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A calcium-dependent rather than a G-protein mechanism is involved in the inward current evoked by muscarinic receptor stimulation in dialysed single smooth muscle cells of small intestine

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A role of internal calcium was found in the inward current response of single, dispersed, smooth muscle cells to muscarinic receptor activation but no obligatory role for guanine nucleotide binding proteins, in contrast to their obligatory role in muscarinic responses in heart and other tissues.

Introduction There is now good evidence that guanine nucleotide binding proteins (G-proteins) mediate the muscarinic receptor responses in several tissues. These include inhibition of cardiac adenylate cyclase (Jakobs *et al.*, 1979) the activation of triphosphoinositide phosphodiesterase (phospholipase C) (Sasaguri *et al.*, 1985) opening of calcium-activated channels in lacrimal cells (Evans & Marty, 1986) and opening of cardiac potassium channels (Codina *et al.*, 1987). In the first two cases the α-subunit of the G-protein is believed to interact with an enzyme; in the last case, the GTP-activated component of the G-protein opens cardiac potassium channels.

Methods Single smooth muscle cells were obtained by collagenase digestion of small pieces of separated longitudinal smooth muscle from the small intestine of 2.5 kg rabbits. Cells were placed at room temperature in a physiological salt solution of the following composition (mM): NaCl 125, KCl 6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, HEPES 10 (titrated to pH 7.2 with NaOH) and glucose 11. They were patch clamped with borosilicate glass pipettes (resistance 1–4 MΩ) filled with a solution of the following composition (mM): KCl 126, $MgCl_2$ 1.2, ATP 1, HEPES 10 (titrated to pH 7.2 with NaOH) glucose 11 and EGTA 0.05 as previously described (Benham & Bolton, 1986).

The following drugs were used: atropine sulphate, carbachol chloride, guanosine 5′-O-(γ-thio) triphosphate (GTPγS), guanosine 5′-O-(β-γ-imido) triphosphate (GPPNHP), guanosine triphosphate (GTP), guanosine 5′-O-(β-thio) diphosphate (GDPβS) (Sigma), ryanodine (Agrisystems International, 125 W. 7th St. Wind Gap, Pennsylvania, U.S.A.).

Results In single cells held under voltage clamp at −50 mV the iontophoretic application of carbachol (usually 0.5 s × 50 nA) elicited a transient inward current (Benham *et al.*, 1985; Benham & Bolton, 1986). In most cells, responses could be elicited at 2 min intervals if the cell could be held, but often declined in size (Figure 1a). If the non-hydrolysable analogue of GTP, GTPγS (0.1–1.0 mM) or GPPNHP (0.5 mM) was present in the patch pipette, no noticeable effect was seen on the inward current in response to carbachol, the differences between the responses shown in Figure 1a being well within normal experimental variation. In contrast, however, spontaneous transient outward currents (Benham & Bolton, 1986) were rapidly blocked, indicating that these analogues had reached the interior of the cell. The presence of GTPγS or GPPNHP in the patch pipette did not activate inward current itself, in contrast to guinea-pig heart cells where 0.1 mM evoked the outward current normally controlled by muscarinic receptor activation (Kurachi *et al.*, 1987); nor was the response to carbachol prolonged or rendered permanent as has been observed in heart with 0.1 mM GPPNHP (Breitwieser & Szabo, 1985). The effects of GTPγS and other stable analogues of GTP included in the patch pipette have been demonstrated in other systems at 0.1 mM or less (e.g. Evans & Marty, 1986; Kurachi *et al.*, 1987). In the case of other receptor responses mediated by G-proteins, permanent opening of receptor-operated channels occurs (e.g. somatostatin, Mihara *et al.*, 1987).

To quantify the effect of GTPγS (0.1 mM) in the pipette on the inward current response, carbachol was applied at known concentrations (10^{-6}, 10^{-5} and 10^{-4} M, Figure 1b,c) in the bathing solution.

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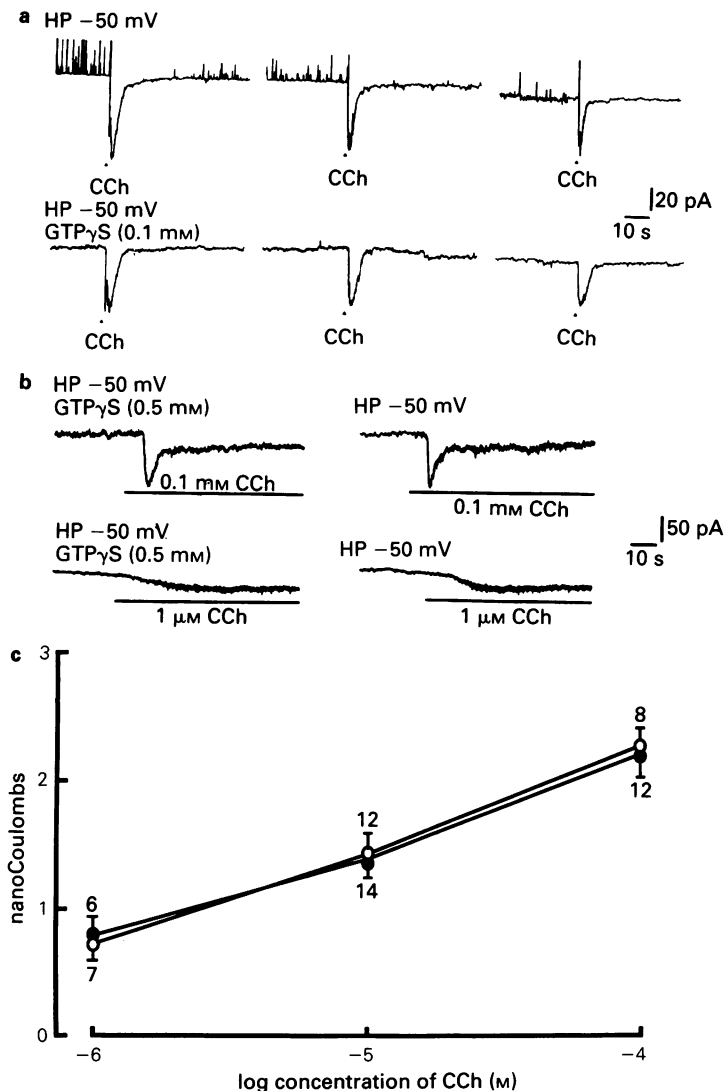

Figure 1 Response to iontophoretic or bath-applied carbachol of single dispersed cells from longitudinal smooth muscle of rabbit jejunum. (a) Three successive responses to iontophoretic carbachol (CCh) (0.5 s × 50 nA) of cell held with a patch pipette filled with normal solution (above) or with a solution containing 0.1 mM guanosine 5′-O-(γ-thio)triphosphate (GTPγS, below). Holding potential (HP) was −50 mV. (b) Responses of single cells held with patch pipettes containing 0.5 mM GTPγS (left) or normal solution (right) to the indicated concentration of bath-applied carbachol. (c) Charge transferred during the first 40 s of the response to bath-applied carbachol in cells dialysed with normal (●) or with 0.1 mM GTPγS solution (○). Mean of responses on the indicated number of cells (one response per cell) of which responses shown in (b) were part; s.e.mean shown by vertical bars.

Only one response was obtained from each cell. The inward charge transferred by the carbachol-evoked current during the first 40 s of the response was estimated by measuring the area of the response (time × current). GTPγS was without effect on the responses to carbachol (Figure 1c).

GTP (0.1 mM) or GDPβS (a GDP analogue which is believed to compete with GTP for binding to the α-subunit of the G-protein) was also without much effect on carbachol inward current. G-proteins may also be activated by a combination of aluminium and fluoride ions. If 10 mM NaF and 10 μM $AlCl_3$

were included in the patch pipette, then the holding inward current sometimes increased slightly initially. However, the response to the iontophoretic application of carbachol was not noticeably changed.

If the EGTA concentration in the patch pipette was increased to 10 mM, or if 10^{-8} M atropine added to the bathing solution, then responses to carbachol were lost. Inclusion of ryanodine (10^{-8}–10^{-4} M) an alkaloid which depletes the calcium stores in smooth muscle was, however, without detectable effect on the inward current response to the iontophoretic application of carbachol. Calcium entry was not important as the inward current was larger in response to carbachol after 5 min in calcium-free solution EGTA (0.1–0.5 mM).

Discussion The experiments described here in smooth muscle cells were done by methods used to study other cell types and indicate that muscarinic receptor stimulation in smooth muscle cells does not have an obligatory requirement for G-proteins in the way that these are required as an essential link between activated muscarinic receptor and channel opening in heart (e.g. Codina *et al.*, 1987) and in other receptor-cell systems (e.g. Mihara *et al.*, 1987). The result is reinforced because, under normal conditions GTP was not usually added to the patch pipette, so that very low intracellular concentrations of GTP probably exist in our cells due to GTP diffusion out into the pipette; despite this, inward current can be evoked several times by muscarinic receptor activation. However, responses to carbachol seemed to be more durable if GTP was included in the patch pipette so that a smaller, modulatory, role for G-proteins in the muscarinic response may be possible.

The experiments in which calcium was strongly buffered in the cell with EGTA suggest a role for calcium in the link between muscarinic receptor activation and inward channel opening confirming results by others (Inoue *et al.*, 1987) although increases in Ca_i do not seem to cause channel opening (Inoue *et al.*, 1987) so that the exact role of calcium is at present obscure. The lack of effect of ryanodine implies that stored calcium release is not involved.

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