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COMMENTARY

Can bethanechol distinguish between different muscarinic signalling pathways in neurones?

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Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BeCh, Bethanechol; OXO-M, Oxotremorine-M; PTX, Pertussis toxin; SCG, Superior cervical ganglion

The reversible modulation of certain types of voltage-dependent Ca^{2+} currents in peripheral and central neurones by activation of muscarinic (acetylcholine) and other various G-protein coupled receptors has been known for some considerable time (Wanke *et al*., 1987; Carbone & Swandulla, 1989; Toselli & Lux, 1989). However, the signal transduction pathways that link such receptor activation to the modulation of Ca^{2+} channels still remain rather elusive. Since intracellular Ca^{2+} is involved in such a multitude of important neuronal functions, that is, neurotransmitter release, control of calcium-dependent enzymes, neuronal development (Linden, 1994), as well as influencing the expression (e.g. Ginty, 1997; Kobayashi *et al*., 2002), and properties of other voltage-sensitive and ligand-gated ion channels involved in controlling membrane excitability, it is quite understandable why so much interest has been devoted over the years, to the elucidation and understanding of such receptor-regulated G-protein signalling pathways, and also, the molecular mechanisms by which Ca^{2+} channel modulation actually occurs. The quest is made even more interesting in the light of recent data showing that Ca^{2+} current modulation by neurotransmitters may show different characteristics in different neuronal compartments (e.g. dendrites; Delmas *et al*., 2000), suggesting a specialized function of Ca^{2+} signalling in important processes such as synaptic integration and long-term synaptic plasticity (for reviews, see Magee *et al*., 1998; Weiss & Burgoyne, 2002).

The adult rat superior cervical ganglion (SCG) neurone has proved a robust and convenient model system for studying signalling cascades linked to Ca^{2+} current (I_{Ca}) inhibition. Based on such experiments, it is now clear that concentration-dependent, muscarinic suppression of Ca^{2+} channel activity in these cells (mainly N- and L-type; Mathie *et al*., 1992) occurs by both rapid- and slow-onset processes; the former (N-type) is sensitive to pertussis toxin (PTX) and acts through a voltage-dependent, membrane-delimited pathway linked to muscarinic M_2/M_4 receptors (via a GoA-type G protein), whereas the latter (N- and L-type) is PTX-insensitive, voltage-independent and utilizes an unknown diffusible cytoplasmic second messenger (not Ca^{2+}, cAMP, cGMP, or protein kinase C) linked to M_1 receptors (via a $G\alpha_q$ G protein) (Bernheim *et al*., 1991, 1992; Hille, 1994; Hille *et al*., 1995; Delmas *et al*., 1998). The cytoplasmic messenger nevertheless, appears to be partly Ca^{2+}-dependent and partly sensitive to the internal divalent cation chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; in whole-cell patch experiments), since a low (0.1 mM) BAPTA, concentration in the recording pipette is necessary to see the response (Beech *et al*., 1991; Mathie *et al*., 1992). A fast PTX-insensitive pathway may also be present in adult ganglia (Beech *et al*., 1992), possibly coupled to a Gαz-type G protein subunit (Jeong & Ikeda, 1998; see also Delmas et al., 1998). The 'fast' PTX-sensitive pathway exhibits two further characteristic properties: voltage-dependence – manifested as a temporary relief of muscarinic I_{Ca} blockade following a positive conditioning prepulse (cf. Elmslie *et al*., 1990; Williams *et al*., 1998), and possibly mediated by a G-protein-dependent blocking particle (Lopez & Brown, 1991), and 'kinetic slowing', seen as a significant slowing of I_{Ca} activation kinetics and positive shift in voltage dependence of Ca^{2+} channels most likely because of the direct binding of 'free' G-protein $\beta\gamma$ subunits to the N-type Ca^{2+} channels (the 'willing-reluctant' model: Ikeda & Dunlap, 1999; Colecraft *et al*., 2000).

Faced with such an extensive collection of existing data, can anything new be added to the already complex muscarinic I_{Ca} modulation story? To date, the two muscarinic signalling pathways in ganglia have been pharmacologically distinguished using established muscarinic receptor antagonists (Bernheim *et al*., 1992) or by utilizing a muscarinic M_1–M_4 knockout approach in mice (Shapiro *et al*., 2001). In this issue of the journal, Liu & Rittenhouse (2003) present some new data showing that a clean discrimination between the signalling cascades can be made by using the conventional muscarinic agonist bethanechol (BeCh). The principal aim of their experiments was to establish whether the fast and slow muscarinic transduction mechanisms originally observed in adult SCG were present in *neonatal* SCG cells; using neonates allowed a better separation of the two signalling pathways, since the fast (membrane-delimited) PTX-*in*sensitive component was absent in these cells (cf. Beech *et al*., 1992). Whole-cell and cell-attached single channel recordings of

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N-and L-type Ca^{2+} currents were made under patch clamp from acutely dissociated neurones, using a protocol designed to optimize L-currents (addition of dihydropyridine L-type channel agonists to the bathing solution, and Ba^{2+} as charge carrier); also by raising or lowering the internal BAPTA concentration in their patch pipettes or pretreating cells with PTX, they could effectively isolate the slow diffusible second messenger pathway from the fast pathway in any given experiment. Under these conditions, the muscarinic agonist oxotremorine-M (OXO-M; 0.01–100 μM) was able to suppress both N- and L-type currents indiscriminately by activating either signalling pathway, thereby confirming their existence in the neonate. In contrast, BeCh (up to 3 mM) was unable to activate the slow (voltage-independent) pathway, but appeared selective for the fast membrane-delimited pathway. This was elegantly demonstrated in PTX-pretreated cells, whereupon the effects of BeCh (but not OXO-M) on N- and L-type currents were eliminated. Interestingly, despite high concentrations of either agonist, the maximal degree of current inhibition by the fast pathway was never greater than 60%, most likely reflecting some basal constitutive G-protein activity. In their concluding series of experiments, the authors showed that the effect of BeCh was not mediated via M_1 or M_4 muscarinic receptors (cf. adult SCG), since it was unaffected by the specific M_1 toxin MT-7 (Adem & Karlsson, 1997) or by pirenzepine (acting here as an M_4 antagonist in the presence of MT-7), respectively, but was blocked by the M_2 antagonist methoctramine; the current inhibition by OXO-M, acting via the slow pathway, was however, abolished by MT-7, confirming it was M_1 mediated (as in the adult).

BeCh, a β-methyl analogue of carbachol, was first synthesized and investigated in the 1930s (Heller Brown & Taylor, 1996). The apparent high selectivity of this agonist for M_2 receptors in the SCG is therefore quite surprising, as the authors themselves admit, since (to my knowledge) no *absolute* selectivity for intact peripheral or central M_2 receptors has previously been demonstrated. In this respect, it would have been interesting to know if other muscarinic agonists (e.g. methacholine, arecoline, pilocarpine, carbachol) could show a similar selectivity in their system. Nevertheless, their paper is novel in that it presents one long-established muscarinic agonist as a new tool for distinguishing between different muscarinic signalling pathways in neurones; whether BeCh exerts a similar selectivity towards M_2 receptors on other neuronal preparations, however, remains to be seen.

On a final note, Beech *et al.*, (1991) drew attention to the fact that muscarinic suppression of the M-current (I_M; a subthresold voltage- and time-dependent K^+ current; Brown & Adams, 1980) in SCG neurons also occurs via a slow BAPTA-sensitive modulatory pathway, implying that the same diffusible second messenger may be involved (see also Mathie *et al.*, 1992). Interestingly, only very recently, Suh & Hille (2002) proposed that the long sought-after 'mystery message' responsible for ganglionic muscarinic suppression of I_M was actually a *depletion* of membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) following G-protein activation of phospholipase C (PLC), and that intracellular ATP and lipid rephosphorylation by phosphoinositide 4-kinase (PI 4-K) were required for I_M recovery from modulation. It will be interesting to see whether the same PIP_2 recycling mechanism can also account for the slow voltage-independent muscarinic modulation of L- and N-type Ca^{2+} (and perhaps other) ion channels in sympathetic neurones.

References

ADEM, A. & KARLSSON, E. (1997). Muscarinic receptor subtype selective toxins. *Life Sci.,* **60,** 1069–1076.

BEECH, D.J., BERNHEIM, L. & HILLE, B. (1992). Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. *Neuron,* **8,** 97–106.

BEECH, D.J., BERNHEIM, L., MATHIE, A. & HILLE, B. (1991). Intracellular Ca^{2+} buffers disrupt muscarinic suppression of Ca^{2+} current and M current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 652–656.

BERNHEIM, L., BEECH, D.J. & HILLE, B. (1991). A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron,* **6,** 859–867.

BERNHEIM, L., MATHIE, A. & HILLE, B. (1992). Characterization of muscarinic receptor subtypes inhibiting Ca^{2+} current and M current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 9544–9548.

BROWN, D.A. & ADAMS, P.R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. *Nature,* **283,** 673–676.

CARBONE, E. & SWANDULLA, D. (1989). Neuronal calcium channels: kinetics, blockade and modulation. *Prog. Biophys. Mol. Biol.,* **54,** 31–58.

COLECRAFT, H.M., PATIL, P.G., YUE, D.T. (2000). Differential occurrence of reluctant openings in G-protein-inhibited N- and P/Q-type calcium channels. *J. Gen. Physiol.,* **115,** 175–192.

DELMAS, P., ABOGADIE, F.C., BUCKLEY, N.J. & BROWN, D.A. (2000). Calcium channel gating and modulation by transmitters depend on cellular compartmentalization. *Nat. Neurosci.,* **3,** 670–678.

DELMAS, P., ABOGADIE, F.C., DAYRELL, M., HALEY, J.E., MILLIGAN, G., CAULFIELD, M.P., BROWN, D.A. & BUCKLEY, N.J. (1998). G-proteins and G-protein subunits mediating cholinergic inhibition of N-type calcium currents in sympathetic neurons. *Eur. J. Neurosci.,* **10,** 1654–1666.

ELMSLIE, K.S., ZHOU, W. & JONES, S.W. (1990). LHRH and GTP-gamma-S modify calcium current activation in bullfrog sympathetic neurons. *Neuron,* **5,** 75–80.

GINTY, D.D. (1997). Calcium regulation of gene expression: isn't that spatial. *Neuron,* **18,** 183–186.

HELLER BROWN, J. & TAYLOR, P. (1996). Muscarinic receptor agonists and antagonists. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics,* 9th ed., Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W. & Goodman Gilman, A., pp. 141–160. New York: McGraw-Hill.

HILLE, B. (1994). Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci.,* **17,** 531–536.

HILLE, B., BEECH, D.J., BERNHEIM, L., MATHIE, A., SHAPIRO, M.S. & WOLLMUTH, L.P. (1995). Multiple G-protein-coupled pathways inhibit N-type Ca channels of neurons. *Life Sci.,* **56,** 989–992.

IKEDA, S.R. & DUNLAP, K. (1999). Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv. Second Messenger Phosphoprotein Res.,* **33,** 131–151.

JEONG, S.W. & IKEDA, S.R. (1998). G protein alpha subunit G alpha z couples neurotransmitter receptors to ion channels in sympathetic neurons. *Neuron,* **21,** 1201–1212.

KOBAYASHI, H., SHIRAISHI, S., YANAGITA, T., YOKOO, H., YAMAMOTO, R., MINAMI, S.I., SAITOH, T. & WADA, A. (2002). Regulation of voltage-dependent sodium channel expression in adrenal chromaffin cells: involvement of multiple calcium signaling pathways. *Ann. NY Acad. Sci.,* **971,** 127–134.

LINDEN, R. (1994). The survival of developing neurones—a review of afferent control. *Neuroscience,* **58,** 671–682.

LIU, L. & RITTENHOUSE, A.R. (2003). Pharmacological discrimination between muscarinic receptor signal transduction cascades with bethanechol chloride. *Br. J. Pharmacol.,* **138**, 1261–1272.

LOPEZ, H.S. & BROWN, A.M. (1991). Correlation between G protein activation and reblocking kinetics of Ca^{2+} channel currents in rat sensory neurons. *Neuron,* **7,** 1061–1068.

MAGEE, J., HOFFMAN, D., COLBERT, C. & JOHNSTON, D. (1998). Electrical and calcium signaling in dendrites of hippocampal pyramidal neurons. *Annu. Rev. Physiol.,* **60,** 327–346.

MATHIE, A., BERNHEIM, L. & HILLE, B. (1992). Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. *Neuron,* **8,** 907–914.

SHAPIRO, M.S., GOMEZA, J., HAMILTON, S.E., HILLE, B., LOOSE, M.D., NATHANSON, N.M., ROCHE, J.P. & WESS, J. (2001). Identification of subtypes of muscarinic receptors that regulate Ca^{2+} and K^+ channel activity in sympathetic neurons. *Life Sci.,* **68,** 2481–2487.

SUH, B.C. & HILLE, B. (2002). Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron,* **35,** 507–520.

TOSELLI, M. & LUX, H.D. (1989). GTP-binding proteins mediate acetylcholine inhibition of voltage dependent calcium channels in hippocampal neurons. *Pflügers Arch.,* **413,** 319–321.

WANKE, E., FERRONI, A., MALGAROLI, A., AMBROSINI, A., POZZAN, T. & MELDOLESI, J. (1987). Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca^{2+} current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 4313–4317.

WEISS, J.L. & BURGOYNE, R.D. (2002). Sense and sensibility in the regulation of voltage-gated Ca(2+) channels. *Trends Neurosci.,* **25,** 489–491.

WILLIAMS, S., SERAFIN, M., MUHLETHALER, M. & BERNHEIM, L. (1998). The serotonin inhibition of high-voltage-activated calcium currents is relieved by action potential-like depolarizations in dissociated cholinergic nucleus basalis neurons of the guinea-pig. *Eur. J. Neurosci.,* **10,** 3291–3294.

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