Muscarnic Inhibition of Calcium Current and M Current in Gαq-Deficient Mice

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Activation of M1 muscarinic acetylcholine receptors (M1 mAChR) inhibits M-type potassium currents (I_{K(M)}) and N-type calcium currents (I_{Ca}) in mammalian sympathetic ganglia. Previous antisense experiments suggested that, in rat superior cervical ganglion (SCG) neurons, both effects were partly mediated by the G-protein Gαq (Delmas et al., 1998a; Haley et al., 1998a), but did not eliminate a contribution by other pertussis toxin (PTX)-insensitive G-proteins. We have tested this further using mice deficient in the Gαq gene.

PTX-insensitive M1 mAChR inhibition of I_{Ca} was strongly reduced in Gαq-/-- mouse SCG neurons and was fully restored by acute overexpression of Gαq. In contrast, M1 mAChR inhibition of I_{K(M)} persisted in Gαq-/-- mouse SCG cells. However, unlike rat SCG neurons, muscarinic inhibition of I_{K(M)} was partly PTX-sensitive. Residual (PTX-insensitive) I_{K(M)} inhibition was slightly reduced in Gαq-/-- neurons, and the remaining response was then suppressed by anti-Gαq11 antibodies.

Bradykinin (BK) also inhibits I_{K(M)} in rat SCG neurons via a PTX-insensitive G-protein (Gq and/or G11; Jones et al., 1995). In mouse SCG neurons, I_{K(M)} inhibition by BK was fully PTX-resistant. It was unchanged in Gαq-/-- mice but was abolished by anti-Gαq11 antibody.

We conclude that, in mouse SCG neurons (1) M1 mAChR inhibition of I_{Ca} is mediated principally by Gαq, (2) M1 mAChR inhibition of I_{K(M)} is mediated partly by Gαq, more substantially by G11, and partly by a PTX-sensitive G-protein(s), and (3) BK-induced inhibition of I_{K(M)} is mediated wholly by G11.

Key words: M current; calcium current; G-protein; superior cervical ganglion neuron; knock-out mouse; muscarinic receptor; bradykinin receptor
Parts of this work have been published previously in abstract form (Haley et al., 1998c).

**MATERIALS AND METHODS**

**q-Deficient mice.** Mice deficient in the q gene (q/) were generated by targeted disruption with a neomycin gene as described previously (Offermanns et al., 1997a). q-Deficient mice used in the experiments were obtained by mating either Gaq knock-out males with heterozygous females or heterozygous males and females (to obtain wild-type and knock-out littermates). Mice were kept on a C57Bl/129Sv background, and genotypes were confirmed by PCR on genomic DNA from tail snips of each mouse.

**Cell culture.** Sympathetic neurons were isolated from Gaq knock-out and wild-type mice that were at least 5 weeks old. Wild-type mice were either C57Bl/6J (Harlan, Bicester, UK) or from litters also containing Gaq knock-out littermates; results obtained were the same from both these groups and so have been pooled. We also used ICR mouse (coding for outbred albino mouse; Harlan) to compare properties of SCG neurons derived from different strain of mouse. SCG were removed, and neurons were cultured using standard procedures as described previously for rat (Delmas et al., 1998b).

**Microinjection.** In a few experiments, Gaq or q antiserum (OC-2 and CQ1, respectively, from G. Milligan) (Caulfield et al., 1994, Delmas et al., 1998a, 1999) or an expression plasmid encoding Gaq were pressure injected into the cytoplasm (antiserum) or nucleus (plasmid) of SCG neurons in culture using a microinjector (Eppendorf, Hamburg, Germany). To allow identification of the injected neurons for recording, FITC-dextran (70,000 MW; Molecular Probes, Leiden, The Netherlands) was added in a final concentration of 0.2% (antiserum) (Jones et al., 1995) or 0.5% (plasmid) (Haley et al., 1998a). Cells injected with the antiserum were recorded at least 2 hr after injection, whereas those injected with the Gaq-encoding plasmid were recorded 24 hr later.

**Electrophysiology.** Currents were measured from SCG neurons cultured for 2–3 d, using the amphotericin-B perforated patch technique (Horn and Marty, 1988; Rae et al., 1991). Patch electrodes (2–5 MΩ) were filled by dipping the tip for 40 sec into the appropriate filtered internal solution, and the pipette was then back-filled with the internal solution containing 0.07–0.1 mg/ml amphotericin-B. High-resistance seals (>2 GΩ) were initially achieved and, after amphotericin-B permeabilization, access resistances were generally <25 MΩ for q recordings and <15 MΩ for q recordings. SCG neurons were perfused (5–10 ml/min) with an external solution consisting of (in mM): NaCl 120, KCl 3, HEPES 5, NaHCO3 23, glucose 11, MgCl2 1.2, CaCl2 2.5, and tetrodotoxin 0.0005, pH 7.4, maintained at 32°C.

**RESULTS**

**M1 mAChR-induced inhibition of ICa and IK(M) in mouse versus rat SCG.**

The mAChR agonist Oxo-M inhibited both ICa and IK(M) (see also Hamilton et al., 1997) in SCG from mouse. However, the dose–response curves were shifted to the right in mouse compared with rat neurons (Fig. 1). ICa values for IK(M) inhibition were 0.4 and 0.7 μM in rat and wild-type mouse, respectively. For M1 mAChR inhibition of ICa, IC50 values were 800 nm and 1.1 μM in rat and mouse, respectively.

**M1 mAChR inhibition of ICa is reduced in Gaq /−/− mouse SCG neurons.**

In rat SCG, inhibition of ICa by mAChR agonists results from the activation of two separate pathways: a voltage- and PTX-sensitive M1 mAChR pathway (M2 receptors in mice) (Shapiro et al., 1999) that requires Gaq and a voltage- and PTX-insensitive M1 mAChR pathway that mostly involves Gaq (Delmas et al., 1998a).

In mice, total mAChR inhibition of ICa was significantly (p < 0.002) reduced when Gaq was deleted. Thus, 1 and 10 μM Oxo-M...
served in the Gαq knock-out mice. All experiments were performed on neurons pretreated with PTX. A. ICa-waveforms in SCG neurons from wild-type (left) and Gαq−/− mice (right) in the absence and presence of 10 μM Oxo-M, using the three-step voltage protocol illustrated above each waveform. The conditioning step to +90 mV did not reverse Oxo-M inhibition in either wild-type or Gαq−/− neurons, confirming the voltage-independent nature of the PTX-insensitive inhibition. Calibration: 5 msec. B. Gαq is expressed in a Gαq−/− neuron after injection of a Gαq expression plasmid (injected cell indicated by arrow). C. In Gαq−/− neurons, Oxo-M inhibition of ICa is restored by exogenous expression of Gαq. D. Summary of M1 mAChR and noradrenergic inhibitions of ICa. Note that NA inhibition cannot be restored in Gαq−/− SCG neurons by expression of Gαq.

Figure 2. M1 mAChR inhibition of ICa is markedly reduced in Gαq−/− mice. Inhibition of ICa by 10 μM loss of the voltage-insensitive M1 mAChR-mediated inhibition became clear that the reduction in M1 plus M2 inhibition of ICa in both wild-type and Gαq−/− mice from PTX-treated cells, with facilitation ratio of 1.4 ± 0.2 (n = 10) and 0.94 ± 0.1 (n = 8), respectively (data not shown). Inhibition by 10 μM noradrenaline (NA) (which requires both Gαo and Gαq in rat) (Caulfield et al., 1994; Delmas et al., 1999) was not altered when Gαq was absent (wild type, 66 ± 3%, n = 8; Gαq−/−, 62 ± 5%, n = 5).

Incubation with 1 μM PTX inactivates members of the Gαq/o G-protein family, removes the PTX-sensitive mACHR inhibition, and isolates the M1 mAChR inhibition, as well as abolishes inhibition by NA (Schofield, 1991; Zhu and Ikeda, 1994). Inhibition by NA was, as expected, abolished in PTX-treated cells from both wild-type and Gαq−/− mice. After PTX treatment, it became clear that the reduction in M1 plus M2 inhibition observed in the Gαq−/− cells resulted from an almost complete loss of the voltage-insensitive M1 mAChR-mediated inhibition (Fig. 2). To be sure that the loss of M1 mAChR inhibition of ICa was attributable to the absence of Gαq, we acutely overexpressed Gαq in cultured SCG neurons from Gαq−/− mice. Twenty-four hours after injection of a plasmid encoding for Gαq, M1 mAChR inhibition was fully restored (Fig. 2C). This reinstated inhibition was specific because NA inhibition was not rescued by Gαq overexpression in PTX-treated cells (Fig. 2). These findings accord with previous data from this laboratory that M1 mAChR inhibition of ICa in rat SCG is primarily mediated by Gαq (Delmas et al., 1998a). It should be noted that the ICa density (normalized to cell capacitance) was significantly lower in Gαq−/− neurons (27 ± 2 pA/pF, n = 10) compared with wild-type mouse SCG (38 ± 2 pA/pF, n = 12, p < 0.001). There was no difference between ICa density in wild-type and rat SCG (rat, 42 ± 2 pA/pF, n = 19).

M1 mAChR inhibition of ICa is not reduced in SCG from Gαq−/− mouse

In rat SCG, inhibition of IK(M) by mACHR agonists is mediated, at least in part, by Gαq (Haley et al., 1998a), so one would predict a loss of inhibition in neurons lacking Gαq. We were surprised, therefore, to discover that inhibition of IK(M) by Oxo-M was not reduced in Gαq−/− SCG compared with wild-type neurons (Fig. 3). Indeed, Oxo-M produced significantly more inhibition in Gαq−/− neurons (p < 0.05), and the dose–response curve lay to the left of the wild-type dose–response (Fig. 3B). IK(M) density (normalized to cell capacitance) was not changed in Gαq−/− mouse SCG compared with wild type, and neither was the resting membrane potential (wild type, 2.5 ± 0.5 mV/PF, n = 8; −58.0 ± 1.3 mV, n = 9; Gαq−/−, −3.3 ± 1.1 mV/PF, n = 9; −56.3 ± 1.3 mV, n = 11), and these did not differ from rat SCG (2.4 ± 0.3 mV/PF, n = 10; −59.9 ± 1.3 mV).

M1 mAChR inhibition of IK(M) partly involves PTX-sensitive G-proteins in mouse SCG

One factor contributing to the persistence of mACHR-induced inhibition of IK(M) in neurons from Gαq-deficient mice became apparent when we tested the effect of PTX. In contrast to previous observations on rat SCG neurons (Brown et al., 1989; Bernheim et al., 1992; Haley et al., 1998a), PTX treatment significantly reduced inhibition by Oxo-M in neurons from both wild-type...
virtually annulled the residual inhibition in neurons from Go,q−/− mice (p < 0.0001). Thus, the residual PTX-insensitive inhibition in neurons from Go,q−/− mice results from activation of Go,11 because the antibody is specific to Go,q and Go,11, and Go,q is absent.

Inhibition of I_{K(M)} by BK is mediated by Go,11 in Go,q−/− neurons

For these experiments, a single application of a low concentration (1 nM) of BK was made to each neuron tested because slow recovery from inhibition precluded repeated applications to the same neuron and desensitization precluded applications of incremental concentrations (Jones et al., 1995; Cruzblanca et al., 1998). At 1 nM, BK inhibited I_{K(M)} in wild-type mouse SCG neurons by 25 ± 4% (n = 8). This was not significantly different from that in rat SCG neurons (29 ± 7%; n = 8; data not shown). Unlike mACHr-induced inhibition, the effect of BK on mouse neurons was not reduced by PTX (Fig. 5). This accords with previous observations on rat neurons (Jones et al., 1995). No inhibition of I_{Ca} by 1 nM BK could be detected.

No significant reduction of BK-induced inhibition of I_{K(M)} was observed in Go,q−/− neurons. However, injection of an antibody against Go,q11 substantially (p < 0.05 vs anti-Go,q antibody) reduced I_{K(M)} inhibition in wild-type mouse neurons (as in rat neurons; Jones et al., 1995) and abolished inhibition in neurons from Go,q−/− mice (p < 0.01) (Fig. 5B).

DISCUSSION

Using mice lacking Go,q, we have demonstrated that M1 mACHr inhibition of I_{Ca} requires Go,q because regulation was essentially lost in Go,q−/− neurons (Fig. 2). This was not a result of secondary effects on transduction mechanisms arising from an absence of Go,q during development because inhibition could be fully restored by acute overexpression of Go,q in Go,q−/− neurons (Fig. 2); thus, the remaining components of the inhibitory pathway were still present and functional in these mutant cells. In contrast, neither the inhibition produced by NA nor the PTX-sensitive (M2) component of mACHr inhibition (Shapiro et al., 1999) were reduced after deletion of the Go,q genes. Hence, these results in mouse neurons are in complete accord with previous conclusions from observations on rat SCG neurons using G-protein antibody injections and antisense depletion (Caulfield et al., 1994; Delmas et al., 1998a) that Go,q is the primary G-protein involved in M1 mACHr-induced inhibition of I_{Ca} but is not involved in the inhibition produced by activating M2,4 mACHRs or α1 adrenergic receptors. It is especially worthy noting (particularly in connection with our observations on I_{K(M)}; see below) that Go,11 appears not to be able to substitute for Go,q in Go,q−/− mice, although it is able to interact with M1 mACHRs (Offermanns et al., 1994; Gudermann et al., 1996) and although expression of a constitutively active form of Go,11 can inhibit I_{Ca}, just like Go,q (unpublished observations; Delmas et al., 1998a).

In contrast [and surprisingly, in view of previous observations in rat SCG neurons using Go,q antisense (Haley et al., 1998a)], mACHr-induced inhibition of I_{K(M)} was not reduced in neurons from Go,q−/− mice. One contributory reason for this seemingly to be that I_{K(M)} inhibition after mACHr stimulation was partly sensitive to PTX. When this component was eliminated, it appeared that some part of the inhibition (particularly at low agonist concentrations) was mediated by Go,q because the dose–response curve for Oxo-M-induced M current inhibition was shifted significantly to the right in Go,q−/− mice. Nevertheless, it was clear

![Figure 3](image_url)

Figure 3. M1 mACHr inhibition of I_{K(M)} is not reduced in Go,q−/− mice. A, I_{K(M)} deactivation relaxation elicited by a -20 mV step for 1 sec from a holding potential of -25 mV. Waveforms (average of 6 traces) are from SCG neurons of wild-type (top) and Go,q−/− (bottom) mice and are shown in the absence and presence of increasing concentrations of Oxo-M (micromolar). Dotted lines indicate 0 pA. Calibration: 250 pA. B, Mean ± SEM data (plus best-fit curves) for Oxo-M inhibition of I_{K(M)} in wild-type and Go,q−/− SCG neurons. Oxo-M dose–response curve in Go,q−/− neurons (n = 8) was significantly different from wild type (p < 0.05). IC_{50} and Hill slope values for inhibition in Go,q−/− cells are 0.6 µM and 1.1, respectively. C, Oxo-M dose–response curves in wild-type and Go,q−/− SCG neurons treated with PTX. IC_{50} and Hill slope values were 1.3 µM and 0.93, and 1.7 µM and 1.16 in wild-type and Go,q−/− cells, respectively.
that a considerable proportion of the inhibition must have been mediated by another PTX-insensitive G-protein. In $\alpha_q$-deficient neurons, this G-protein was identifiable as $\alpha_{11}$, because the residual PTX-insensitive inhibition was virtually annulled using an antibody directed against the unique but common C terminus to $\alpha_q$ and $\alpha_{11}$. Thus, in the $\alpha_q$-deficient mice, we conclude that mACHR-induced M current inhibition is maintained by $\alpha_{11}$ and by an increased contribution of the (unidentified) PTX-sensitive pathway.

Thus, the present experiments reveal two clear differences from the inferences drawn from previous work on mACHR-induced M current inhibition in rat SCG neurons: the additional involvement of a PTX-sensitive G-protein and the greater potentiating involvement of $\alpha_{11}$. One possible explanation for the apparent involvement of a PTX-sensitive G-protein is that, in mouse ganglion cells, stimulation of $M_4$ or $M_2$ receptors might also inhibit $I_{K\text{M}}$. However, this seems unlikely, because Hamilton et al. (1997) found that mACHR-induced M current inhibition was...
completely annulled in mice deficient in the M₁ receptor gene, implying that, as in rat neurons (Marriott et al., 1989; Bernheim et al., 1992), muscarinic inhibition of Iₖ(M) was mediated entirely by M₁ mAChRs. Although M₁ receptors are usually considered to couple exclusively to PTX-insensitive G-proteins, there have been occasional reports of at least partial PTX-sensitive responses after expression of cloned M₁ receptors (Stein et al., 1988; Ashkenazy et al., 1989). Because this PTX-sensitive pathway seems unique to mouse neurons and may have limited general significance, we have not so far made any serious attempt to identify the species of G-protein involved.

Regarding the involvement of Gα₁₁, in previous experiments on rat neurons, anti-Gαq antisense produced a rightward shift of the dose–response curve for inhibition of Iₖ(M) by Oxo-M (Haley et al., 1998a), not dissimilar to that seen in Gαq −/− mouse neurons. However, although this might also suggest the involvement of another PTX-insensitive G-protein, antisense to Gα₁₁ had no effect on the inhibitory effect of Oxo-M in rat neurons. Hence, it was concluded that the limited effect of Gαq antisense probably resulted from incomplete protein suppression rather than to the additional effect of another G-protein. Nevertheless, it is necessary to point out that, although Gα₁₁ can sustain mAChR-induced M current inhibition in Gαq-deficient mice, we have no direct evidence from the present experiments that Gα₁₁ mediates any part of the response of normal (wild-type) mouse neurons to Oxo-M; the reduced inhibition seen in the presence of the Gαq antisense could equally well have been attributable to antagonism of either endogenous Gαq or Gα₁₁. The large contribution of Gα₁₁ in Gαq −/− neurons might then be an adaptive change, perhaps resulting from a redistribution of G-proteins in the plasma membrane, because there is no evidence for compensatory overexpression of Gα₁₁ in the nervous system of these mice (Offermanns et al., 1997b). Hence, the present observations do not necessarily negate our previous conclusion that mAChR-induced M current inhibition in normal rat neurons results primarily from activation of Gαq. In this context, it should be noted that previous observations have revealed differences between the coupling of muscarinic receptors to ion channels in mouse and rat SCG neurons. Thus, in the mouse, inhibition of IₖCa is mediated by M₂ receptors (Shapiro et al., 1999) whereas the corresponding response in rat SCG neurons is mediated by M₁ receptors (Bernheim et al., 1992); indeed, although rat neurons possess functional M₂ receptors capable of activating PTX-sensitive G-proteins, they appear not to inhibit IₖCa (Fernandez-Fernandez et al., 1999).

The effects of BK on Iₖ(M) in mouse ganglion cells seem to match those on rat SCG neurons much more closely. Thus, 1 nM BK produced the same amount of inhibition in both, and neither showed any sensitivity to PTX (unlike muscarinic inhibition). Previous experiments on rat neurons using G-protein antibodies (Jones et al., 1995) strongly suggested that inhibition was mediated by either Gαq or Gα₁₁ (or both) but could not identify which. Because in the present experiments inhibition was unchanged in neurons from Gαq −/− mice and then annulled by anti-Gαq/α₁₁ antibody, we infer that inhibition in normal SCG neurons is probably mediated exclusively by Gα₁₁ (although with the caveat regarding possible adaptive responses after Gαq deletion expressed above). Lack of any involvement of Gαq would accord with the fact, that, unlike mAChR stimulation, BK did not inhibit IₖCa in the mouse ganglion cells.

The reason for this apparent selectivity of BK receptors for Gα₁₁ in mouse neurons (and possibly in rat neurons) is unclear.

Although it is usually assumed that BK can activate Gαq, we are unaware of any experiments that unequivocally distinguish effects mediated by Gαq from those that might equally be mediated by Gα₁₁. On the contrary, Ricupero et al. (1997) have reported that BK-induced stimulation of phospholipase C was enhanced, rather than inhibited, in mouse embryonic stem cells lacking Gαq, whereas Wilk-Blaszczak et al. (1994b) identified G₁₃ as the G-protein responsible for the PTX-insensitive component of IₖCa inhibition in neuroblastoma hybrid cells. [In previous experiments, these authors (Wilk-Blaszczak et al., 1994a) had identified Gα₁₁ as mediating the phospholipase C-driven activation of Iₖ(Ca) in these cells but, because this was from anti-Gα₁₁ antibody infusion, it could have resulted from activation of Gα₁₁/G₁₃, or both.] In conclusion, the present results point to a surprising degree of divergence at the G-protein level in the coupling of M₁ mAChR and B₂ BK receptor-induced inhibition of IₖCa and Iₖ(M) channels in rat SCG neurons and in wild-type and Gαq-deficient mouse SCG neurons.

Figure 6. Diagram to summarize projected G-protein involvement for M₁ mAChR- and B₂ BK receptor-induced inhibition of IₖCa and Iₖ(M) channels in rat SCG neurons and in wild-type and Gαq-deficient mouse SCG neurons.
al., 1998b). The difference in G-protein coupling from M1 mACHRs to Ca\(^{2+}\) channels and K\(_M\) \(^{2+}\) channels might equally imply that, contrary to previous inferences (Hille, 1994), the modulation of these two channels may also involve different transduction pathways.

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