Protein kinase C requirement of Ca^{2+} channel stimulation by intracellular ATP in guinea-pig basilar artery smooth muscle cells

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1. Smooth muscle cells were isolated from guinea-pig basilar artery and conventional whole-cell recordings of Ca^{2+} channel activity were made at room temperature within 7 h of the isolation procedure. The purpose of the study was to investigate the mechanism of the stimulatory action of intracellular ATP on Ca^{2+} channels.

2. High (millimolar) concentrations of ATP were needed to produce stimulation of Ca^{2+} channels, and neither ADP nor AMP mimicked the action of ATP.

3. The ATP effect was not mimicked by stable ATP derivatives (AMP-PNP or AMP-PCP) and was abolished by incubation of cells in non-specific protein kinase inhibitors (staurosporine or H-7) or specific protein kinase C inhibitors (GF109203x, calphostin C or chelerythrine) but not by tyrosine kinase inhibitors (tyrphostin B42 and genistein).

4. The data suggest that ATP-induced stimulation of L-type Ca^{2+} channels requires functional activity of a protein kinase C isozyme.

Intracellular ATP stimulates voltage-gated Ca^{2+} channels in arterial, venous and intestinal smooth muscle cells (Ohya, Kitamura & Kuriyama, 1987; Ohya & Sperelakis, 1989; Okashiro, Tokuno, Fukumitsu, Hayashi & Tomita, 1992; McHugh & Beech, 1996), cardiac and skeletal muscle cells (reviewed by McDonald, Pelzer, Trautwein & Pelzer, 1994), neurones (Pearson & Dolphin, 1993), and chromaffin cells (Elhamdani, Bossu & Feltz, 1995). The effect is expected to be important for maintenance of normal Ca^{2+} channel function, is suggested to underlie metabolic regulation of Ca^{2+} channels during hypoxia and ischaemia (O'Rourke, Backx & Marban, 1992; McHugh & Beech, 1996), and is a key aspect of Ca^{2+} channel 'run-down' in whole-cell voltage-clamp recordings (McDonald *et al.* 1994). We have reported previously (McHugh & Beech, 1996) that the stimulatory action of ATP in basilar artery smooth muscle cells occurs only if the Ca^{2+} channels are in the presence of a blocking concentration of intracellular Mg^{2+} ions (more than about 50 μM Mg^{2+}); it appears as if ATP acts to protect the Ca^{2+} channels against Mg^{2+} block. We now report observations which suggest that the stimulatory effect of ATP in basilar artery cells occurs via a mechanism requiring phosphorylation and the tonic activity of a protein kinase C (PKC).

METHODS

Male guinea-pigs (350–400 g) were killed by 100% CO_2 inhalation and the basilar artery was dissected free and placed into ice-cold Hanks' solution. Single smooth muscle cells were isolated by an enzymatic and mechanical procedure and stored in Kraft-Brühe (KB) medium as described previously (McHugh & Beech, 1996). Cells were stored at 2 °C and used within 7 h. A suspension of cells was allowed to settle to the base of a modified culture dish and the KB medium was replaced by 1·5 mM Ca^{2+} solution and recordings were attempted 15 min later.

Recordings were made at 23–25 °C using an Axopatch 200A or Axopatch-1D patch-clamp amplifier (Axon Instruments, Inc., Foster City, CA, USA) and the conventional whole-cell configuration of the patch-clamp technique. Current signals were filtered (4-pole Bessel) at 1 kHz and then digitized at 2 kHz by a 1401-plus CED analog-to-digital converter (Cambridge Electronic Design Ltd, Cambridge, UK) and stored on a 486 computer. Voltage-clamp commands and data sampling were controlled by CED software. Patch pipettes were made from borosilicate glass capillaries (Clark Electromedical Instruments, Reading, UK) and had resistances of 1–3 MΩ after fire polishing and when filled with pipette solution. Cell capacitance values were determined from capacity current (filtered at 10 kHz and sampled at 20 kHz) elicited by a square voltage step from −80 to −75 mV. Liquid junction potentials between bath and pipette solutions were ≤ 2 mV. The solution in the recording chamber was exchanged using a gravity-flow perfusion system with multiple input reservoirs and solutions were fully exchanged in < 1 min. Results are expressed as means ± S.E.M., and 'n' values indicate the number of observations from different cells. Statistical significance was determined using Student's unpaired t test (* indicates significance at the $P < 0{\cdot}05$ level). Data presentation and mathematical fitting of functions to data were performed using the program Origin 3.5 (MicroCal Inc., Northampton, MA, USA).

Hanks' (modified) solution contained (mM): NaCl, 137; KCl, 5·4; $CaCl_2$, 0·01; NaH_2PO_4, 0·34; K_2HPO_4, 0·44; glucose, 8; and Hepes, 5. Modified KB medium contained (mM): KCl, 85; K_2PO_4, 30; $MgSO_4$, 5; sodium pyruvate, 5; glucose, 8; taurine, 20; creatine, 5;

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and β-hydroxybutyrate, 5 (with 0·1% fatty acid-free bovine serum albumin). The 1·5 mM Ca^{2+} bath solution contained (mM): NaCl, 130; KCl, 5; $CaCl_2$, 1·5; $MgCl_2$, 1·2; Hepes, 10; and glucose, 8. The divalent cation-free bath solution contained (mM): NaCl, 120; EDTA, 10; Hepes, 10; tetraethylammonium chloride, 4; 3,4-diaminopyridine (3,4-DAP), 1; 2-deoxy-D-glucose, 8; and 2,4-dinitrophenol, 0·2. Solutions were titrated to pH 7·4 using KOH (KB medium) or NaOH (all other extracellular solutions).

All recording pipette solutions contained (mM): CsCl, 130; Hepes, 10; EDTA, 5; and BAPTA, 1. To this solution, various concentrations of nucleotide and $MgCl_2$ were added. The solution was then titrated to pH 7·4 using CsOH, filtered (pore size, 0·2 μm) and frozen in aliquots at −18 °C. Pipette solutions were stored on ice prior to experiments. To prepare the various pipette solutions the following additions were made (all mM), each giving a calculated (Eqcal, Biosoft, Cambridge, UK) unbound Mg^{2+} concentration of 1–1·04 mM. (a) Mg^{2+} alone: 6 $MgCl_2$ and 10 NaCl (17·5 LiCl for comparison with (i)). (b) 5 mM ATP: 5 Na_2ATP and 10·3 $MgCl_2$. (c) 1 mM ATP: 1 Na_2ATP and 6·86 $MgCl_2$. (d)* 7·8 mM ADP: 7·8 NaADP and 9·3 $MgCl_2$. (e) 5 mM AMP: 5 NaAMP and 6·3 $MgCl_2$. (f)† 1 mM GDP-β-S: 1 Li_3GDP-β-S and 7 $MgCl_2$. (g)† 5 mM ATP with 1 mM GDP-β-S: 5 Na_2ATP, 1 Li_3GDP-β-S and 11·42 $MgCl_2$. (h) 5 mM AMP-PCP: 5 Na_2AMP-PCP and 10·65 $MgCl_2$. (i) 5 mM AMP-PNP: 5 Li_3AMP-PNP and 10·65 $MgCl_2$. * These experiments were performed with 3·95 mM EDTA in the pipette solution and thus for the ADP-free pipette solution only 4·98 mM $MgCl_2$ was added. † We do not have an affinity constant (K_a) value for Mg^{2+} binding to GDP-β-S: assuming the K_a is the same as that for Mg^{2+}-GDP the free Mg^{2+} concentration for (f) and (g) would be 1·25 mM, and if it is the same as that for Mg^{2+}-ATP the Mg^{2+} concentration would be 1·14 mM. K_a values (logM) used to describe nucleotide–Mg^{2+} affinities were: ATP, 3·84; ADP, 3·11; GDP, 3·4; AMP, 1·69; AMP-PCP, 4·11; AMP-PNP, 4·11 (Sillén & Martell, 1964; Yount, 1975).

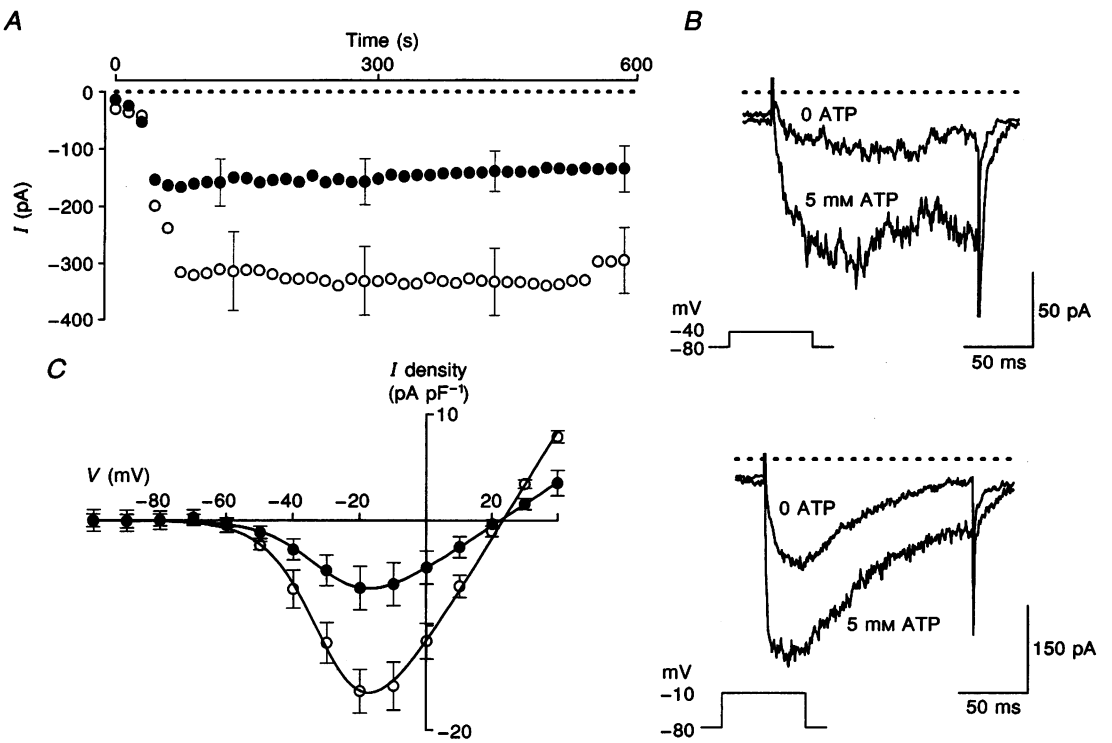

Figure 1. Stimulation of Ca^{2+} channel current by intracellular ATP

A, mean inward Na^+ current amplitude through L-type Ca^{2+} channels measured (alternately from different cells) with a recording pipette solution containing 5 mM ATP and 1 mM free Mg^{2+} (○, $n = 6$), or zero ATP and 1 mM free Mg^{2+} (●, $n = 8$). The x-axis is time (t) after breaking the cell-attached patch to begin whole-cell recording. Current amplitude increased in the first 1–2 min as the bath solution was changed to the divalent cation-free medium. The $I_{Ca(Na)}$ was elicited by stepping to −10 mV from a holding potential of −80 mV for 150 ms every 15 s; this is also the case for Figs 2 and 3. The inward current was primarily $I_{Ca(Na)}$ because leakage currents were small at −10 mV (Fig. 3*B*). *B*, example current traces for an experiment included in *A*. Leakage and capacity currents have not been subtracted and the dashed line marks zero current. Current records were taken 10 min after starting whole-cell recording. *C*, plot of mean current density against test potential for the recordings described in *A*. ○, 5 mM ATP; ●, 0 ATP. Linear leakage currents were substracted and the smooth curve was described by the equation: $g_{max}(V - V_{rev})/\{1 + \exp((V - V_{½})/dV)\}$, where the mid-point of the Boltzmann function ($V_{½}$) is −28·3 and −28·0 mV for recordings with ($n = 6$) and without ATP ($n = 5$), respectively, V is membrane potential, dV is the slope of the Boltzmann function, V_{rev} the reversal potential and g_{max} the maximum conductance. Current amplitudes were measured at 10 min after the start of whole-cell recording for this and subsequent figures.

EDTA, Hepes, 2-deoxy-D-glucose, staurosporine, nicardipine, 2,4-dinitrophenol, GDP-β-S, AMP-PNP (adenylylimidodiphosphate), AMP-PCP (β,γ-methyleneadenosine 5'-triphosphate), ATP, ADP and AMP were from Sigma. H-7 (1-(5-isoquinolinesulphonyl)-2-methyl-piperazine), BAPTA, tyrphostin B42, genistein and calphostin C were from Calbiochem. 3,4-DAP was from Fluka Chemie AG (Buchs, Switzerland), and GF109203x was from Research Biochemicals International (Natick, MA, USA). Salts were purchased from BDH (Poole, UK), Sigma, or Aldrich. Staurosporine, tyrphostin B42, genistein, GF109203x, calphostin C, chelerythrine and nicardipine stock solutions were in 100% dimethylsulphoxide (Sigma). The final dimethylsulphoxide concentration in experiments was always $\leq 0{\cdot}1\%$. H-7 was dissolved directly in water. Calphostin C was stored in the dark but exposed to fluorescent lighting and daylight (but not the microscope light) during recordings.

RESULTS

Ca^{2+} current recorded in the presence of 1·5 mM extracellular Ca^{2+} averages about −10 pA in basilar artery smooth muscle cells. We have found this amplitude too small for routine quantitative comparisons between current amplitudes in different cells and so in this study we have used an extracellular solution devoid of divalent cations, permitting inward flux of Na^+ through Ca^{2+} channels. This inward current ($I_{Ca(Na)}$) is about 30 times larger than the Ca^{2+} current and is completely blocked by the dihydropyridine Ca^{2+} antagonist nicardipine (1 μM), demonstrating it is through L-type Ca^{2+} channels (Fig. 3*B*).

Action of ATP

Figure 1 shows the stimulatory effect of intracellular ATP on Ca^{2+} channels. The amplitude of $I_{Ca(Na)}$ was compared for recordings made with 5 mM or no ATP in the patch pipette solution and such that the identity of the pipette solutions was revealed to the experimenter only after the series was completed (Fig. 1*A* and *B*). Ca^{2+} channel current density was about 2·5 times larger when ATP was in the pipette but the voltage dependence of channel activity remained unchanged (Fig. 1*C*). The stimulatory effect of ATP was not explained by Mg^{2+} chelation by ATP^{4-} because the unbound Mg^{2+} concentration was 1 mM for both pipette solutions.

If an intracellular ATP effect on Ca^{2+} channels has a regulatory role it might be expected to occur only at quite high (1–5 mM) ATP concentrations. We have tested whether this is the case by comparing $I_{Ca(Na)}$ for two patch pipette solutions; one containing 1 mM ATP and the other 5 mM ATP. The $I_{Ca(Na)}$ was observed to be 2·4 times larger for the 5 mM ATP group (Fig. 2*A*), and so the ATP effect on Ca^{2+} channels did occur at high ATP concentrations.

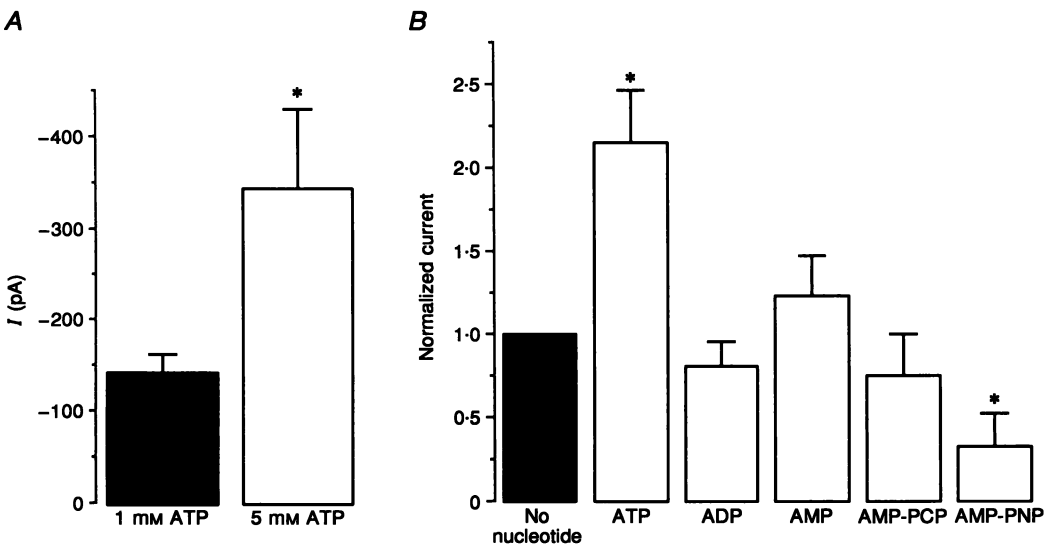

Figure 2. ATP acts at high concentrations and ADP, AMP and stable ATP derivatives do not mimic ATP action

A, mean amplitudes of $I_{Ca(Na)}$ recorded alternately with 1 mM ATP ($n = 8$) or 5 mM ATP ($n = 8$) in the recording pipette (1 mM free Mg^{2+} in both cases). *B*, mean amplitudes of $I_{Ca(Na)}$ recorded alternately with or without a nucleotide in the recording pipette (1 mM free Mg^{2+} in each case). For each pair of experiments, $I_{Ca(Na)}$ amplitude was normalized to the mean amplitude in the nucleotide-free group. The number of cells recorded from was: 8 and 6 (no ATP and 5 mM ATP); 9 and 7 (no ADP and 7·8 mM ADP); 6 and 7 (no AMP and 5 mM AMP); 5 and 5 (no AMP-PCP and 5 mM AMP-PCP); 7 and 6 (no AMP-PNP and 5 mM AMP-PNP). In this figure and Fig. 3, * indicates a statistically significant difference ($P < 0{\cdot}05$) between the mean Ca^{2+} channel current amplitudes (before normalization) observed in two groups of cells recorded from alternately using different pipette solutions (for example: no ATP compared with 5 mM ATP).

ADP, AMP and stable ATP derivatives

Metabolites of ATP were tested to see if they could mimic ATP action. Neither ADP nor AMP could stimulate Ca^{2+} channel current (Fig. 2*B*). To investigate whether hydrolysis of ATP was required for the stimulatory effect, stable ATP analogues (AMP-PCP and AMP-PNP; Yount, 1975) were tested for activity. The effect of each analogue was compared alternately with its own Mg^{2+} control, which contained no nucleotide. AMP-PCP was without effect (Fig. 2*B*) and AMP-PNP inhibited rather than stimulated $I_{Ca(Na)}$ (Fig. 2*B*), suggesting that cleavage of the γ-phosphate of ATP is required for the stimulatory action of ATP on Ca^{2+} channels. The mechanism of the inhibitory action of AMP-PNP was not investigated further.

Broad specificity kinase inhibitors and selective PKC inhibitors prevent ATP action

The inability of AMP-PNP and AMP-PCP to mimic the stimulatory action of ATP suggested that ATP hydrolysis and protein kinase activity might be required for the action of ATP on Ca^{2+} channels. This possibility was investigated further by using the compounds staurosporine and H-7, which inhibit a wide range of protein kinases (Hidaka & Kobayashi, 1992).

The amplitude of $I_{Ca(Na)}$ was compared for cells which were alternately pre-incubated in the presence of protein kinase inhibitor or vehicle control. In each case the recording pipette solution contained 5 mM ATP and 1 mM free Mg^{2+},

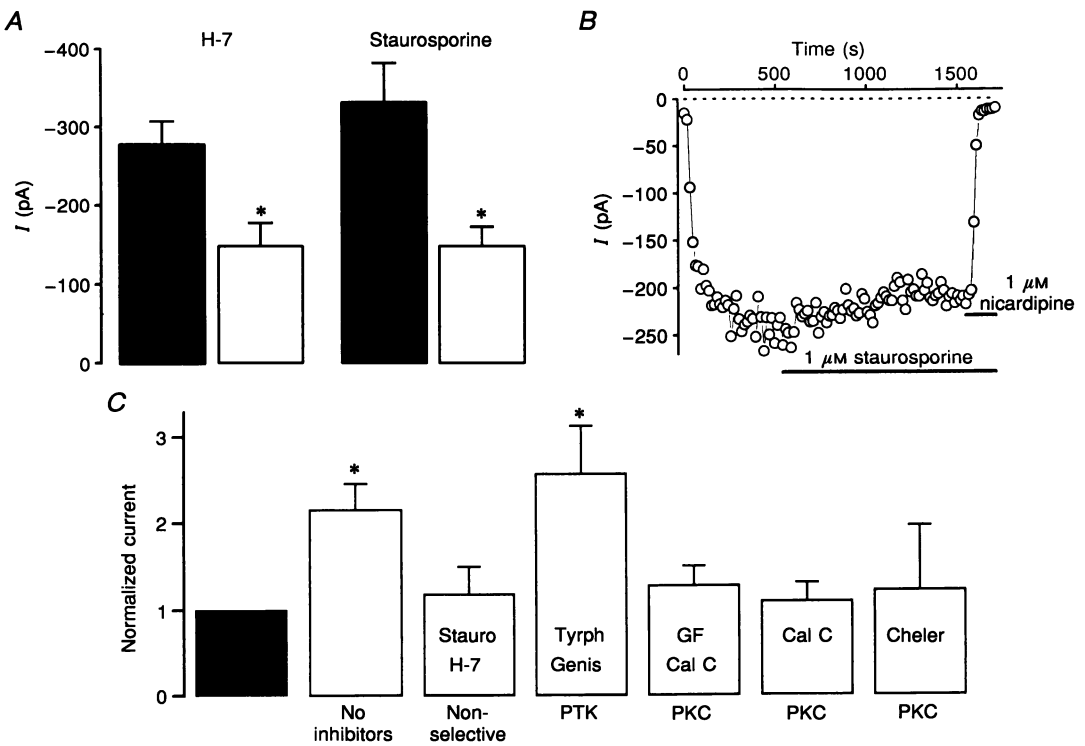

Figure 3. Protein kinase C requirement of ATP action

A, mean amplitudes of $I_{Ca(Na)}$ in the absence or presence of H-7 (50 μM), and the absence or presence of staurosporine (0·3 μM). ATP (5 mM) and Mg^{2+} (1 mM free) were in the recording pipette for all experiments. Cells were pre-incubated in and recorded from in the presence of H-7 (□), or staurosporine (□), or vehicle control (■) in the pipette and bath solutions. *B*, plot of the amplitude of $I_{Ca(Na)}$ without ATP or Mg^{2+} in the recording pipette solution. Staurosporine (1 μM) was bath applied, as was nicardipine (1 μM). *C*, mean amplitudes of $I_{Ca(Na)}$ recorded alternately with (□) or without (■) 5 mM ATP in the recording pipette (1 mM free Mg^{2+} in each case) and such that both groups of cells were in the presence of inhibitor(s). Cells were pre-incubated in the presence of inhibitor, and inhibitors were in the bath and pipette solutions during recordings. For each pair of experiments, $I_{Ca(Na)}$ amplitude was normalized to the mean amplitude of the ATP-free group (■). The number of cells recorded from in 0 ATP and 5 mM ATP, respectively, was: (a) no inhibitors, $n = 8$ and 6; (b) 0·3 μM staurosporine (Stauro) with 50 μM H-7, $n = 6$ and 6; (c) 10 μM tyrphostin B42 (Tyrph) with 100 μM genistein (Genis), $n = 7$ and 8; (d) 350 nM GF109203x (GF) with 50 nM calphostin C (Cal C), $n = 7$ and 8; (e) 500 nM calphostin C, $n = 10$ and 11; (f) 10 μM chelerythrine (Cheler), $n = 5$ and 5. The $I_{Ca(Na)}$ amplitude in the ATP-free control groups was: (a) -151 ± 35 pA, $n = 8$; (b) -152 ± 49 pA, $n = 6$; (c) -112 ± 25 pA, $n = 7$; (d) -132 ± 28 pA, $n = 7$; (e) -115 ± 23 pA, $n = 10$; (f) -33 ± 7 pA, $n = 5$. 'Non-selective' indicates the non-selective inhibition of all protein kinases. PTK, protein tyrosine kinase (inhibited). PKC, protein kinase C (inhibited). In all experiments described in this figure, cells were pre-incubated with the inhibitor(s) or vehicle control for at least 20 min and for not more than 2 h.

but for the 'inhibitor group' the protein kinase inhibitor was in the bath solution during the recording and in the recording pipette solution. It was observed that staurosporine and H-7 halved the amplitude of $I_{Ca(Na)}$ (Fig. 3*A*). Thus, both kinase inhibitors appeared to prevent the stimulatory action of ATP. Neither 1 μM staurosporine (Fig. 3*B*, $n = 5$) nor 50 μM H-7 ($n = 2$, data not shown) had a direct blocking action on Ca^{2+} channels. Staurosporine and H-7 also prevented ATP action in experiments using a different protocol where the inhibitors were present for two groups of cells but 5 mM ATP was in the pipette for one group and not the other (Fig. 3*C*).

To identify which protein kinase family is involved in ATP action we tested compounds which selectively inhibit protein tyrosine kinases or PKC isozymes. We focused on these kinases because of the reported stimulatory effects of $pp60^{c\text{-}src}$ (Wijetunge & Hughes, 1995) and PKC (Leprêtre, Mironneau & Morel, 1994) on smooth muscle Ca^{2+} channels. cAMP- and cGMP-dependent protein kinases seemed less likely candidates. cAMP-dependent protein kinase has only a small stimulatory action or no effect on smooth muscle Ca^{2+} channels (reviewed in Beech, 1996). cGMP-dependent protein kinase inhibits Ca^{2+} channel activity in vascular smooth muscle cells (Ishikawa, Hume & Keef, 1993; reviewed by Beech, 1996) and so seems unlikely to be involved in the stimulatory action of ATP.

A combination of 10 μM tyrphostin B42 and 100 μM genistein will inhibit a variety of tyrosine kinases but not protein kinase C (Gazit, Osherov, Yaish, Poradosu, Gilon & Levitzki, 1991; Akiyama & Ogawara, 1991). Cells were pre-incubated in the presence of these inhibitors at concentrations which block tyrosine kinases and the inhibitors were present in the bath and pipette solutions during recordings. These agents did not, however, affect the action of ATP on Ca^{2+} channels (Fig. 3*C*). Therefore, although we have no independent test to indicate that the blockers were indeed effective against tyrosine kinases in the basilar artery cells the result makes it unlikely that a tyrosine kinase is involved in the effect of ATP.

GF109203x, calphostin C and chelerythrine are structurally unrelated but they all inhibit most isozymes of PKC without affecting other protein kinases at the concentrations used in our experiments (Kobayashi, Nakano, Morimoto & Tamaoki, 1989; Herbert, Augereau & Maffrand, 1990; Toullec *et al.* 1991). In the first series of experiments we pre-incubated and recorded from cells in the presence of a combination of 350 nM GF109203x and 50 nM calphostin C to broadly but selectively inhibit PKC isozymes, and alternated between making recordings with and without ATP in the pipette. The combination of inhibitors completely prevented the action of ATP on Ca^{2+} channels. We also investigated the effect of a higher concentration of calphostin C (0·5 μM) without the inclusion of GF109203x. We did this to investigate whether PKM (a constitutively active form of PKC; see Discussion) was involved in ATP action, and because 50 nM calphostin C produces only 25–50% inhibition of PKC activity (Kobayashi *et al.* 1989; Bruns *et al.* 1991). At the concentration of 0·5 μM, calphostin C alone prevented ATP action on Ca^{2+} channels (Fig. 3*C*). We also observed that 10 μM chelerythrine prevented the action of ATP on Ca^{2+} channels (Fig. 3*C*). This PKC inhibitor appeared to have a direct blocking effect on the Ca^{2+} channels (Fig. 3, legend) but it was present for ATP-containing and ATP-free groups and the remaining current amplitude was sufficient to allow a comparison between groups. Neither GF109203x nor calphostin C had an obvious inhibitory action on the Ca^{2+} channels in the absence of ATP. We have not investigated whether the PKC inhibitory peptide PKC(19–36) is effective at preventing ATP action on Ca^{2+} channels because this peptide may inhibit kinases in addition to PKC (Smith, Colbran & Soderling, 1990; Hvalby *et al.* 1994). The abilities of GF109203x, calphostin C and chelerythrine to prevent ATP action on Ca^{2+} channels indicates strongly that PKC activity was required.

Tonic PKC activity

It was apparent that we were observing an absolute PKC requirement for ATP action in the absence of any exogenous activator of PKC such as a diacylglycerol. It seemed plausible that there might be tonic GTP-binding protein activity in the cells which was activating PKC via a phospholipase C-type pathway. We, therefore, investigated whether a high concentration of GDP-β-S in the recording pipette could prevent ATP stimulation of Ca^{2+} channels. The amplitude of $I_{Ca(Na)}$ was compared for two recording pipette solutions, both containing 1 mM GDP-β-S and 1 mM free Mg^{2+} but one without ATP and the other with 5 mM ATP. ATP action was not inhibited: $I_{Ca(Na)}$ was -445 ± 26 pA with 5 mM ATP present, and -146 ± 47 pA in the absence of ATP ($n = 4$ for each).

DISCUSSION

The stimulatory action of intracellular ATP on L-type voltage-gated Ca^{2+} channels was investigated in basilar artery smooth muscle cells. It was observed that high concentrations of ATP are needed to produce the effect and that ADP and AMP cannot mimic ATP action. The ATP effect requires protein phosphorylation by a PKC isozyme because it cannot be mimicked by stable ATP derivatives in the absence of ATP, and it is blocked by non-specific protein kinase inhibitors and specific PKC inhibitors but not by tyrosine kinase inhibitors.

Preceding reports generally support the view that PKC activation enhances Ca^{2+} channel activity in smooth muscle cells. Phorbol esters which activate PKC stimulate Ca^{2+} channel current in smooth muscle cells from rat portal vein, rabbit saphenous artery, rat aorta, guinea-pig ileum and human umbilical vein (Loirand, Pacaud, Mironneau & Mironneau, 1990; Oike, Kitamura & Kuriyama, 1992; Schuhmann & Groschner, 1994; Unno, Komori & Ohashi, 1995; Hirakawa, Kuga, Kobayashi, Kanaide & Takeshita,

1995), as does 1,2-dioctanoyl-*sn*-glycerol (a diacylglycerol analogue) in toad stomach smooth muscle cells (Vivaudou, Clapp, Walsh & Singer, 1988). Phorbol ester or diacylglycerol derivatives which do not activate PKC are ineffective and effects of phorbol esters have been shown to be prevented by PKC inhibitors (Leprêtre *et al.* 1994; Hirakawa *et al.* 1995). We have observed that 1 μM phorbol 12,13-dibutyrate stimulated Ca^{2+} channel current in some (3 out of 9 cells tested) but not all basilar artery smooth muscle cells recorded from with 5 mM ATP in the pipette (data not shown). This supports the view that PKC has a stimulatory effect on these Ca^{2+} channels but suggests that in many basilar artery smooth muscle cells the PKC isozyme involved was already maximally active before the phorbol ester was applied.

In contrast to previous studies describing PKC-induced stimulation of Ca^{2+} channels in smooth muscle it was not necessary for us to apply a diacylglycerol or phorbol ester to activate PKC. It appears that a PKC is tonically active in guinea-pig basilar artery smooth muscle cells and that this activity allows ATP action. We do not know the mechanism by which PKC becomes tonically active but have evidence against two possibilities. Firstly, activated GTP-binding proteins (and thus G-protein-coupled receptors) are not involved because ATP action is not inhibited by GDP-β-S. The second possibility is that a PKM is involved. PKM is the catalytic subunit of PKC, cleaved from PKC by the protease calpain (Kishimoto *et al.* 1989). Several isozymes of PKC are susceptible to this proteolysis and the action makes a constitutively active PKC. PKM, however, seems unlikely to underlie the ATP effect on Ca^{2+} channels because ATP action is abolished by calphostin C, which acts on the regulatory domain of PKC and does not inhibit PKM (Kobayashi *et al.* 1989). Other explanations for the tonic PKC activity could be that high concentrations of ATP stimulate PKC (directly or via an intermediate), or that a form of cell stress has induced PKC activity (Yamazaki *et al.* 1995).

Although we cannot exclude the possibility that ATP stimulates Ca^{2+} channels solely by acting as a phosphate donor for PKC this explanation is unlikely because PKC has a high affinity for ATP. The K_m for ATP interaction with PKC is about 6 μM (Kikkawa, Takai, Minakuchi, Inochura & Nishizuka, 1982) and yet the half-effective concentration of ATP for stimulation of Ca^{2+} channels is 1 mM or more (Fig. 1; Ohya & Sperelakis, 1989). An explanation could be that high concentrations of ATP directly stimulate Ca^{2+} channels, but only when there is PKC-dependent phosphorylation. In this hypothesis, ATP would still act as a phosphate donor for PKC but the concentration dependence of the ATP effect would arise from a separate direct action of ATP. A stimulatory nucleotide site on Ca^{2+} channels has been suggested previously for cardiac myocytes (O'Rourke *et al.* 1992) and chromaffin cells (Elhamdani *et al.* 1995).

The PKC isozyme involved in ATP action on Ca^{2+} channels is unknown. However, of the isozymes cloned to date only α, β, δ, ϵ and ζ have been reported to be expressed in vascular smooth muscle cells (reviewed by Harrington & Ware, 1995). PKCα and PKCβ may seem unlikely to be involved because they are Ca^{2+}-dependent enzymes and our measurements were made in Ca^{2+}-free conditions, and PKCζ may seem unlikely to be involved because it is in the perinuclear region (Khalil, Lajoie, Resnick & Morgan, 1992) and because it has low sensitivity to inhibition by staurosporine (Ishii *et al.* 1996). Nevertheless, there are no water-tight arguments against the involvement of any of the five PKC isozymes in ATP action, making it premature to indicate which isozyme is involved.

The function of the ATP effect on Ca^{2+} channels may be to provide a mechanism for metabolic regulation of vascular tone. The results of this study suggest that the effect should be of particular significance when tonic activity of PKC is high, for example, when there is vasospasm (Minami, Tani, Maeda, Yamaura & Fukami, 1992) or myogenic tone (reviewed by Harder, Narayanan, Gebremedhin & Roman, 1995).

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