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ON THE MECHANISM OF M-CURRENT INHIBITION BY MUSCARINIC m1 RECEPTORS IN DNA-TRANSFECTED RODENT NEUROBLASTOMA × GLIOMA CELLS

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SUMMARY

1. Acetylcholine (ACh) produces two membrane current changes when applied to NG108-15 mouse neuroblastoma × rat glioma hybrid cells transformed (by DNA transfection) to express m1 muscarinic receptors: it activates a Ca^{2+}-dependent K^+ conductance, producing an outward current, and it inhibits a voltage-dependent K^+ conductance (the M conductance), thus diminishing the M-type voltage-dependent K^+ current ($I_{K(M)}$) and producing an inward current. The present experiments were undertaken to find out how far inhibition of $I_{K(M)}$ might be secondary to stimulation of phospholipase C, by recording membrane currents and intracellular Ca^{2+} changes with indo-1 using whole-cell patch-clamp methods.

2. Bath application of 100 μM ACh reversibly inhibited $I_{K(M)}$ by $47{\cdot}3 \pm 3{\cdot}2$ % ($n = 23$). Following pressure-application of 1 mM ACh, the mean latency to inhibition was 420 ms at 35 °C and 1·79 s at 23 °C. Latencies to inhibition by Ba^{2+} ions were 148 ms at 35 °C and 92 ms at 23 °C.

3. The involvement of a G-protein was tested by adding 0·5 mM GTP-γ-S or 10 mM potassium fluoride to the pipette solution. These slowly reduced $I_{K(M)}$, with half-times of about 30 and 20 min respectively, and rendered the effect of superimposed ACh irreversible. Effects of ACh were not significantly changed after pretreatment for 24 h with 500 ng ml^{-1} pertussis toxin or on adding up to 10 mM GDP-β-S to the pipette solution.

4. The role of phospholipase C and its products was tested using neomycin (to inhibit phospholipase C), inositol 1,4,5-trisphosphate (InsP_3) and inositol 1,3,4,5-tetrakisphosphate (InsP_4), heparin, and phorbol dibutyrate (PDBu) and staurosporin (to activate and inhibit protein kinase C respectively). Both neomycin (1 mM external) and InsP_3 (100 μM intrapipette) inhibited the ACh-induced outward current and/or intracellular Ca^{2+} transient but did not block ACh-induced inhibition of $I_{K(M)}$. Intrapipette heparin (1 mM) blocked activation of $I_{K(Ca)}$ and reduced Ach-induced inhibitions of $I_{K(M)}$, but also reduced inhibition of I_{Ca} via endogenous m4 receptors. PDBu (with or without intrapipette ATP) and staurosporin had no significant effects.

5. ACh induced a transient rise in intracellular [Ca^{2+}] but this did not appear to be responsible for inhibition of $I_{K(M)}$ since (a) the latter preceded the rise in [Ca^{2+}] by

3·6 s, and (b) ACh still inhibited $I_{K(M)}$ when the rise in $[Ca^{2+}]$ was suppressed by (i) repetitive ACh applications, (ii) addition of 100 μM InsP_3 to the pipette solution, and (iii) buffering with 20 mM BAPTA. All three procedures inhibited the ACh-induced outward current.

6. Inhibitors of phospholipase A_2, lipoxygenase, cyclo-oxygenase or nitric oxide synthase had no significant effect on ACh-induced inhibition of $I_{K(M)}$.

7. The presence of GTP (2 mM), ATP (2 mM), dibutyryl-cAMP (1 mM), ATP-γ-S, (500 μM), adenylyl-imidodiphosphate (AMP-PNP, 2 mM), calmodulin (10 μM), calmodulin antipeptide (10 μM) or the growth-associated protein, GAP-43 (2·5 mM) in the pipette did not affect M-current or modify responses to acetylcholine.

8. It is concluded that ACh-induced inhibition of $I_{K(M)}$ is unlikely to be mediated by products of phospholipase C stimulation, either individually or in combination. It is suggested that activation of phospholipase C and inhibition of $I_{K(M)}$ represent parallel pathway responses to ACh, and that $I_{K(M)}$ might be inhibited by a direct effect of the activated G-protein(s) or through another (unidentified) enzymatic product.

INTRODUCTION

In NG108-15 mouse neuroblastoma × rat glioma hybrid cells transformed by DNA transfection to express m1 (or m3) muscarinic acetylcholine receptors, application of acetylcholine produces two membrane current changes: it activates a Ca^{2+}-dependent K^+ current, $I_{K(Ca)}$, and inhibits an M-type voltage-dependent K^+ current, $I_{K(M)}$ (Fukuda *et al.* 1988; Neher, Marty, Fukuda, Kudo & Numa, 1988; Robbins, Caulfield, Higashida & Brown, 1991).

Activation of $I_{K(Ca)}$ probably results from the formation of inositol 1,4,5-trisphosphate (InsP_3) and subsequent release of intracellular Ca^{2+}, since (i) both m1 and m3 receptor activation stimulated inositol phosphate production (Fukuda *et al.* 1988), (ii) outward current activation coincides with a rise in intracellular $[Ca^{2+}]$ (Neher *et al.* 1988), and (iii) a comparable effect is induced by the intracellular iontophoretic injection of either InsP_3 or Ca^{2+} ions (Higashida & Brown, 1986; Robbins, Cloues & Brown, 1992*a*).

The mechanism responsible for $I_{K(M)}$ inhibition is less clear. Experiments on the analogous effect of bradykinin have led to the suggestion that it might result from the parallel formation of diacylglycerols (DAG) and subsequent activation of protein kinase C (PKC), since the effect of bradykinin could be partly replicated by phorbol esters (Higashida & Brown, 1986; Schäfer, Béhé & Meves, 1991) and partly blocked by inhibitors of protein kinase C (Schäfer *et al.* 1991). However, in other cells where $I_{K(M)}$ is inhibited by muscarinic receptor agonists, this mechanism has been discounted because agonist-induced inhibition was not prevented by protein kinase C inhibitors (e.g. frog ganglion cells, Bosma & Hille, 1989). Alternative suggestions include (i) a response to the rise in intracellular $[Ca^{2+}]$ (Kirkwood, Simmons, Mather & Lisman, 1991; but see Pfaffinger, Leibowitz, Subers, Nathanson, Almers & Hille, 1988; Beech, Bernheim, Mathie & Hille, 1991; Marrion, Zucker, Marsh & Adams, 1991), (ii) a Ca^{2+}-independent effect of InsP_3 (Dutar & Nicoll, 1988), or (iii) a direct effect of the activated G-protein (Lopez, 1992; Chen & Smith, 1992).

In the present experiments, therefore, we have attempted to find out more about

the mechanism responsible for $I_{K(M)}$ inhibition by acetylcholine in m1-transformed NG108-15 cells, using whole-cell patch-electrode recording in combination with intracellular [Ca^{2+}] measurements with indo-1. These transformed cells have several advantages for such a study. First, they have a homogeneous population of expressed m1 receptors, obviating some of the complexities of primary neurones. Second, a great deal is already known about their biochemical responses to muscarinic receptor stimulation. Third, the activation of $I_{K(Ca)}$ provides a useful electrophysiological measure of InsP_3-induced Ca^{2+} release against which to judge the effectiveness of procedures designed to suppress this (or an antecedent) component of response. We would hope that the results obtained may be relevant to mechanisms for $I_{K(M)}$ inhibition in other cells.

METHODS

Tissue culture. Neuroblastoma × glioma (NG108-15) cells transfected with the porcine brain acetylcholine muscarinic m1 receptor (subclone PM1-8, Fukuda *et al.* 1988) were continuously grown at 37 °C in Dulbecco's minimal essential medium (DMEM, high glucose) containing 5% fetal calf serum (FCS), hypoxanthine (30 μM), aminopterine (1·2 μM), thymidine (4·8 μM) and L-glutamine (2 mM) and in the presence of 10% CO_2. Cells were grown to 70–80% confluence then passaged 1:3 every 3–4 days. For electrophysiological recording, cells were transferred to 35 mm Petri dishes (density 2000–5000 cells per dish) which had been precoated with polyornithine; for microfluorimetry, they were plated onto polyornithine-coated glass coverslips (22 mm × 22 mm). The cells were differentiated by changing the medium 24 h later to one in which the FCS had been reduced to 1%, aminopterine omitted, and 10 μM prostaglandin E_1 and 50 μM 3-isobutylmethylxanthine (IBMX) added.

Electrophysiology. The whole-cell variant of the patch-clamp technique was used in discontinuous voltage-clamp mode (Axoclamp-2, Axon Instruments, Foster City, CA, USA) as detailed in Robbins, Trouslard, Marsh & Brown (1992*b*). Cells were superfused with a modified Krebs solution at 35 °C, of composition (mM): NaCl, 120; KCl, 3; glucose, 11·1; $NaHCO_2$, 22·6; $MgCl_2$, 1·2; Hepes, 5; $CaCl_2$, 2·5; tetrodotoxin, 0·0005; pH was 7·36 when gassed with 95% O_2–5% CO_2. Flow rates were between 5 and 10 ml min^{-1}. Electrodes (3–5 MΩ) were normally filled with a solution containing (mM); $KOOCCH_3$, 90; KCl, 20; Hepes, 40; $MgCl_2$, 3; EGTA, 3; $CaCl_2$, 1. The calculated free calcium concentration was 40 nM (programme REACT 2·01; G. L. Smith, Physiology Department, Glasgow University); measured resting levels of calcium were around 45 nM (see below). Access resistance was between 4 and 9 MΩ. Cells were voltage clamped at between −20 and −30 mV and M-current deactivation tails were evoked by hyperpolarizing steps for 1 s to −50 or −60 mV (see Robbins *et al.* 1992*b*). Current–voltage relationships were obtained using incremental voltage steps of 10 mV between −110 and −10 mV. Calcium currents were recorded under similar conditions except that the electrode solution was CsCl based and the external medium contained tetraethylammonium chloride instead of NaCl. Currents were evoked by stepping to 0 mV for 500 ms from a holding potential of −90 mV (see Caulfield, Robbins & Brown, 1992, for details).

Pressure ejection. A separate micropipette was used to pressure-apply $BaCl_2$ (100 mM), $CdCl_2$ (1 mM) or acetylcholine (1–10 mM) dissolved in the external superfusate. The pressures routinely used were 138 kPa for 50–300 ms. The pipette was placed upstream of the cell and as close to the cell as possible without inducing pressure artifacts.

Intracellular iontophoresis. In some experiments cells were also impaled with a second electrode filled with either InsP_3 (100–500 μM) or $CaCl_2$ (100–200 mM), and InsP_3 or Ca^{2+} injected by iontophoresis using current pulses of ±10–40 nA for 0·1–1 s (see Robbins *et al.* 1992*a*).

Microfluorimetry. The method used for measuring intracellular [Ca^{2+}] has been previously described in detail (Robbins *et al.* 1992*b*). In brief, cells loaded with either indo-1 AM (the acetoxymethyl ester of indo-1) or indo-1 (via the patch-pipette) were excited with UV light (360 nm) and the emission at 408 and 488 nm measured simultaneously to produce a ratio (R) 408/488. This was converted to free calcium by the expression: $[Ca^{2+}] = [(R - R_{min})/(R_{max} - R)] \times K_d(F_o/F_s)$, where $R_{max} = 4{\cdot}0$, $R_{min} = 0{\cdot}38$ and $K_d(F_o/F_s) = 1400$ nM. Electrode solutions

were as detailed above but with the inclusion of indo-1 tetrapotassium salt (0·1 mM) plus 0·1 mM BAPTA, with no added calcium or EGTA. Some experiments were done on cells preloaded for 30–40 min with 5 μM indo-1-AM ester, to make calcium measurements during the transition from cell-attached to whole-cell recording mode.

Drugs and chemicals. The following were used (with sources): acetylcholine chloride (ACh), apamin, bradykinin (BK), tetrodotoxin (TTX), ethylene glycol bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), 1,2-bis(2-aminophenoxy)ethane -*N,N,N′,N′*-tetraacetic acid (BAPTA), atropine sulphate, adenosine triphosphate (ATP), dibutyryl-cAMP, adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), guanosine triphosphate (GTP), guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S), mastoparan, arachidonic acid (AA), 4-bromophenacyl bromide (BPB), indomethacin, neomycin sulphate, potassium fluoride, doxorubicin (Doxo), phorbol dibutyrate (PDBu), oleic acid, linoleic acid, elaidic acid, heparin low molecular weight, nordihydroguaiaretic acid (NDGA), N^G-nitro-L-arginine (L-NOARG), 8-bromo-cyclic guanosine monophosphate (8-bromo-cGMP), sodium nitroprusside and 2,3-butanedione monoxime (BDM) (all from Sigma, Dorset, UK); 1,4,5-inositol trisphosphate (InsP_3), 1,3,4,5-inositol tetrakisphosphate (InsP_4), BAPTA AM, indo-1, indo-1 AM and calmodulin (CAM) (from Calbiochem, CA, USA); staurosporine and adenylyl-imidodiphosphate (AMP-PNP) (Boehringer Mannheim, Germany); *Bordatella pertussis* (PTX) toxin (Porton Products, Dorset, UK); charybdotoxin (ChTX; Latoxan, Rosans, France); DL-muscarine chloride (RBI, MA, USA); eicosatetraynoic acid (ETYA; Cayman Chemical, MI, USA); and okadaic acid (Scientific Marketing Associates, Herts, UK). Drugs that were not water soluble were dissolved in ethanol or dimethyl sulphoxide (DMSO), and diluted at $\geqslant 1:1000$ in Krebs solution for use. (Control dilutions of DMSO or ethanol were without effect.) GAP-43 C-terminal decapeptide was a gift from Dr G. Milligan (Department of Biochemistry, Glasgow University). Calmodulin binding peptide (CBP), a twenty-nine amino acid polypeptide (Kelly, Weinberger & Waxman, 1988) was synthesized by Dr S. Bansal (Department of Pharmaceutical Chemistry, Kings College, London). Purified protein kinase C (PKC) was a gift from Dr P. J. Parker (ICRF, London). The cyclophilin–cyclosporin A (Cp–CsA) was kindly supplied by Dr R. J. Docherty (Sandoz Institute, London).

RESULTS

Control perfusion of a just-submaximal ACh concentration (100 μM) to a sample of twenty-three m1-transformed cells produced an inward current at the standard holding potential of about −30 mV, and inhibited $I_{K(M)}$ deactivation relaxations at −60 mV by 47·3 ± 3·2% (mean ± S.E.M.; $n = 23$; see Fig. 1 and Table 1). This agrees with previous observations that the $I_{K(M)}$ deactivation relaxations in these cells were inhibited maximally by about 50% at $\geqslant$ 1 mM acetylcholine (Robbins *et al.* 1991). Current–voltage curves (Fig. 1*C*) showed that the inward current resulted from a reduction in the normal outward rectification observed at potentials positive to −60 mV, with little or no change in the linear component of the curve negative to −60 mV. This accords with the supposition that the inward current results from inhibition of $I_{K(M)}$ (Robbins *et al.* 1992*b*). In twelve out of the twenty-three cells, the inward current was preceded by a transient outward current, as previously reported by Fukuda *et al.* (1988) and Neher *et al.* (1988) (Fig. 1).

In a separate sample of eight cells impaled with potassium citrate-filled microelectrodes (cf. Fukuda *et al.* 1988), 100 μM ACh inhibited $I_{K(M)}$ by 60 ± 11·2%; an initial outward current was observed in six of these. Hence, whole-cell patch clamping, and consequent exchange of the normal intracellular solution with the electrode solution, did not materially alter the effect of ACh.

Bradykinin (BK) also activates $I_{K(Ca)}$ and inhibits $I_{K(M)}$ in NG108-15 cells, by activating endogenous BK receptors (Higashida & Brown, 1986; Schäfer *et al.* 1991). In contrast to ACh, the effect of BK wanes rapidly during continued exposure, as shown in Fig. 2*A*. However, in the continued presence of BK, ACh could still inhibit

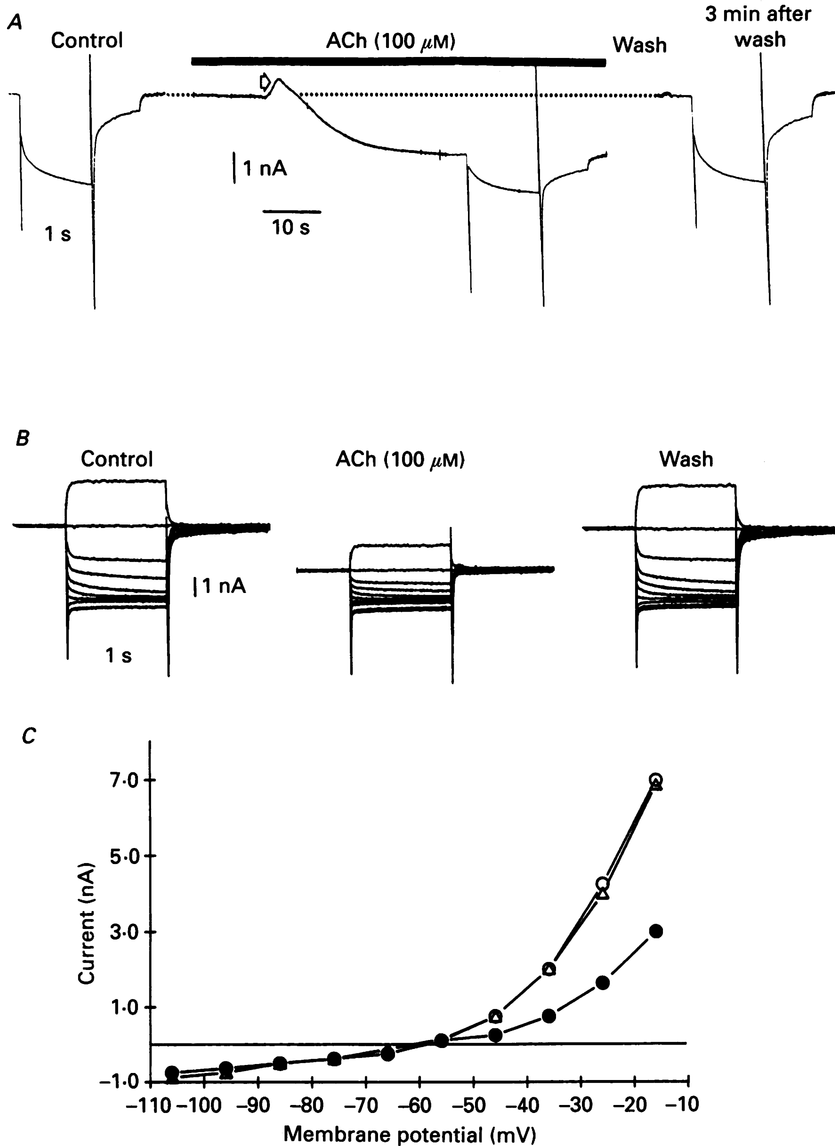

Fig. 1. Effects of ACh on potassium currents in m1 transformed NG108-15 cells. The cell in *A* was clamped at −28 mV (V_H) and stepped to −58 mV for 1 s before, during and after superfusion with 100 μM ACh (at bar). During these hyperpolarizing steps (lower trace) the recorded current shows a slow inward relaxation, due to deactivation of $I_{K(M)}$ (see Robbins *et al.* 1992*b*); the transient inward current at the end of the step is a Ca^{2+} current. Note that ACh induced a transient outward current (at arrow), then an inward current; during the latter the amplitude of the $I_{K(M)}$ deactivation relaxation was reduced. Records in *B* show current responses of another cell to a series of voltage steps from V_H −26 mV to command potentials between −16 and −106 mV in 10 mV increments, recorded before, during and after superfusion with 100 μM ACh. The graphs in *C* show the absolute current level attained at the end of each voltage step in *B* (ordinates, nA) plotted against the command potential (abscissae, mV) before (○), during (●) and after (△) superfusion with ACh.

TABLE 1. Effects of transduction pathway modifiers on M-current inhibition evoked by acetylcholine (100 μM)

Compound	Concentration (mM)	Route†	M-current‡ amplitude	M-current§ inhibition	Number of cells
Control	—	—	330±34	47·3±3·2	23
G-proteins					
GTP-γ-S	0·5	Int	118±24*	53·3±6·9	6
GDP-β-S	10·0	Int	477±173	56·6±6·2	5
Mastoparan	0·1	Int	400±106	47·8±1·4	4
PTX	(500 ng ml^{-1})	Ext	578±107	41·5±5·0	8
Calcium					
Calcium free		Ext	650±216	45·0±4·9	5
BAPTA	20·0	Int	317±73	42·8±4·5	6
BAPTA AM	0·1	Ext	519±62	39·8±5·2	4
Phospholipase C pathway					
Heparin	1·0	Int	546±131	13·0±3·8**	7
Neomycin	1·0	Ext	311±37	40·8±4·7	6
Neomycin	1·0	Int	413±99	49·5±4·5	4
Doxorubicin	0·1	Int	300±67	49·0±2·5	4
InsP_3	0·1	Int	268±86	74·6±6·7**	9
InsP_4	0·1	Int	371±56	42·0±5·9	6
PDBu	0·001	Ext	424±71	33·2±3·7*	11
PDBu (+5 mM ATP)	0·001	Ext	819±180	42·5±5·4	4
PDBu (sharp)	0·001	Ext	700±253	42·6±3·6	5
Staurosporine	0·002	Int	236±42	40·0±1·2	5
Phospholipase A_2					
BPB	0·01	Ext	488±102	45·0±6·9	4
ETYA	0·01	Ext	320±96	49·8±4·9	5
NDGA	0·05	Ext	338±99	60·8±5·3	5
Indomethacin	0·05	Ext	260±80	55·8±6·8	5
Arachidonic acid	0·05	Ext	288±65	41·6±7·5	5
NO synthase pathway					
L-NOARG	0·1	Ext	600±195	41·7±5·5	6
8-Bromo-cGMP	1·0	Ext	510±195	—	5
Sodium nitroprusside	1·0	Ext	490±177	—	5
Nucleotides					
ATP	5·0	Int	725±130	45·8±6·1	4
GTP	2·0	Int	225±38	54·0±4·0	3
AMP-PNP	2·0	Int	300±63	61·8±7·4	5
ATP-γ-S (+1 mM GTP)	0·5	Int	320±42	45·8±6·9	5
Phosphorylation–dephosphorylation					
BDM	20·0	Ext	590±176	66·6±6·1	5
Okadaic acid	0·005	Ext	1250±164	52·6±7·3	8
Cp–CsA	0·00002–0·00005	Int	588±226	53·8±5·2	4
Calmodulin					
CAM	0·01	Int	413±99	44·0±8·3	4
CBP	0·01	Int	808±249	46·3±9·6	3

Significant difference from control: *$P < 0·05$, **$P < 0·0005$.
† Int, internal; Ext, external.
‡ Mean ± S.E.M. amplitude (pA), measured at −50 to −60 mV.
§ Mean ± S.E.M. percentage inhibition.

$I_{K(M)}$. Conversely, superimposition of BK on the partial inhibition produced by ACh induced further inhibition (Fig. 2*B*). Thus, these two agonists seem to inhibit $I_{K(M)}$ in a non-interactive manner, and do not cross-desensitize.

Response latency

To find out how rapidly ACh could inhibit $I_{K(M)}$, we tested the effect of focal pressure-application of high concentrations (1–10 mM) of ACh (Fig. 3; see Methods).

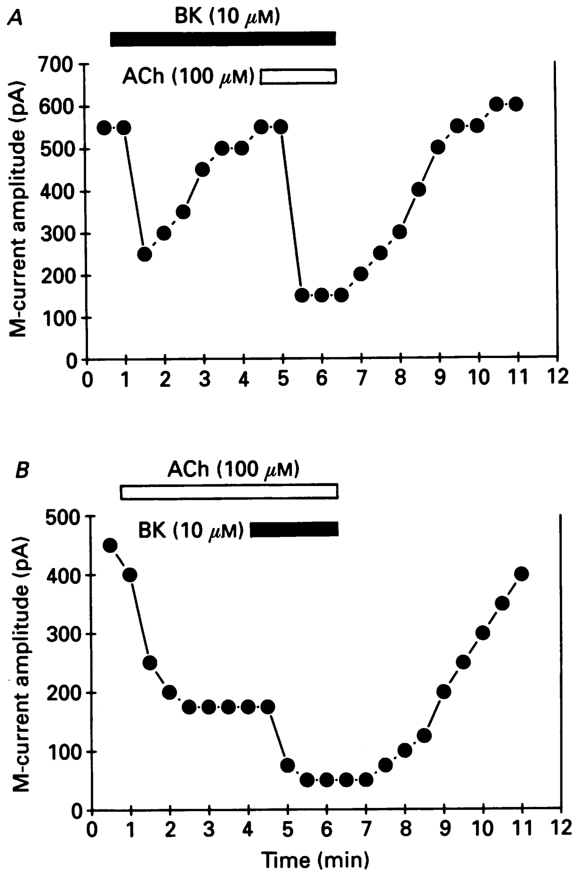

Fig. 2. Effects of ACh and bradykinin on $I_{K(M)}$ in two m1-transformed NG108-15 cells. Graphs show M-current deactivation relaxation amplitudes at the command potential of −60 mV evoked from a holding potential of −30 mV for 1 s every 30 s. Bradykinin (BK, 10 μM) and ACh (100 μM) were applied by bath perfusion for the duration of the filled and open bars respectively. In *A*, BK was applied first and produced an inward current which then waned in the continued presence of BK. Superimposed ACh then induced a second inward current. In *B*, ACh produced a steady inward current, which was increased on subsequent application of BK.

In these experiments, the initial outward current (due to activation of $I_{K(Ca)}$, see Fukuda *et al.* 1988, and Neher *et al.* 1988) was suppressed by adding 100 nM apamin and 10 nM charybdotoxin to the bathing fluid and pressure-pipette solution. The inward current produced by these ACh applications was fully blocked by 1 μM atropine, hence resulted exclusively from muscarinic receptor activation. To control

for the time taken for ejected ACh to reach the cell, we also pressure-applied Ba^{2+} ions: this probably blocks the M-channels directly (Robbins *et al.* 1992*b*). As shown in Fig. 3, the effect of Ba^{2+} was clearly more rapid than that of ACh. The mean latency to observable inward current development after the onset of a pressure-pulse

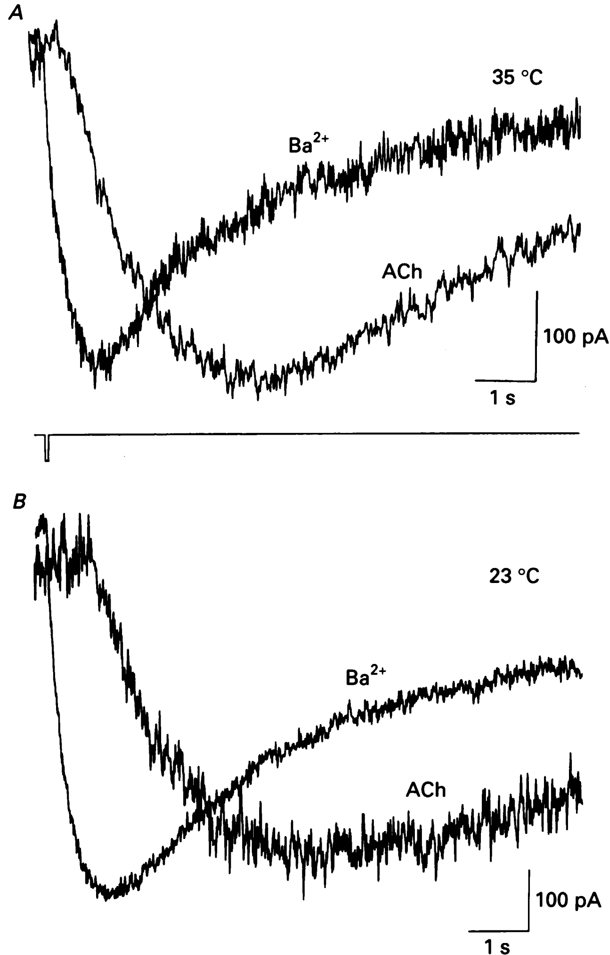

Fig. 3. Time course of $I_{K(M)}$ inhibition by barium (Ba^{2+}) and ACh recorded at 35 °C (*A*) and 23 °C (*B*) (two cells). Micropipettes containing 100 mM Ba^{2+} or 1 mM ACh were placed close to the cell surface and ejected using 100 ms, 138 kPa pressure pulses. Cells were held at -20 mV and bathed in a solution containing apamin (100 nM) and charybdotoxin (10 nM) in order to block contaminating $I_{K(Ca)}$.

of Ba^{2+} was $147 \cdot 7 \pm 14 \cdot 1$ ms ($n = 12$) at 35 °C (minimum latency, 81 ms) and $91 \cdot 7 \pm 8 \cdot 3$ ms ($n = 3$) at 23 °C. In contrast, the mean latencies for the inward current induced by 1 mM ACh were $419 \cdot 5 \pm 63 \cdot 7$ ms ($n = 10$) at 35 °C (minimum latency, 202 ms) and 1793 ± 288 ms at 23 °C ($n = 8$). Increasing the concentration of ACh to 10 mM did not shorten the latency ($412 \cdot 5 \pm 51 \cdot 9$ ms at 35 °C; $n = 4$). Thus, assuming ACh and Ba^{2+} reach the cell membrane at the same time, there is an additional delay of about 270 ms at 35 °C between activation of the m1 receptors and inhibition of

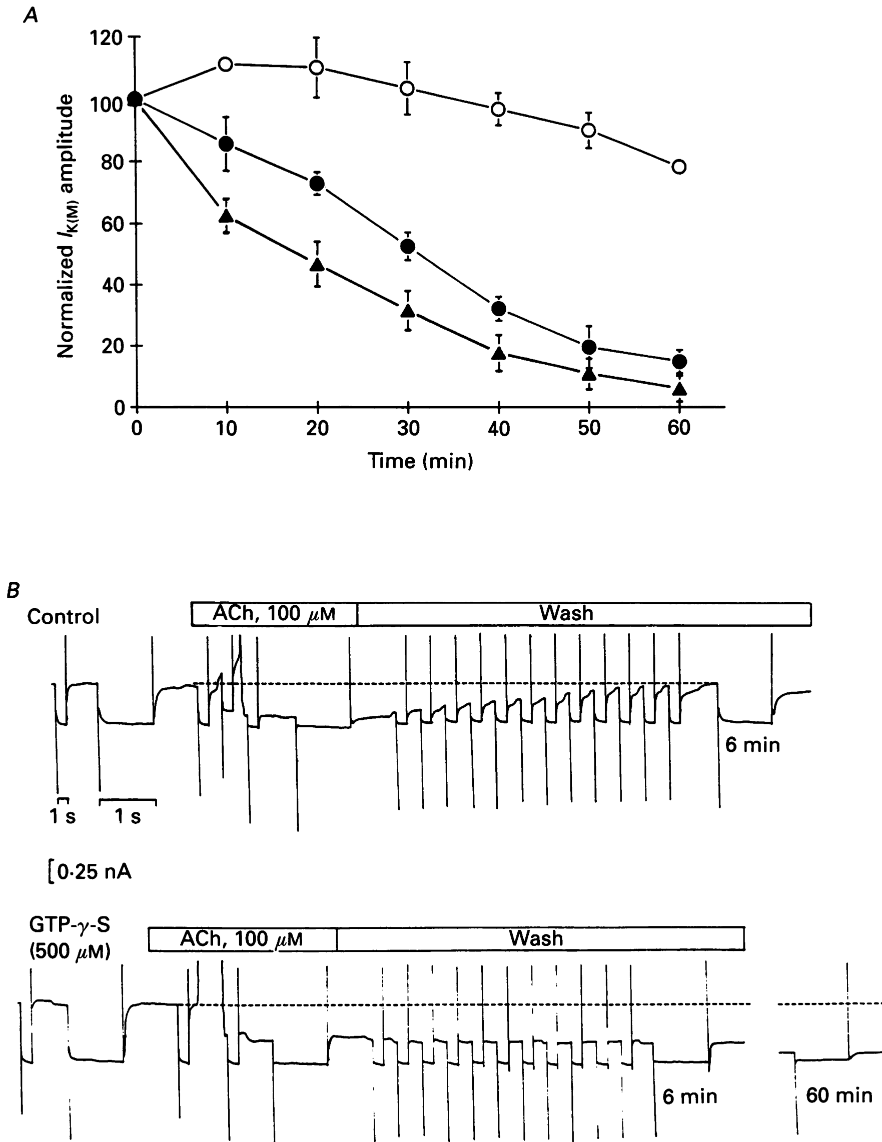

Fig. 4. Effect of irreversible G-protein activation on $I_{K(M)}$. The graph in *A* shows the change in M-current ($I_{K(M)}$) amplitude with time following breakthrough with pipettes containing the normal intracellular solution (○, see Methods), or with the normal solution supplemented with 500 μM GTP-γ-S (●) or 10 mM potassium fluoride (▲). Cells were held at −30 mV and stepped to −60 mV for 1 s every 30 s. M-current amplitude was measured as the amplitude of the deactivation relaxations induced by the hyperpolarizing steps (see Methods and Fig. 1), and expressed as a fraction of that recorded immediately after breakthrough (time 0). Each point is the mean from five cells; bars show S.E.M. Records in *B* show responses of two cells to a brief bath application of ACh, recorded with the normal pipette solution (upper trace) and with 500 μM GTP-γ-S in the pipette (lower trace). Note that $I_{K(M)}$ recovers within 6 min of removing ACh in the absence of GTP-γ-S, but remains depressed 60 min after removing ACh in the presence of GTP-γ-S.

$I_{K(M)}$. This lengthens to about 1·7 s at 23 °C, giving a temperature coefficient, Q_{10}, over the range 22–35 °C, for the transduction process of about 3·6.

NG108-15 cells possess endogenous m4 muscarinic acetylcholine receptors, activation of which inhibits an ω-conotoxin-sensitive component of Ca^{2+} current (Caulfield *et al.* 1992). There is no evidence to suggest that this involves any intracellular transduction process; instead, by analogy with equivalent effects in sympathetic neurones (Mathie, Bernheim & Hille, 1992), it seems more likely that this effect involves a local ('membrane-delimited') response. It therefore seemed interesting to find out how rapidly ACh could inhibit the Ca^{2+} current. To test this, we applied long (2 s) depolarizing steps to 0 mV from −90 mV using Cs^{+}-filled electrodes (see Methods), and then pressure-applied either 1 mM ACh or 1 mM Cd^{2+} (which blocks the Ca^{2+} channels directly) at a predetermined time (usually 50–100 ms) after the onset of the voltage step. Mean latencies to detectable reduction in I_{Ca} (at 35 °C) were: Cd^{2+}, 69·3 ± 3·5 ms ($n = 4$); ACh, 68·1 ± 3·3 ms ($n = 6$). These were indistinguishable, and probably within the limits of the pressure-ejection system. Notwithstanding, the effect of ACh on I_{Ca} was clearly much more rapid than its effect on $I_{K(M)}$.

G-proteins

To test whether a G-protein was involved in the transduction pathway between muscarinic receptor activation and M-current inhibition, we added GTP (2 mM), GTP-γ-S (0·5 mM), GDP-β-S (up to 10 mM) or potassium fluoride (10 mM) to the pipette solution. Addition of GTP did not modify the response to 100 μM ACh (see Robbins *et al.* 1991). Addition of GTP-γ-S or potassium fluoride caused a slow run-down of the current, with half-times of about 20 min (for potassium fluoride) and 30 min (for GTP-γ-S) (Fig. 4*A*). When ACh (100 μM) was applied in the presence of 0·5 mM intracellular GTP-γ-S, maximum inhibition of residual $I_{K(M)}$ was not significantly increased (Table 1), but there was no longer any recovery following wash-out of ACh (Fig. 4*B*). These effects agree qualitatively with those previously reported in sympathetic ganglion cells (Pfaffinger, 1988; Brown, Marrion & Smart, 1989; Lopez & Adams, 1989). However, and unlike previous observations on sympathetic ganglion cells where a partial block of the agonist-evoked response was seen (see above, and Simmons & Mather, 1991), GDP-β-S, even at 10 mM, had no significant inhibitory effect on the action of ACh (Table 1). (GDP-β-S was also ineffective in preventing inhibition of I_{Ca} by noradrenaline; I. McFadzean, Department of Pharmacology, Kings' College London, personal communication. Resistance to GDP-β-S might result from a high rate of GTP production in these cells, as evidenced by the lack of any requirement for added GTP in the pipette solution.)

In agreement with previous observations on sympathetic neurones (Pfaffinger, 1988), pretreatment for 24 h with 500 ng ml^{-1} pertussis toxin did not significantly reduce the effect of ACh (Table 1). In parallel experiments on these cells, the same pretreatment regimen suppressed the inhibition of I_{Ca} by acetylcholine (Higashida, Hashii, Fukuda, Caulfield, Numa & Brown, 1990).

We also tested the effects of adding to the pipette solution 100 μM mastoparan (which directly activates certain G-proteins; Higashijima, Uzu, Nakajima & Ross, 1988) or 2·5 mM of the C-terminal decapeptide of GAP-43 (which has been reported

to modify the activity of one G-protein, G_o; Strittmatter, Valenzuela, Kennedy, Neer & Fishman, 1990). Neither modified responses to ACh (Table 1).

Phospholipase C and products of its activation

ACh activates phospholipase C and induces the formation of inositol phosphates in m1- and m3-transformed cells (Fukuda *et al.* 1988). We tested the role of phospholipase C and products of its activation in mediating the inhibition of $I_{K(Ca)}$ by ACh as follows.

Phospholipase C (PLC). Neomycin and doxorubicin inhibit the production of inositol trisphosphate by PLC and block Ca^{2+} transients in other cells resulting from agonist-induced InsP_3 formation (e.g. Penner, 1988; Oakes, Schlager, Santone, Abraham & Powis, 1990). In the present experiments, 1 mM neomycin prevented the generation of the outward current $I_{K(Ca)}$ by ACh (which provides an index of Ca^{2+} release, see below) in five out of six cells tested, but did not significantly affect ACh-induced inhibition of $I_{K(M)}$ (Table 1). Doxorubicin (100 μM) did not significantly affect either the amplitude of the initial outward current induced by ACh ($n = 4$) or the amount of $I_{K(M)}$ inhibition (Table 1). Further, even at a concentration 100 times higher than that reported to suppress histamine-induced increases in intracellular Ca^{2+} in N1E-115 neuroblastoma cells (Oakes *et al.* 1990), doxorubicin did not reduce the intracellular Ca^{2+} transient resulting from ACh application in NG108-15 cells (see Fig. 10 below).

Inositol trisphosphate (InsP$_3$). As in microelectrode-impaled cells (Higashida & Brown, 1986), intracellular iontophoretic injection of InsP_3 induced a transient outward current (Robbins *et al.* 1992*a*), and so replicated the initial response to ACh, but this was not succeeded by inhibition of $I_{K(M)}$. However, this may result from rapid metabolism, or rapid dilution into the patch pipette. We therefore tested whether a more sustained application of InsP_3 via the patch pipette might either inhibit $I_{K(M)}$ or occlude the effect of subsequent applications of ACh. When 100 μM InsP_3 was added to the pipette solution, breakthrough induced a transient rise in intracellular Ca^{2+} which then returned to resting levels after some 2 min (Fig. 9). (In some cells, a transient outward current could also be detected.) Thereafter, a subsequent application of ACh no longer induced a rise in intracellular Ca^{2+} (see below and Fig. 10). Notwithstanding, the M-current deactivation relaxations were not significantly reduced in the presence of InsP_3, and the inhibitory action of ACh was significantly *increased* (from 47 to 74%; Table 1.) Thus, InsP_3 augmented, rather than blocked, the action of ACh. Inclusion of 100 μM inositol 1,3,4,5-tetrakisphosphate (InsP_4) in the pipette solution had no significant effect on the action of ACh (Table 1).

Heparin has been reported to prevent InsP_3-induced Ca^{2+} release (e.g. Ghosh, Eis, Mullaney, Ebert & Gill, 1988). Inclusion of 1 mM heparin in the patch pipette solution prevented the elevation of intracellular Ca^{2+} by ACh (Fig. 10) and also largely suppressed the inhibitory action of ACh on $I_{K(M)}$ (Table 1). However, heparin is far from being a specific inhibitor of the InsP_3 receptor, since it also inhibits receptor coupling to certain G-proteins (e.g. Willuwett & Aktories, 1988). We therefore tested the effect of intracellular heparin on the inhibition of I_{Ca} by ACh and noradrenaline, which is mediated by a different (pertussis toxin sensitive) G-protein (see above) and so is unlikely to involve InsP_3. Heparin (1 mM) reduced the inhibitory effect of both

agonists on I_{Ca}, from 23·6 ± 1·7 % ($n = 8$) to 13·2 ± 2·0 % ($n = 5$) in the case of ACh, and from 22·4 ± 1·4 % ($n = 5$) to 10·3 ± 3·7 % ($n = 6$) with noradrenaline as agonist (means ± S.E.M.).

Protein kinase C (PKC). It has previously been suggested that inhibition of $I_{K(M)}$ in NG108-15 cells by bradykinin might be mediated by diacylglycerol, rather than

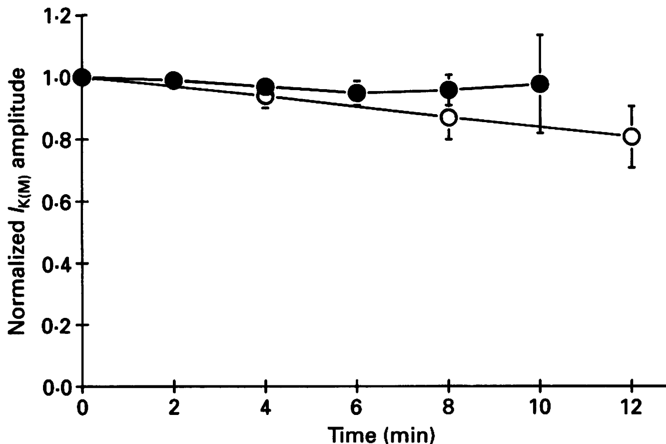

Fig. 5. Effect of PDBu (1 μM) on $I_{K(M)}$ recorded using whole-cell patch electrodes (●, 10 cells) or 25–40 MΩ potassium citrate-filled microelectrodes (○, 4 cells). M-current amplitude was normalized to the control response (at time 0) before application of PDBu. Bars show S.E.M.

by InsP_3, since the effect of bradykinin was partly mimicked by phorbol esters such as phorbol dibutyrate (PDBu; Higashida & Brown, 1986; Schäfer *et al.* 1991). We attempted to test this further, but could not detect any significant inhibition of $I_{K(M)}$ in the present experiments after 10 min perfusion with 1 μM PDBu (Fig. 5). To test whether we had dialysed out some essential intracellular ingredient with the patch pipette, we repeated this test using the citrate-filled microelectrodes as employed by Higashida & Brown (1986), but could detect no more than 20 % inhibition after 12 min perfusion (Fig. 5). In whole-cell recordings, the inhibitory effect of ACh on $I_{K(M)}$ was reduced (at the 5 % level of significance) from 47 to 33 % in the presence of 1 μM PDBu, but no significant reduction was observed in the presence of PDBu with 5 mM ATP in the pipette, or following pre-incubation in 1 μM PDBu for 24 h (to down-regulate PKC). Further, 10–30 min application of 2 μM staurosporin (which inhibits PKC; Rüegg & Burgess, 1989) did not reduce the effect of ACh (Table 1). Finally, in two cells, we added 100 nM purified PKC plus 0·1 mM dithiothreitol, 5 μg ml^{-1} bovine serum albumin, 0·5 mM GTP and 2 mM ATP to the patch-pipette solution and raised [Ca^{2+}] to 120 nM. However, this had no obvious effect on the amplitude of the M-current deactivation relaxations, and did not inhibit the effect of ACh.

Calcium

It has been suggested that muscarinic inhibition of $I_{K(M)}$ in frog sympathetic ganglia results from elevation of intracellular [Ca^{2+}] (Kirkwood *et al.* 1991). This also seems a possible mechanism for $I_{K(M)}$ inhibition in NG108-15 cells because previous

observations suggested that a transient rise in intracellular [Ca^{2+}] produced (for example) by a voltage-activated Ca^{2+} current could produce a temporary inhibition of $I_{K(M)}$ (Robbins *et al.* 1992*b*). Hence, we have explored the relationship between intracellular [Ca^{2+}] and $I_{K(M)}$ in more detail, by recording indo-1 fluorescence and membrane current under voltage clamp.

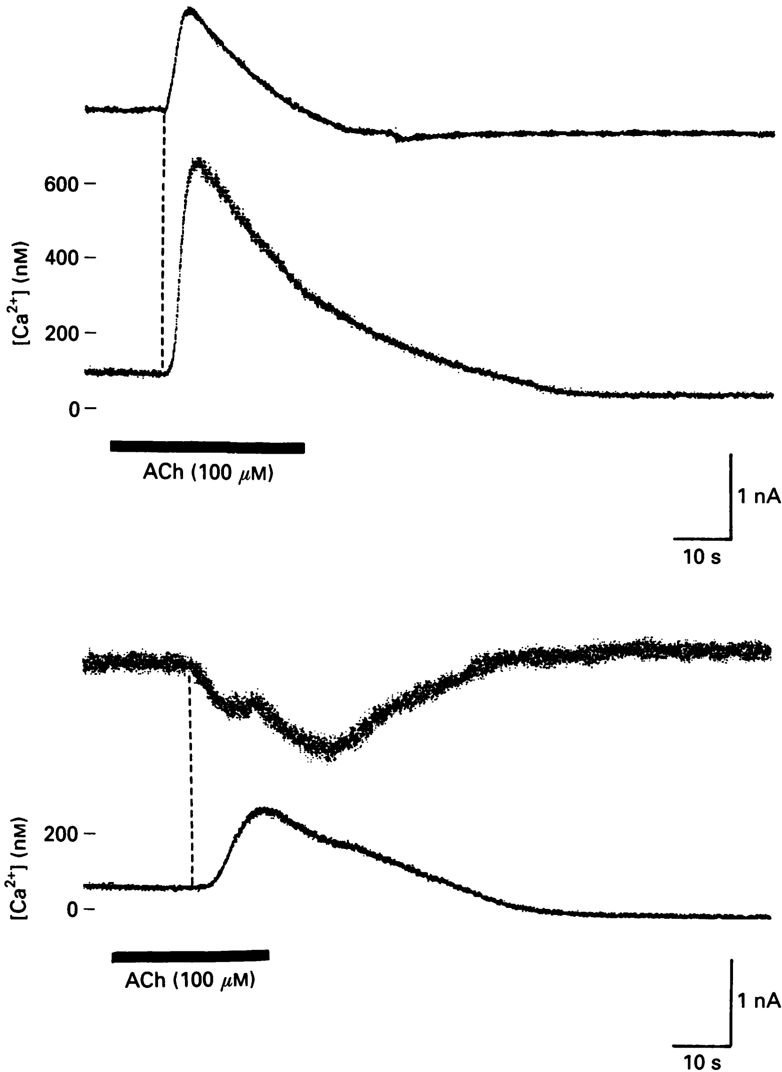

Fig. 6. Simultaneous recording of membrane current (upper traces) and intracellular [Ca^{2+}] (lower traces, calculated from indo-1 fluorescence; see Methods) in two NG108-15 cells during applications of 100 μM ACh. Cells were clamped at −20 mV using patch pipettes containing 100 μM indo-1. In *A*, ACh induced an outward current coincident with the rise in [Ca^{2+}], followed by an inward current. In *B*, ACh induced an initial inward current which *preceded* the rise in [Ca^{2+}]; this is interrupted by an outward current deflection during the rise in [Ca^{2+}].

Under resting conditions (estimated membrane potential between −60 and −80 mV) cells preloaded with indo-1 AM yielded an estimated free Ca^{2+} concentration of 44·8 ± 7·1 nM ($n = 13$). Cells patched with electrodes containing 0·1 mM indo-1 and 0·1 mM BAPTA and initially clamped at −60 mV, then

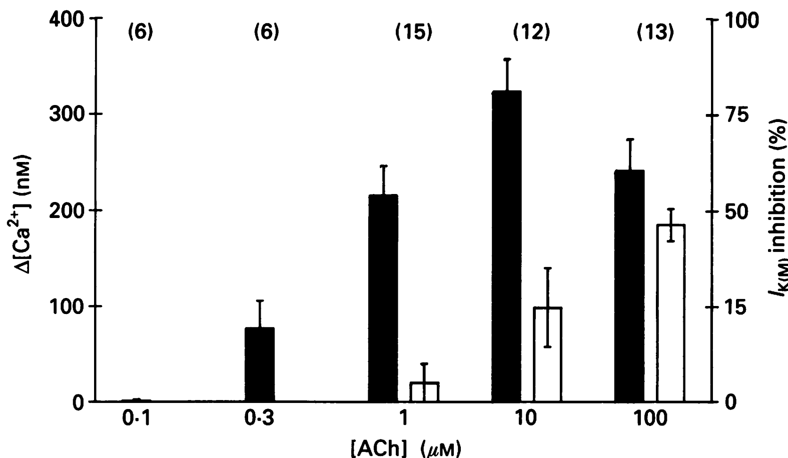

Fig. 7. Concentration–response relationship for ACh-stimulated $[Ca^{2+}]$ rise (filled bars) and $I_{K(M)}$ inhibition (open bars). Values for $I_{K(M)}$ inhibition are taken from Robbins *et al.* (1991). Values for $[Ca^{2+}]$ rises are means of the number of determinations shown above the bars. All values are means ± S.E.M.

depolarized to between −20 and −30 mV, gave stable resting free Ca^{2+} concentrations of 63·5 ± 5·7 nM ($n = 23$). (The depolarization itself did not consistently change the fluorescence ratio.)

Bath application of ACh (100 μM) to cells clamped at between −30 and −20 mV consistently produced a transient (30–60 s) increase in intracellular $[Ca^{2+}]$ of between 100 and 461 nM (e.g. Fig. 6). This declined to normal or subnormal levels during continued superfusion with ACh. In many (but not all) cells the rise in Ca^{2+} was accompanied by a transient outward current, as previously described by Neher *et al.* (1988) (Fig. 6*A*). The onset of this outward current slightly preceded the onset of the Ca^{2+} transient by 0·6 ± 0·4 s ($n = 10$). This may reflect the difference between the rate of change of the submembrane calcium compared to that of the whole cell as monitored by indo-1. In other cells (e.g. Fig. 6*B*), little or no outward current was seen, leaving only an inward current (which presumably resulted from inhibition of $I_{K(M)}$, see above). The onset of this inward current also preceded the rise in $[Ca^{2+}]$, by 3·9 ± 0·9 s ($n = 4$).

Tests using different concentrations of ACh showed that the threshold for detectable increases in intracellular $[Ca^{2+}]$ was about 0·1 μM, and that a maximum increase was obtained with 10 μM. Thus, the concentrations of ACh required to raise intracellular $[Ca^{2+}]$ were at least 10 times lower than those needed to inhibit $I_{K(M)}$ (Fig. 7; see also Robbins *et al.* 1991).

When ACh was applied repeatedly at intervals of 10–15 min, the Ca^{2+} response progressively diminished, becoming undetectable after four or five such applications (Fig. 8*A* and *C*). Correspondingly, the initial outward current induced by ACh also

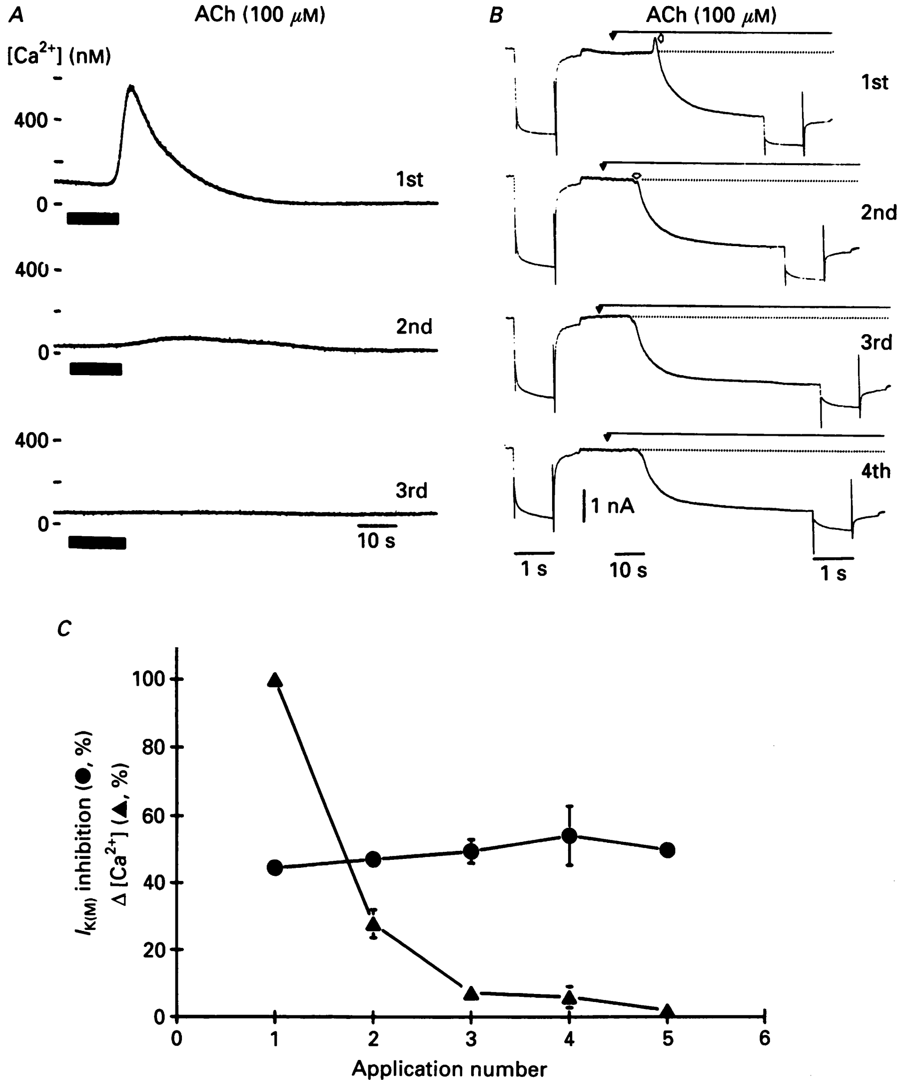

Fig. 8. Effect of repeated applications of ACh on intracellular [Ca^{2+}] (*A*) and membrane currents (*B* and *C*). Cells were held between −30 and −20 mV. ACh (100 μM) was applied for 1 min at approximately 10–15 min intervals. In *A*, such applications produced rapidly diminishing increments in intracellular [Ca^{2+}]. The records in *B* (from another cell) show a corresponding decline in the initial outward current, with no substantial change in the subsequent inward current or inhibition of $I_{K(M)}$ (indicated by the inward currents generated by 1 s hyperpolarizing steps before and after application of ACh, cf. Fig. 1). The graph in *C* shows the mean percentage inhibition of $I_{K(M)}$ (●) and mean rise in [Ca^{2+}] (▲ expressed as a percentage of that produced by the first application of ACh) recorded simultaneously following up to five successive applications of ACh (pooled data from 17 cells; bars shown S.E.M.).

declined (Fig. 8*B*). In contrast, there was no apparent diminution in the inward current response (Fig. 8*B*), nor in the amount of inhibition of $I_{K(M)}$ as measured from the amplitudes of the deactivation relaxations (Fig. 8*C*).

As noted above, when InsP_3 (100 μM) was included in the pipette solution, a transient rise in [Ca^{2+}] occurred on breakthrough into cells preloaded with indo-1 AM, which subsequently declined (Fig. 9). A similar (though smaller) effect was seen on adding 0·5 mM GTP-γ-S to the pipette solution (Fig. 9*B*). In the continued presence of

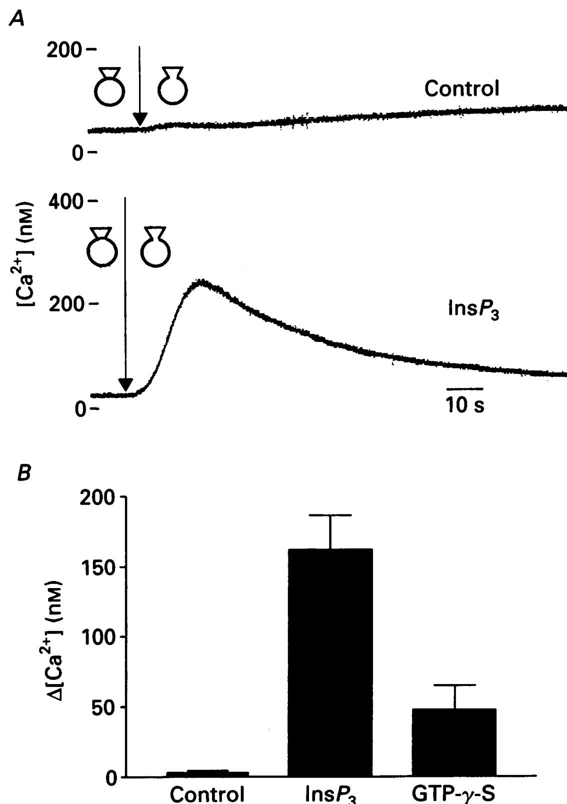

Fig. 9. Changes in intracellular [Ca^{2+}] on the transition between cell-attached and whole-cell recording mode ('breakthrough'). Records in *A* show intracellular [Ca^{2+}] signals following breakthrough (at the arrows) in two cells which were preloaded with indo-1 AM (5 μM, 40 min). In the upper record, the pipette solution contained 100 μM indo-1 and 100 μM BAPTA. In the lower record, an additional 100 μM InsP_3 was added to the electrode solution. The histogram in *B* shows the mean rise in intracellular [Ca^{2+}] on breakthrough using electrodes containing 100 μM indo-1 plus 100 μM BAPTA without addition (controls; $n = 4$), with additional InsP_3 (100 μM; $n = 4$) and with additional GTP-γ-S (500 μM; $n = 3$). Bars show S.E.M.

intrapipette InsP_3, ACh no longer increased intracellular [Ca^{2+}] (Fig. 10), though it still inhibited $I_{K(M)}$ (Table 1). The increase in intracellular [Ca^{2+}] by ACh was also prevented by adding 1 mM heparin to the pipette solution, but not by 100 μM internal doxorubicin (Fig. 10; see also above).

We also tested whether ACh-induced inhibition of $I_{K(M)}$ could be prevented by buffering intracellular Ca^{2+}. Addition of 20 mM BAPTA to the pipette solution or 100 μM BAPTA AM to the bathing solution not only prevented ACh-induced rises in

intracellular [Ca^{2+}] as measured with indo-1 fluorescence, but also suppressed the Ca^{2+}-dependent outward current induced by ACh ($n = 4$). Notwithstanding, there was no significant change in ACh-induced M-current inhibition following either procedure (Table 1). ACh-induced M-current inhibition also persisted in a Ca^{2+}-free external medium containing 5 mM Mg^{2+} and 0·1 mM EGTA (Table 1).

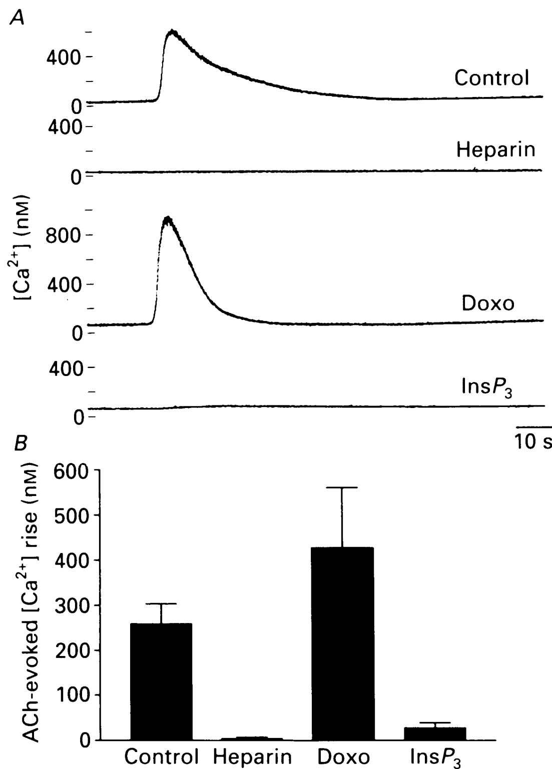

Fig. 10. ACh-evoked calcium rises in the presence of some internally applied compounds. Cells were held at −20 mV. Records in *A* show (from above downwards) responses to bath applications of ACh (100 μM) in control cell (patched with an electrode containing 100 μM indo-1) and in cells patched with electrodes containing 100 μM indo-1 with additional heparin (1 mM), doxorubicin (Doxo; 100 μM), and InsP_3 (100 μM). The histogram in *B* shows the mean ACh-induced rises in [Ca^{2+}] in controls ($n = 10$) and in the presence of internal heparin (1 mM; $n = 5$), doxorubicin (100 μM; $n = 5$) and InsP_3 (100 μM; $n = 8$). Bars show S.E.M.

Phospholipase A_2 pathway

In transformed fibroblasts, activation of m1 and m3 receptors stimulates phospholipase A_2, leading to the production of arachidonic acid (Conklin, Brann, Buckley, Ma, Bonner & Axelrod, 1988). Further, arachidonic acid (AA), or its metabolites, have been reported to modify M-currents in NG108-15 cells (Béhé & Meves, 1992). Hence, we tested whether the formation of AA or its metabolites might contribute to the inhibition of $I_{K(M)}$ by ACh. Addition of AA itself inhibited $I_{K(M)}$ at concentrations above 10 μM (Fig. 11). This appeared not to be due to metabolism by

the lipoxygenase or cyclo-oxygenase pathways since ETYA, (an AA analogue which is not a substrate for these enzymes, but instead inhibits them) produced the same effect, inhibiting $I_{K(M)}$ by 26·4 ± 7·0 %, $n = 5$; and the action of AA was not prevented by indomethacin (an inhibitor of cyclo-oxygenase) or nordihydroguaretic acid

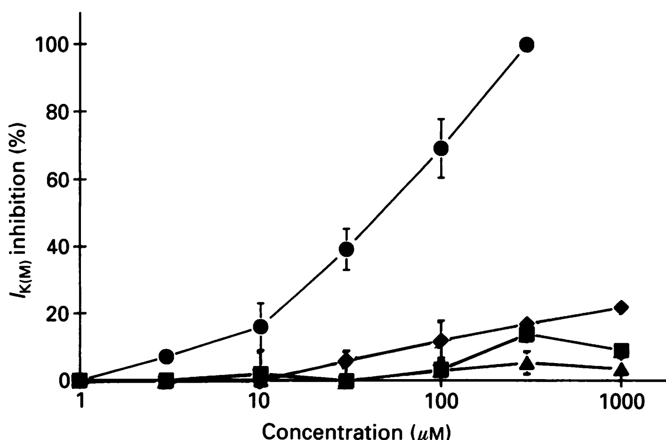

Fig. 11. Effect of fatty acids on $I_{K(M)}$. Graphs show mean percentage inhibition of $I_{K(M)}$ (measured from deactivation relaxations, see Methods) produced by increasing concentrations of arachidonic acid (●, $n = 12$), oleic acid (◆, $n = 5$), linoleic acid (■), $n = 3$) and elaidic acid (▲, $n = 2$). Bars show S.E.M.

(NDGA, an inhibitor of lipoxygenase). Other fatty acids which have been reported to modify K^+ currents in neuroblastoma cells, such as linoleic, oleic and elaidic acids (Rouzaire-Dubois, Gerard & Dubois, 1991), had no substantial effect on $I_{K(M)}$ at concentrations up to 1 mM (Fig. 11).

In the presence of 50 μM AA, ACh still reduced the residual M-current. Further, the inhibitory effect of ACh was not significantly reduced in the presence of 50 μM ETYA, 50 μM NDGA, 50 μM indomethacin, or 10 μM 4-bromophenacylbromide (BPB), which inhibits phospholipase A_2. (NDGA itself partly inhibited $I_{K(M)}$ by 23·4 ± 10·5 % ($n = 5$). This may be a channel-blocking effect, since it also inhibits Ca^{2+} currents at comparable concentrations; Korn & Horn, 1990.)

Hence, we conclude that neither AA nor its metabolites contribute significantly to the inhibitory action of ACh. The depressant effect of AA itself might arise from a direct effect on the M-channels or the surrounding lipid (Ordway, Singer & Walsh, 1991). An alternative possibility is that AA activated PKC (see McPhail, Clayton & Snyderman, 1984) but this seems less likely because its effect was not imitated by oleic or linoleic acids.

Nitric oxide synthase pathway

Agents which stimulate phospholipase C in NG108-15 cells and raise intracellular Ca^{2+} also activate guanylate cyclase and increase intracellular cyclic GMP, probably through the Ca^{2+}-induced formation of nitric oxide (NO; Reiser, 1990). To find out if this pathway might be involved in ACh-induced inhibition of $I_{K(M)}$ we tested the

effects of sodium nitroprusside (which liberates NO and stimulates cGMP production), cGMP and N^{G}-nitro-L-arginine (L-NOARG), which inhibits NO production from L-arginine by nitric oxide synthase (Moore, al-Swayeh, Chong, Evans & Gibson, 1990). Neither sodium nitroprusside (1 mM) nor 8-bromo-cGMP (1 mM) significantly inhibited $I_{K(M)}$, nor did L-NOARG (100 μM) significantly reduce the inhibitory effect of ACh (Table 1).

Phosphorylation–dephosphorylation

Activation of PLC by bradykinin in NG108-15 cells induces the phosphorylation of several proteins and the dephosphorylation of others (Higashida *et al.* 1991). Staurosporin is a broad-spectrum kinase inhibitor (Rüegg & Burgess, 1989), so the lack of effect of staurosporin (Table 1) suggests that the inhibitory action of ACh does not result from phosphorylation by most common kinases. Notwithstanding, we pursued this possibility further by testing the effect of promoting phosphorylation by adding 0·5 mM ATP-γ-S to the pipette solution (with excess GTP, to preclude effects resulting from transphosphorylation to GDP; Otero, 1990), or inhibiting phosphorylation with AMP-PNP (in the absence of added ATP). Neither of these substances significantly affected $I_{K(M)}$ or the inhibitory effect of ACh (Table 1). Unlike Chen & Smith (1992), we detected no increased 'run-down' of $I_{K(M)}$ in cells loaded with ATP-γ-S.

An alternative possibility is that ACh might induce *de*phosphorylation via a protein phosphatase. We tested this by (i) applying a chemical phosphatase (2,3-butanedione monoxime, BDM; see Huang & McArdle, 1992) and (ii) okadaic acid, a phosphatase inhibitor. BDM (20 mM) reduced $I_{K(M)}$, but this was rapid and not reversed by cAMP (cf. Huang & McArdle, 1992) so probably reflected a channel-blocking action. Okadaic acid did not affect the inhibitory action of ACh (Table 1) in a concentration (5 μM) which has been reported to enhance and prolong the effect of isoprenaline on cardiac Ca^{2+} currents (Hescheler, Mieskes, Rüegg, Takai & Trautwein, 1988). (Although the mean current amplitude recorded in these experiments was larger than normal, this merely reflects endogenous variations, since the addition of okadaic acid did not reduce $I_{K(M)}$.)

We also tested the effect of adding calmodulin or the calmodulin-inhibitory binding peptide (Kelly *et al.* 1988) to the pipette solution and found no significant effect on the action of ACh (Table 1). Finally inhibition of the Ca^{2+} and calmodulin-dependent phosphatase, calcineurin, by a cyclophilin–cyclosporin A complex (Liu, Farmer, Lane, Friedman, Weissman & Schreiber, 1991) did not alter $I_{K(M)}$ or the nature of the agonist responses (Table 1).

Multiple pathways

It is possible that no one pathway dominates the inhibitory effect of ACh. Therefore we tested the effect of a 'cocktail' of potential inhibitors, using InsP_3 (100 μM), staurosporin (2 μM) and neomycin (1 mM) in the pipette solution (with 2 mM ATP and 0·25 mM GTP), and neomycin (1 mM), staurosporin (2 μM) and BPB (10 μM) in the bathing solution. In eight such cells the mean amplitude of $I_{K(M)}$ was 628 ± 105 pA and ACh inhibited $I_{K(M)}$ by $49{\cdot}4 \pm 8{\cdot}7$ %, i.e. not significantly different from control cells.

DISCUSSION

The most obvious electrophysiological manifestation of phospholipase C stimulation by ACh in these transformed NG108-15 cells is the initial outward current (see Fukuda *et al.* 1988; Neher *et al.* 1988). This is a Ca^{2+}-activated K^+ current, which results from the formation of InsP_3 and consequent release of intracellular Ca^{2+}. Thus, in the present experiments, this current (or the associated rise in intracellular [Ca^{2+}]), was readily prevented by buffering intracellular Ca^{2+} with 20 mM BAPTA, by intracellular perfusion with high concentrations of InsP_3 or with heparin, and (partly) by inhibiting phospholipase C with neomycin. This response also proved rather labile, and was readily desensitized with repeated applications of ACh.

The effectiveness of these procedures on the outward current thus provides a useful yardstick by which we can evaluate the role of the phospholipase C–InsP_3–Ca^{2+} cascade in mediating the inhibition of $I_{K(M)}$ by ACh. By this criterion such a role seems most unlikely. Thus, only one of these procedures clearly affected the inhibitory action of ACh on $I_{K(M)}$, namely, perfusion with heparin. However, since heparin also reduced the inhibition of I_{Ca} by ACh (or noradrenaline), inhibition of $I_{K(M)}$ might well have resulted from an effect of heparin on the primary step of G-protein activation (e.g. Willuwett & Aktories, 1988).

None of the other procedures significantly reduced $I_{K(M)}$ inhibition. Thus, intracellular perfusion with 100 μM InsP_3 or InsP_4 neither inhibited $I_{K(M)}$ nor reduced the inhibitory action of ACh. This concentration of InsP_3 probably exceeds that likely to be formed following receptor-mediated stimulation of phospholipase C by at least a factor of 4 (e.g. Bird, Oliver, Horstman, Obie & Putney, 1991), so would be expected both to replicate and occlude any action of ACh due to InsP_3 formation; and indeed, it completely occluded both the rise in [Ca^{2+}] and the outward current produced by ACh. In contrast, the inhibitory effect of ACh on $I_{K(M)}$ was *augmented* by InsP_3, rather than inhibited (cf. Dutar & Nicoll, 1988). (This is interesting, but of uncertain significance.)

As previously noted in frog ganglion cells (Pfaffinger *et al.* 1988), there was also a clear dissociation between the rise in [Ca^{2+}] and the inhibition of $I_{K(M)}$ (or the consequential inward current) in these NG108-15 cells. First, when ACh was applied repeatedly, the Ca^{2+} signal dissipated (along with the outward current), without any corresponding reduction in the amount of M-current inhibition. Second, simultaneous recording of inward current and Ca^{2+} transients showed that the inward current *preceded* the rise in [Ca^{2+}] by 3–4 s. This lag was much greater than that (0·6 s) preceding the outward current. Third, inhibition of $I_{K(M)}$ was not prevented by adding 20 mM BAPTA to the pipette solution. While the concentration of BAPTA in the cell might well not achieve that in the pipette (Beech *et al.* 1991), this procedure was clearly sufficient to prevent activation of $I_{K(Ca)}$ in our NG108-15 cells. It might be argued that the amount of Ca^{2+} required to initiate M-current inhibition is much less than that necessary to activate $I_{K(Ca)}$. This would have some force if the dose–response curve for ACh-induced Ca^{2+} release lay to the right of that for M-current inhibition, but in fact the opposite is the case (see Fig. 7), implying that, if the M-current is sensitive to Ca^{2+}, it is less so than $I_{K(Ca)}$. In previous experiments where $I_{K(M)}$ was inhibited following a voltage-gated Ca^{2+} current (Robbins *et al.*

1992*b*), we might suppose that the submembrane Ca^{2+} concentration attained much higher levels than those following release of Ca^{2+} from internal stores by InsP_3.

An alternative messenger pathway (previously suggested to mediate the inhibition of $I_{K(M)}$ by bradykinin in these cells: Higashida & Brown, 1986; Schäfer *et al.* 1991) might be through the formation of diacyglycerol and consequent activation of protein kinase C (PKC). However, we could obtain no clear evidence to support this as a potential mechanism for ACh-induced inhibition. First, the effects of activating PKC with phorbol dibutyrate (PDBu) were small and marginally significant. Since the action of PDBu was not enhanced by increasing the supply of ATP, by raising intracellular [Ca^{2+}], or by adding purified PKC to the pipette solution, we have no ready explanation for the weaker effect of PDBu than that detected in previous experiments on these cells. We should add that equivalent exposures of hippocampal cells to the same samples of PDBu under essentially similar conditions inhibited the slow Ca^{2+}-activated K^+ current in these cells (Sim, Gerber, Knöpfel & Brown, 1992), suggesting that it should have effectively activated PKC. Second, the effect of ACh was not reduced by concentrations of staurosporin which, in these parallel experiments in hippocampal cells (Sim *et al.* 1992), effectively blocked the action of PDBu. Third, pretreatment with PDBu, which would be expected to 'down-regulate' PKC in NG108-15 cells (Fu, Okano & Nozawa, 1988), did not prevent the action of ACh. Thus, although PKC might potentially be capable of modulating $I_{K(M)}$, it seems that activation of PKC did not contribute strongly to the inhibitory effect of ACh.

This raises the question whether the pathway for inhibition of $I_{K(M)}$ by ACh might differ from that used by bradykinin. Unfortunately, as reported previously (Robbins *et al.* 1992*b*), we found that, under the present patch-clamp recording conditions, the effects of bradykinin were too variable and desensitized too rapidly to allow an adequate range of experimental tests. Nevertheless, it was clear that bradykinin could still inhibit $I_{K(M)}$ in the presence of staurosporin, though we were unable to determine whether the amount of inhibition might or might not have been attenuated to the extent reported by Schäfer *et al.* 1991). To this extent, therefore, it seems likely that the effect of bradykinin recorded under the present experimental conditions, like that of ACh, does not depend to any major extent on protein kinase C activation.

We also tested a number of 'downstream' messengers which (in other cells) have been reported to be generated as a consequence of phospholipase C (PLC) activation, such as products of phospholipase A_2, nitric oxide synthase and guanylate cyclase, but could obtain no firm evidence to implicate any of these in ACh-induced inhibition of $I_{K(M)}$. For example, although high concentrations of arachidonic acid inhibited $I_{K(M)}$, this could not be taken as evidence for a role for phospholipase A_2, or for a function for the cyclo-oxygenase/lipoxygenase pathways, since inhibitors of these enzyme systems did not prevent the action of ACh. Instead, we suspect that the effect of arachidonic acid resulted from some non-specific membrane action, since similar effects were observed on other voltage-activated K^+ currents (Rouzaire-Dubois *et al.* 1991; J. Robbins, unpublished observations).

Finally, we tested in a more general way whether phosphorylation–dephosphorylation reactions might be involved in the effect of ACh using intracellular AMP-PNP to inhibit phosphorylation, extracellular okadaic acid to inhibit phosphatases, and calcineurin antagonists, but, in agreement Chen & Smith (1992) could obtain no positive evidence to support such mechanisms.

Thus, although there seems to be a clear relationship between PLC stimulation and M-current inhibition, in the sense that M-current inhibition appears to be restricted to those agonist receptors which activate PLC, we have been unable to obtain firm evidence for the participation of any of the known primary or secondary messengers formed through PLC activation in the response of the M-current to ACh. This raises the question of whether PLC activation and M-current inhibition might be parallel, rather than sequential, responses to ACh, mediated by the same (or a similar) G-protein. This would account for the fact that M-current inhibition could precede (rather than follow) the rise in $[Ca^{2+}]_i$ generated through PLC activation.

We then have two further possibilities: that the transducing G-protein might interact directly with the M-channels (as suggested by Lopez, 1992), or that it might generate a cytoplasmic messenger, as yet unidentified. We have as yet no hard evidence to distinguish between these possibilities, but our measurements of the latency to $I_{K(M)}$ inhibition following pressure-applications of high concentrations of ACh might provide some clue. Thus, the mean latency at 35 °C was about 270 ms. This is much longer than, for example, the opening of cardiac K^+ channels by ACh (about 30 ms; Noma, 1987), which probably does not involve a cytoplasmic messenger. As reported by Jones (1991) in frog ganglion cells, the latency to M-current inhibition was also longer than that required for ACh or noradrenaline to inhibit I_{Ca} in these neuroblastoma cells, which also probably involves a local (membrane delimited) action (see Lipscombe, Kongsamut & Tsien, 1989). Hence, this rather suggests that some additional steps are involved in M-current inhibition.

However, this is far from conclusive, since a long latency might equally result from a sparsity of G-proteins and M-channels. Thus, adapting the calculations of Lamb & Pugh (1992) for the initial steps in phototransduction (i.e. the collision coupling of rhodopsin, transducin and phosphodiesterase) to the lower densities of receptors, G-protein and effector molecules in NG108-15 cells, we calculate a minimal latency of around 180 ms (see below). This amounts to a substantial fraction of the recorded latency (270 ms at 35 °C), without the need to postulate any additional enzymic steps. The shorter latency for inhibition of I_{Ca} might then be simply attributed to a 4-fold higher density of Ca^{2+} channels than M-channels (see below). The much longer latency to $I_{K(M)}$ inhibition at 25 °C might result from a disproportionate increase in membrane viscosity on cooling (perhaps involving a transition temperature break).

To calculate the first latency between activation of the muscarinic receptor and closure of the M-channel, we have adapted eqn (A16) of Lamb & Pugh (1992) for the sequential steps: rhodopsin–transducin–phosphodiesterase, to read:

$$t = \{3(D_R + D_G)\,C_G\}^{-1} + \{3(D_{G\alpha} + D_M)\,C_M\}^{-1},$$

where D_R = receptor diffusion coefficient, D_G and $D_{G\alpha}$ are the diffusion coefficients for the G-protein and its activated α-subunit, D_M is the diffusion coefficient for the M-channel, and C_G and C_M are the densities of G-protein and M-channels respectively. We assume that the time taken for the initial diffusion and binding of ACh to the muscarinic receptors is approximately represented by the latency to the Ba^{2+} block of M-channels, and that conformational changes in receptors, G-proteins and channels are fast (< 10 ms) and not rate limiting. For D_R, we have used the value of Lamb & Pugh (1992) for rhodopsin (0·7 $\mu m^2\ s^{-1}$) since the m1 receptor is of similar size. Similarly, we have assumed the diffusion coefficient for the M-channel-coupling G-protein to be similar to that for transducin (G_T = 1·2 $\mu m^2\ s^{-1}$, $G_{T\alpha}$ = 1·5 $\mu m^2\ s^{-1}$; Lamb & Pugh, 1992). For D_M we used a value of 0·4 $\mu m^2\ s^{-1}$, assuming arbitrarily a channel molecular weight about twice that of the receptor. (We

recognize that diffusion of the M-channel might be constrained to a greater extent than that of phosphodiesterase molecules by structural proteins, but this may be offset by the greater mobility of the muscarinic receptor.)

The principle determinants of the latency to 'first hit' of the ion channel are the densities of the receptor and G-protein. Photoreceptors possess an unusually high density of both transducin and phosphodiesterase (about 2500 and 167 molecules per square micrometre respectively; Lamb & Pugh, 1992). As a result, the first-encounter latency to phosphodiesterase activation is about 1·3 ms. (Even with the subsequent enzymatic step and diffusion of cGMP, the latency to photocurrent onset at saturating light intensities is only 5–10 ms; Lamb & Pugh, 1992). However, the latency increases steeply as the concentration of G-protein and effector diminishes. Thus, keeping the same ratio of fifteen G-protein molecules per effector molecule, the latency would increase to 12·7 ms at one-tenth the concentration of each, and to 94 ms at one-hundredth the concentration.

In NG108-15 cell membranes, the number of expressed m1 receptors is about 50000 per cell (Robbins *et al.* 1991). The G-protein responsible for M-current inhibition has not yet been positively identified, but each NG108-15 cell contains about 255000 molecules of the principal pertussis-insensitive G-protein ($G_{q/11}$) linking receptors to phospholipase C (G. Milligan, personal communication). Since the maximum M conductance is around 50 nS per cell (Robbins *et al.* 1992*b*) we may estimate about 10000 channels per cell, assuming a mean single-channel conductance of around 10 pS and maximum P_{open} of 0·5 (Selyanko, Stansfeld & Brown, 1992). Then, given a surface area of around 10000 μm^2, we obtain G-protein and channel densities of around 25 and 1 μm^{-2}, and a first-encounter latency of around 182 ms.

Similar calculations for the m4-mediated inhibition of calcium currents in these cells suggests that the conotoxin-sensitive (therefore transmitter sensitive; Caulfield *et al.*, 1992) calcium channels are at a higher density than M-channels, at around 4 μm^{-2}. If so, then there would be a delay of around 50 ms in this system consistent with our experimental value of less than 70 ms (see Results). It should be noted that, because of the apparently high ratio of G-proteins to ion channels, the calculated first latency to channel collision depends primarily on the estimates of channel density. Thus, if the true M-channel density were 2 μm^{-2} instead of the calculated 1 μm^{-2}, first-encounter latency drops to 95 ms, and then to 48 ms at a channel density of 5 μm^{-2}.

In recent experiments on sympathetic neurones M-current transduction has been largely resolved in favour of a diffusion step involving a cytoplasmic messenger, by recording closures of single M-channels in cell-attached patch pipettes following extra-patch application of agonist (Selyanko, Stansfeld & Brown, 1992). Comparable experiments on NG108-15 cells, coupled with further attempts to identify the G-protein responsible for M-channel closure, should help to resolve some of these questions.

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