the continued presence of wortmannin failed to activate a current. D illustrates a cell where 20 μM wortmannin did not evoke a current and subsequent application of noradrenaline (in the continued presence of wortmannin) failed to activate a current. The duration of application of noradrenaline and wortmannin is indicated by the horizontal open and filled bars, respectively.

Effect of MLCK inhibitors on noradrenaline-evoked Ca^{2+}-activated K^+ conductance

We carried out experiments to test whether the inhibitors of MLCK have general non-specific effects on cell function. For these studies we investigated whether these agents inhibited the Ca^{2+}-activated K^+ current evoked by 100 μM noradrenaline with K^+-containing pipette and bathing solutions at a holding potential of 0 mV. It was observed that $MLCK_{(11-19)}$amide (5 μM, in the pipette), AV25 (10 μM, in the pipette) and 5 μM ML-9 had no effect on the noradrenaline-induced $I_{K(Ca)}$. Thus the control and test amplitudes of noradrenaline-induced $I_{K(Ca)}$, respectively, were 0.85 ± 0.2 nA ($n = 4$) and 0.86 ± 0.1 nA ($n = 5$) for

$MLCK_{(11-19)}$amide, 2.0 ± 0.6 nA ($n = 4$) and 2.0 ± 0.3 nA ($n = 4$) for AV25, and 2.0 ± 0.5 nA ($n = 4$) and 2.0 ± 0.2 nA ($n = 5$) for ML-9.

In order to test whether the peptide inhibitors of MLCK had an inhibitory effect on non-selective cation currents we investigated their action on the inward current activated by ATP in rabbit ear artery cells, using the same bathing and pipette solutions as those used to record I_{cat} in portal vein cells. The response to ATP is mediated by a ligand-gated receptor but is similar to noradrenaline-evoked I_{cat} in portal vein cells in respect of a significant permeability to divalent cations (cf. Benham et al. 1987; Benham & Tsien, 1987; Wang & Large, 1991). With 5 μM $MLCK_{(11-19)}$amide in the
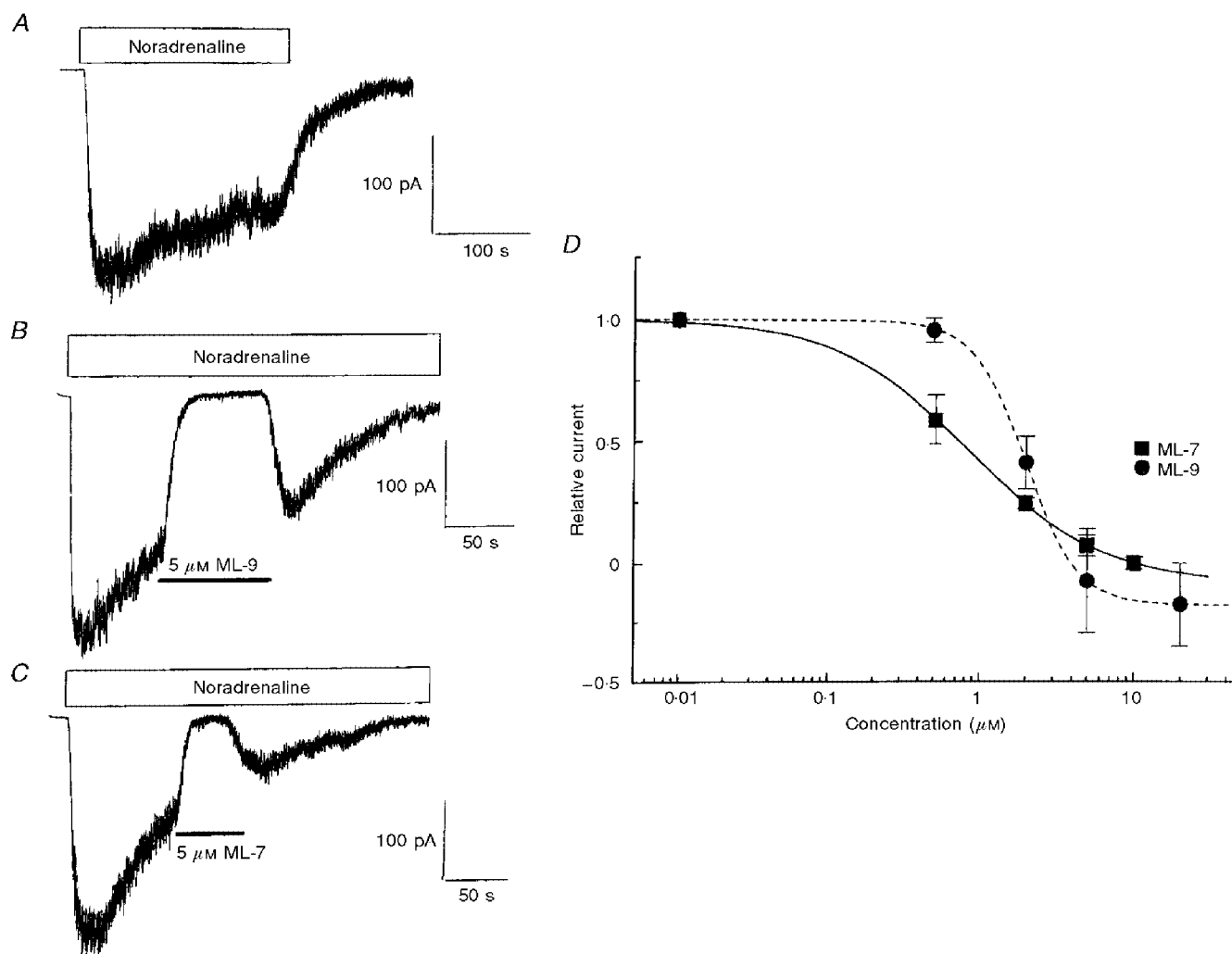

Figure 4. Effects of ML-9 and ML-7 on I_{cat}

A, I_{cat} recorded by bath application of 100 μM noradrenaline in the absence of ML-9 and ML-7. B and C show the inhibition of I_{cat} by 5 μM ML-9 (B) and 5 μM ML-7 (C) applied in the continued presence of noradrenaline. D shows the concentration-dependent inhibitory effects of ML-9 and ML-7, where the current amplitude (pA) in the presence of ML-9 or ML-7 was normalized to the current amplitude immediately prior to application (Relative current). The relative current is plotted against the concentrations of ML-9 and ML-7 on a logarithmic scale. The data could be fitted with a logistic equation of the following form: $y = y_{max}[x^{n_H}/(IC_{50}^{n_H} + x^{n_H})]$, where x denotes the concentration of ML-9 or ML-7 and the slope (n_H) was 2.5 and 0.9 for ML-9 and ML-7, respectively. This gave estimated IC_{50} values (the concentrations of ML-9 and ML-7 required to inhibit the current amplitude by 50%) of 2 and 0.8 μM for ML-9 and ML-7, respectively. Each data point represents the mean of 5–6 cells.

pipette, the amplitude of the ATP-induced current (I_{ATP}) in ear artery cells was 340 ± 70 pA ($n = 3$), compared with a control current of 322 ± 74 pA ($n = 3$). With 10 μM AV25 in the pipette solution, I_{ATP} was 168 ± 20 pA ($n = 4$), compared with a control I_{ATP} of 161 ± 20 pA ($n = 4$). It can be concluded that the inhibitory peptides of MLCK do not have a non-specific effect on non-selective cation channels.

Effect of increasing intracellular Ca²⁺ and including calmodulin in the pipette solution on noradrenaline-evoked I_{cat}

The above data suggest that MLCK may be involved in the activation of noradrenaline-evoked I_{cat}. With regard to smooth muscle contraction MLCK is activated by an increase in the intracellular calcium concentration, with subsequent binding of Ca²⁺ to the Ca²⁺-binding protein calmodulin. This sequence leads to activation of the kinase and therefore we investigated whether increasing the pipette Ca²⁺ concentration ($[Ca^{2+}]_i$) in the presence of calmodulin could activate I_{cat}. Figure 6A illustrates noradrenaline-evoked I_{cat} with $[Ca^{2+}]_i$ at 14 nM. When 400 nM Ca²⁺ and 9 μM calmodulin were included in the pipette solution there was an increase in the background

'noise' but no net inward current over a period of 5–10 min. Subsequent application of noradrenaline evoked I_{cat} (Fig. 6B). With 400 nM Ca^{2+}_i and 9 μM calmodulin the amplitude of noradrenaline-evoked I_{cat} was 109 ± 15 pA ($n = 6$), compared with 148 ± 25 pA ($n = 8$) with 14 nM Ca²⁺ in the pipette solution.

Intracellular dialysis with 2 μM Ca²⁺ and 9 μM calmodulin induced a large 'noisy' inward current with a mean amplitude of 60 ± 14 pA ($n = 7$). However, this current developed relatively slowly and had a mean time to peak of 334 ± 40 s ($n = 7$). Thus it can be concluded that I_{cat} can be activated by increasing the intracellular calcium concentration.

Role of phosphorylation in activation of noradrenaline-evoked I_{cat}

In the final series of experiments we carried out a simple test to ascertain whether phosphorylation was important in the activation of noradrenaline-evoked I_{cat}, which would be predicted if a kinase was involved in the transduction mechanism. In this study cells were dialysed with a pipette solution containing 5 mM AMP-PNP, a non-hydrolysable analogue of ATP. It can be seen that after 5 min dialysis with AMP-PNP the amplitude of the noradrenaline-induced
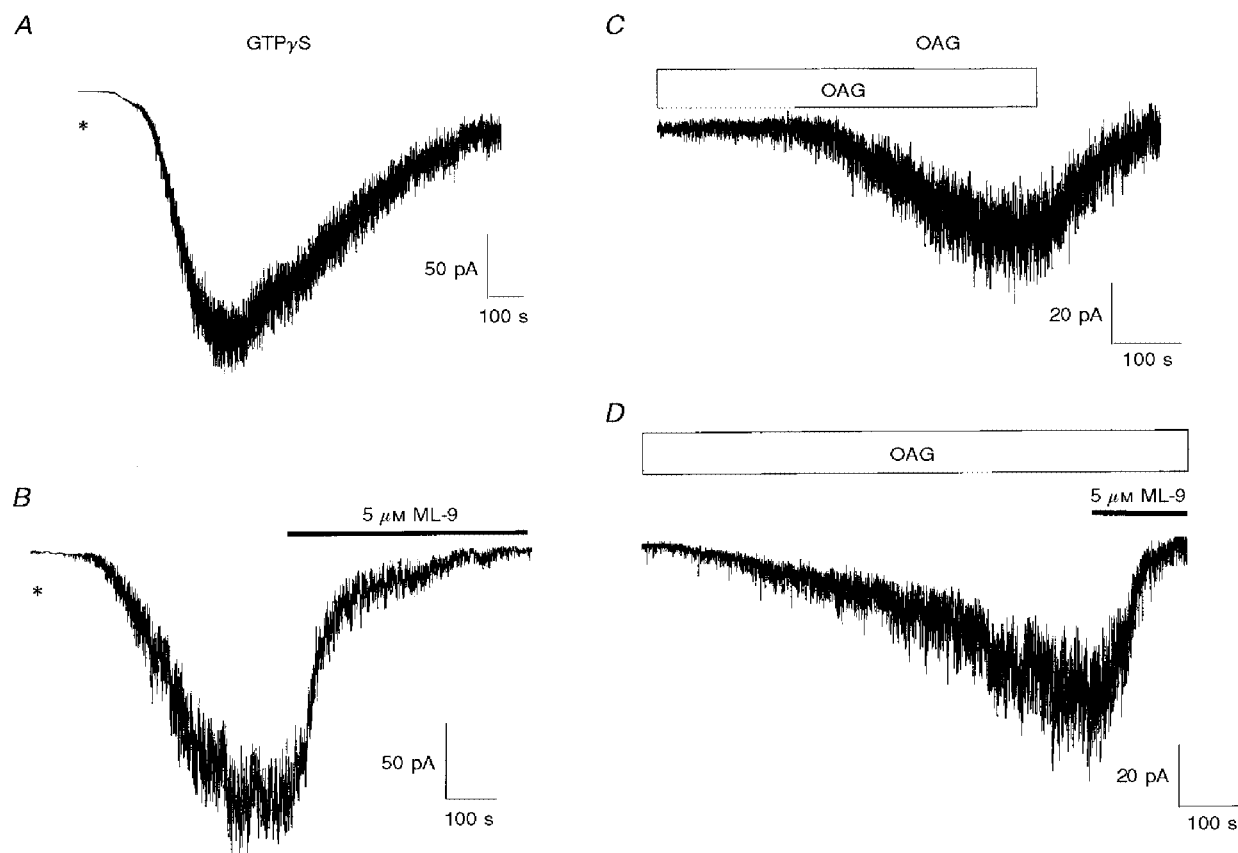

Figure 5. Effect of ML-9 on GTPγS- and OAG-activated I_{cat}

A, representative trace of I_{cat} evoked by inclusion of 500 μM GTPγS in the pipette solution 2–4 min after achieving whole-cell configuration (denoted by *). B illustrates the effect of ML-9 applied during the sustained phase of the GTPγS-evoked I_{cat}. C, bath application of OAG (10 μM, horizontal bar) activated a slowly developing 'noisy' inward current. D illustrates the effect of ML-9 applied during the sustained phase of the OAG-activated I_{cat}.

I_{cat} (Fig. 7*B*) was much smaller than the control response (Fig. 7*A*, no AMP-PNP). The mean data from three cell populations are shown in Fig. 7*C* and as with the peptide inhibitors AMP-PNP also significantly decreased the rate of activation of I_{cat} (Fig. 7*D*). Therefore it can be concluded that protein phosphorylation plays a role in the activation of I_{cat}.

DISCUSSION

The present work provides evidence for a role for MLCK in the activation of noradrenaline-evoked I_{cat} in rabbit portal vein smooth muscle cells. This conclusion is based on the observed marked reduction in amplitude and rate of activation of I_{cat} by several chemically different inhibitors

which act at different sites on the MLCK molecule. In our experiments we used concentrations of the MLCK inhibitors that are close to the estimated K_i values obtained in biochemical studies where the ability of MLCK to phosphorylate myosin was assessed. Thus when myosin kinase activity was assayed the K_i values of MLCK$_{(11-19)}$-amide and AV25 were about 10 μM (Pearson *et al.* 1986) and 0·2 μM (Weber *et al.* 1999), respectively. In our studies we used 5 μM MLCK$_{(11-19)}$amide and 10 μM AV25 in the pipette solution. Also the K_i values for smooth muscle MLCK of ML-7 and ML-9 were 0·3 and 3·8 μM, respectively (Saitoh *et al.* 1987). In the present study the IC$_{50}$ values of ML-7 and ML-9 for inhibition of noradrenaline-evoked I_{cat} were 0·8 and 2·0 μM, respectively, i.e. similar to the K_i values obtained in the biochemical experiments. Consequently, the
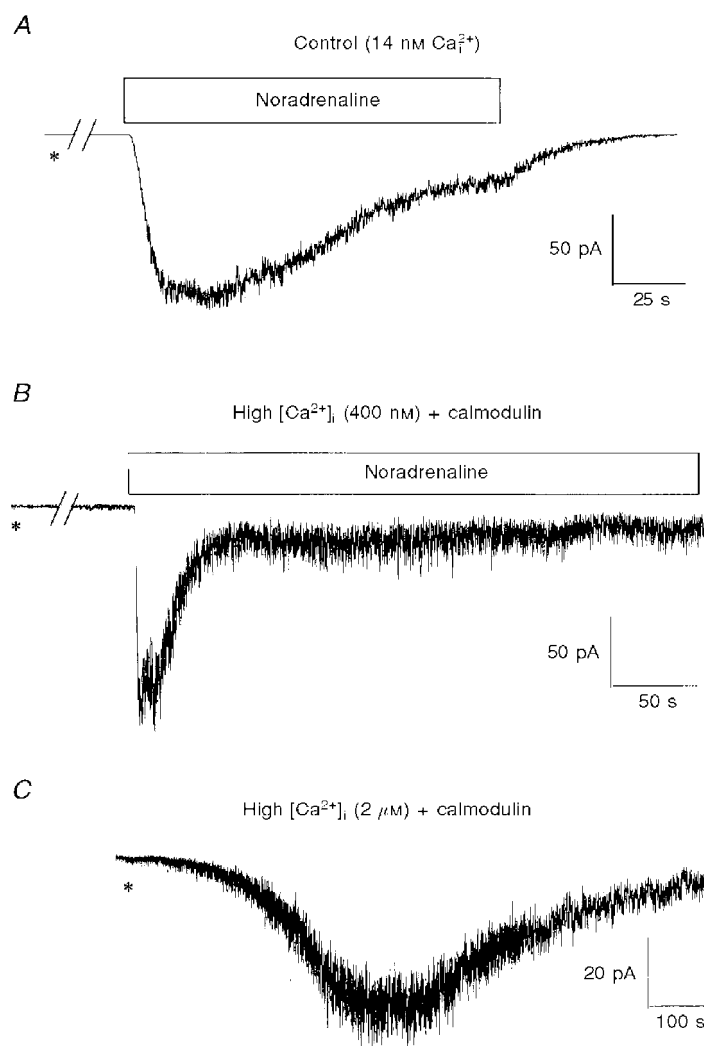

Figure 6. Effect of high intracellular calcium concentration and calmodulin on noradrenaline-evoked I_{cat}

A, example of I_{cat} activated by bath application of noradrenaline 5 min after achieving whole-cell configuration (*) using a pipette solution containing a free calcium concentration of 14 nM. *B*, I_{cat} evoked with 400 nM Ca^{2+} plus 9 μM calmodulin in the pipette solution. *C* illustrates the activation of I_{cat} with a pipette solution containing 2 μM Ca^{2+} and 9 μM calmodulin. In *A* and *B*, noradrenaline (100 μM) was applied for the period denoted by the horizontal bars. The breaks in the records in *A* and *B* represent about 4 min. Note the differences in time scale.

concentrations of inhibitors used in the present study are in the range expected to produce inhibition of MLCK. Wortmannin at micromolar concentrations also inhibits MLCK and the concentration used (20 μM) has little effect on cAMP-dependent protein kinase, cGMP-dependent protein kinase and calmodulin-dependent protein kinase II (Nakanishi *et al.* 1992).

$MLCK_{(11-19)}$amide, AV25 and ML-9 did not reduce the noradrenaline-evoked $I_{K(Ca)}$. Since this conductance involves the well-known α_1-adrenoceptor–G-protein–phospho-inositide–IP_3–Ca^{2+}-store pathway (see Large & Wang, 1996), it can be deduced that these compounds do not compromise (a) binding of noradrenaline to α_1-adreno-ceptors, (b) G-protein activation, (c) hydrolysis of PIP_2 by PLC to produce IP_3 and DAG, (d) release of Ca^{2+} ions from the sarcoplasmic reticulum and (e) the opening of Ca^{2+}-activated K^+ channels. Therefore these inhibitors of MLCK do not have general non-specific effects on cell function. Moreover the inhibitory peptides did not inhibit the non-selective cation currents induced by ATP in ear artery cells. Therefore it is apparent that the compounds used inhibited noradrenaline-evoked I_{cat} in portal vein cells without

compromising cellular function or blocking non-selective cation channels. It is not possible to state unequivocally that the compounds used did not affect other kinases, but since $MLCK_{(11-19)}$amide, AV25 and the ML-compounds (and wortmannin) act at three distinct sites on the MLCK molecule, namely the substrate site ($MLCK_{(11-19)}$amide), the auto-inhibitory site (AV25) and the ATP-binding site (ML-compounds and wortmannin), the most likely explanation for our data is that activation of I_{cat} by noradrenaline involves MLCK. It has also been suggested that MLCK is involved in the bradykinin-stimulated Ca^{2+} influx in endothelial cells (Watanabe *et al.* 1998).

If MLCK is involved in the activation of I_{cat} it might be expected that the introduction of high concentrations of Ca^{2+} and calmodulin into the cell via the pipette would activate I_{cat}. It was observed that 2 μM Ca^{2+} in the pipette solution (with calmodulin) did induce I_{cat}, indicating that an increase in $[Ca^{2+}]_i$ does activate I_{cat}. However, the relative slowness of this response (a time to peak of about 5 min) shows that this is not a classical Ca^{2+}-activated conductance. For example, with 1 μM Ca^{2+} in the pipette solution a Ca^{2+}-activated chloride current is evoked immediately (within
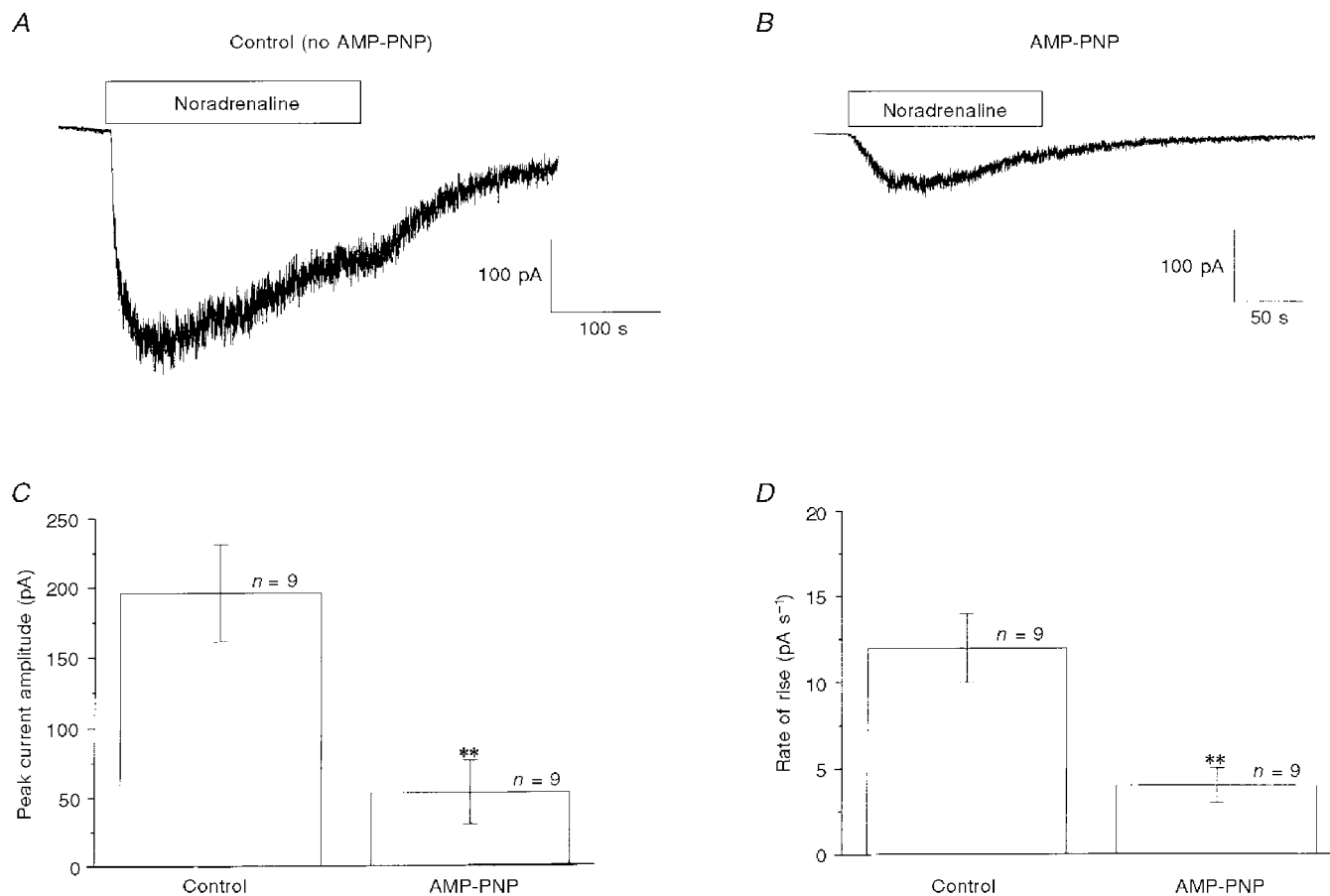

Figure 7. Effect of AMP-PNP on I_{cat}

A, I_{cat} evoked by bath-applied noradrenaline (100 μM, horizontal bar) in the absence of AMP-PNP. *B*, I_{cat} evoked 5 min after achieving whole-cell configuration with a pipette solution containing 5 mM AMP-PNP. *C* and *D* show the quantitative effect of AMP-PNP on the peak amplitude (*C*) and the rate of activation (*D*) of I_{cat}. $**P < 0.01$.

1 s) after rupture of the cell membrane in rabbit portal vein cells (I. A. Greenwood, personal communication). Therefore, our data indicate that I_{cat} may be activated by a Ca^{2+}-activated process (e.g. a calcium-sensitive kinase) although we feel that noradrenaline can evoke I_{cat} without a rise in $[Ca^{2+}]_i$. Interestingly, a high molecular weight isoform of MLCK has been identified in bovine endothelial and smooth muscle cells, which can be activated in the absence of a rise in cytosolic $[Ca^{2+}]$ (Verin et al. 1998; Gilbert-McClain et al. 1998).

It was observed that the ML-compounds also inhibited I_{cat} evoked by GTPγS and OAG. This is in contrast to the result of Kim et al. (1997), who demonstrated that whereas the carbachol-induced non-selective cation current in guinea-pig gastric myocytes was inhibited by micromolar concentrations of ML-7, the same current evoked by GTPγS was not inhibited by ML-7. These authors concluded that the inhibitory site of ML-7 was upstream of the G-protein and was either the muscarinic receptor or a closely related molecule. Our data indicate that the target for the ML-compounds is downstream of GTPγS and OAG. The lack of effect of the inhibitors on noradrenaline-evoked $I_{K(Ca)}$ also suggests that the inhibitory effect on I_{cat} occurs at a stage between the production of DAG, which is produced simultaneously with IP_3, and the opening of the non-selective cation channel. Taking together the previous results of Helliwell & Large (1997; see Introduction) and the present work a plausible transduction mechanism is: α_1-adrenoceptor → G-protein → PLC activation → DAG → MLCK → cation channel opening.

At present we are uncertain of the links between DAG and MLCK, and between MLCK and the ion channel, but AMP-PNP also produced a substantial reduction of the amplitude of I_{cat}, which indicates that protein phosphorylation is involved in activation of the conductance. MLCK may phosphorylate myosin light chain leading to channel opening by some unknown mechanism or alternatively MLCK may phosphorylate directly the channel protein, or perhaps an intermediate substrate, to produce channel opening.

There are some effects of the inhibitors of the binding of ATP to MLCK that require further comment. Firstly, in some cells wortmannin itself activated a current as well as inhibiting the response to noradrenaline applied subsequently and it is possible that wortmannin may act as a partial agonist at MLCK, i.e. possess the ability to stimulate and block the kinase. Secondly, in some cells the ML-compounds appeared to reduce the holding current. This observation suggests that these agents may also affect conductances other than I_{cat} but it should be noted that in some unstimulated cells there seemed to be a low resting activity of I_{cat} (as manifested by the background 'noise') before noradrenaline was applied. Thus I_{cat} may be active before application of noradrenaline in some cells, which may,

in part at least, account for the reduction of the holding current by ML-compounds, although other actions cannot be ruled out.

In conclusion, these experiments suggest that MLCK is involved in the activation of noradrenaline-evoked non-selective cation channels in rabbit portal vein smooth muscle cells.

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