The M₃ Receptor-mediated K⁺ Current (IₖM₃), a Gq Protein-coupled K⁺ Channel*  

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Stimulation of muscarinic acetylcholine receptors (mAChRs) can activate an inward rectifier K⁺ current (IₖACh), which is mediated by the M₂ subtype of mAChR in cardiac myocytes. Recently, a novel delayed rectifier-like K⁺ current mediated by activation of the cardiac M₃ receptors (designated IₖM₃) was identified, which is distinct from IₖACh and other known K⁺ currents. While IₖACh is known to be a Gᵢ protein-gated K⁺ channel, the signal transduction mechanisms for IₖM₃ activation remained unexplored. We studied IₖM₃ with whole-cell patch clamp and macropatch clamp techniques. Whole cell IₖM₃ activated by choline persisted with minimal rundown over 2 h in presence of internal GTP. When GTP was replaced by guanyl-5'-yl thiophosphate, IₖM₃ demonstrated rapid and extensive rundown. While IₖACh (induced by ACh) was markedly reduced in cells pretreated with pertussis toxin, IₖM₃ was unaltered. Intracellular application of antibodies targeting α-subunit of G₁o protein suppressed IₖACh without affecting IₖM₃. Antibodies targeting the N and the C terminus, respectively, of G₂ protein α-subunit substantially depressed IₖM₃ but failed to alter IₖACh. The antibody against β-subunits of G proteins inhibited both IₖACh and IₖM₃. IₖM₃ activated by choline in the cell-attached mode of macropatches persisted in the cell-free configuration. Application of purified G₄ protein α-subunit or βγ-subunit of G proteins or guanosine 5'-O-(thiotriphosphate) to the external solution activated IₖM₃-like currents in inside-out patches. Our findings revealed a novel aspect of receptor-channel signal transduction mechanisms, and IₖM₃ represents the first Gq protein-coupled K⁺ channel. We propose that the G protein-coupled K⁺ channel family could be divided into two subfamilies: Gq protein-coupled K⁺ channel subfamily and Gq protein-coupled K⁺ channel subfamily.

While M₃ receptors are commonly believed to be the only functional mAChRs1 in cardiac tissues, this concept has been challenged by recent findings revealing the presence of M₃ receptors in the hearts of various species including guinea pig (1, 2), rat (3), dog (4–6), and human (7–11). We discovered that the cardiac M₃ receptors mediate the activation of a novel delayed rectifier K⁺ current (we have named it IₖM₃) distinct from IₖACh and other known K⁺ currents. IₖACh is characterized by strong inward rectification, whereas IₖM₃ conducts a delayed rectifier-like K⁺ current. We also found that M₃ receptors and IₖM₃ play a significant role in regulating heart rates, cardiac resting membrane potential, and membrane repolarization (1, 2). The findings suggest that we are no longer able to consider parasympathetic control of the heart as due to a simple ACh-M₂ interaction; we have to understand cholinergic effects in terms of the consequence of activating multiple subtypes of mAChRs, with potentially varying signal transduction and effector systems (such as different K⁺ channels) (6).

The M₂ and M₃ receptors are characterized biochemically by stimulation of a large inositol phosphate response while having a small stimulatory effect on adenylyl cyclase activity. The M₃ and M₄ isoforms are typically linked to an inhibition of adenylyl cyclase activity and only a modest stimulation of inositol phosphate release (12, 13). IₖACh is a G protein-gated K⁺ channel and its activation is a result of interactions between M₂ receptors and K⁺ channels coupled directly via PTX-sensitive G₄ proteins (14–16). i.e. activation of IₖACh, is critically determined by G₂ protein activity and is independent of the downstream components of the G₂ protein signaling pathway. It remained unknown how the M₃ receptors mediate activation of IₖM₃. Since M₃ receptors have been found to mainly activate the G₂-phospholipase C (PLC)-protein kinase C (PKC) pathway, we hypothesized that G₂ protein plays a critical role in IₖM₃ activation upon M₃ receptor stimulation in cardiac cells. The present study was designed to examine this hypothesis so as to elucidate the signal transduction mechanisms of M₃-mediated activation of IₖM₃, as compared with M₂-IₖACh coupling.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture—Single canine atrial myocytes were isolated as previously described (1, 2, 4–6). The dispersed cells were stored in KB medium (20 mM KCl, 10 mM KH₂PO₄, 2.5 mM glucose, 70 mM potassium glutamate, 10 mM β-hydroxybutyric acid; 20 mM taurine, 10
results and discussion

Depolarizing voltage steps activated \(I_{\text{KM3}}\) in the presence of continued stimulation of \(M_2\) receptors by choline (10 mM) in the bathing solution, which was otherwise absent before choline application. The current was highly sensitive to \(M_2\)-selective antagonists \(p\)-FIHISI (20 mM), darifenac (20 mM), or 4-DAMP (10 mM) (Fig. 1B). To test whether G protein is required for \(I_{\text{KM3}}\) activation by \(M_2\) receptor stimulation, GTP of the pipette solution was replaced by GDP\(\beta\)S (1 mM, a nonphosphorylatable analogue of GDP) to deplete the cytosolic GTP. Recordings were made right after membrane rupture before dialysis took place as base-line control data, and the same recordings were repeated 20 min after membrane rupture with complete dialysis, and the data reflected the effects of antibodies. Cloning of Cardiac \(M_3\) Receptor DNA Fragment—Gene-specific primer pairs (tggaacaacaatgatgctgc (forward) and ccttttccgcttagtgatctg (reverse)) were designed based on published sequence of human \(M_3\). Total RNA was isolated from dog atrium as previously described in detail (19). First-strand cDNA resulting from reverse transcription was applied to the cytosol of the cells by dialysis through the pipette delivering antibodies into the cytoplasm of cells by pipette solution dialysis have been frequently employed by other researchers (21, 22). To confirm the role of \(G_\alpha_q\) protein in the coupling between \(M_3\) receptors and \(I_{\text{KM3}}\), a nonphosphorylatable analogue of GDP was used to deplete the cytosolic GTP. Recordings were made right after membrane rupture before dialysis took place as base-line control data, and the same recordings were repeated 20 min after membrane rupture with complete dialysis, indicating a requirement of G proteins for \(I_{\text{KM3}}\) activation.

It is known that stimulation of \(M_3\) receptors activates the PTX-sensitive \(G\) protein, which is in turn interacts with \(I_{\text{KM3}}\) to open up the channels. That is, \(M_3\) receptors are coupled to \(I_{\text{KM3}}\) via the membrane-delimited \(G\) proteins, or in other words, \(I_{\text{KM3}}\) is a \(G\) protein-gated \(K^+\) channel (14–16). Our experiments confirmed this point. The possible role of PTX-sensitive \(G\) proteins in \(M_3\)-\(I_{\text{KM3}}\) coupling was also investigated. As shown in Fig. 1B, while \(I_{\text{KM3}}\) was clearly suppressed in cells pretreated with PTX (2 \(\mu\)g/ml) for 90 min in the recording solution, \(I_{\text{KM3}}\) was unaltered. Similar results were obtained when PTX (2 \(\mu\)g/ml) was applied intracellularly by dialysis (data not shown). In another set of experiments, anti-\(G_\alpha_{16}\) antibody targeting the C terminus of \(G_\alpha_q\) protein \(\alpha\)-subunit was applied to the cytosol of the cells by dialysis through the pipette solution. Consistent with the PTX data, anti-\(G_\alpha_{16}\) antibody (15 \(\mu\)g/ml) remarkably diminished \(I_{\text{KM3}}\), whereas \(I_{\text{KM3}}\) was unchanged (Fig. 1C). The results from the above experiments, while confirming the role of \(G_\alpha_q\) protein in the coupling between \(M_3\) receptors and \(I_{\text{KM3}}\), ruled out contribution of \(G_\alpha_q\) protein to \(I_{\text{KM3}}\) activation by choline. Studies on mAChR function by delivering antibodies into the cytoplasm of cells by pipette solution dialysis have been frequently employed by other researchers (21, 22).

We then turned to test the possible role of \(G_\beta\) protein. We used two different antibodies: anti-\(G_\beta_{24}\)(N) and anti-\(G_\beta_{24}\)(C), antibodies targeting the N and the C-terminal regions of a \(G\) protein \(\alpha\)-subunit, respectively. Clearly, both anti-\(G_\beta_{24}\)(N) and anti-\(G_\beta_{24}\)(C) at a concentration of 10 \(\mu\)g/ml produced substantial suppression of \(I_{\text{KM3}}\), whereas \(I_{\text{KM3}}\) was unaffected. Elevating anti-\(G_\beta_{24}\)(N) antibody concentration to 50 \(\mu\)g/ml nearly abolished \(I_{\text{KM3}}\), and the inhibitory effects were not reproduced when the antibody was preinactivated by boiling (Fig. 2A). There is evidence indicating that the C-terminal region of \(G_\beta\) protein \(\alpha\)-subunits is responsible for interaction with receptor proteins (14, 23–24). Thus, the results from anti-\(G_\beta_{24}\)(C) antibody would attest the requirement of whole \(G_\beta\) protein for \(I_{\text{KM3}}\) activation but do not allow us to distinguish which \(G_\beta\) protein heterotrimer subunit (\(G_\alpha\) or \(G_\beta\) or \(G_\gamma\)) transduces the signal from \(M_3\) receptors to \(I_{\text{KM3}}\). It is also known that the N terminus of \(G_\beta\) protein bears a region critical for effector function (15,
The inset shows the suppressing effects of anti-Gq(C) antibody (10 µg/ml) on I_{KMS} (n = 3). B, effects of anti-Gqcom antibody (10 µg/ml) targeting various β-subunits of G protein on I_{KACH} and I_{KMS}. t test indicates significant reduction of both I_{KACH} (p < 0.05 for all potentials tested, n = 3) and I_{KMS} (p < 0.05 for potentials from −20 to +50 mV, n = 4). C, effects of various components belonging to the Gq-PKC signaling pathway on I_{KMS} activation. Data shown are from at least three cells for each group. *, p < 0.05 versus Ctrl and +p < 0.05 versus phenylephrine (Phen), OAG, or PMA.

25–26) and βγ-subunit binding (23). The anti-Gαq(N) antibody is designed to target the “active” α-subunit-GTP complex. The ability of this antibody to suppress I_{KMS} would therefore suggest the importance of the α-subunit of Gq protein in interacting with I_{KMS}.

The data from anti-Gαq(N) antibody experiments indicated a role of α-subunit of Gq protein for I_{KMS} activation, because the antibody targets the N terminus of α-subunit, which is thought to be responsible for effector interactions and for βγ-subunit binding (25, 26). The βγ-subunit of Gq protein is known to play a major role over α-subunit for I_{KACH} activation (14–16). We then further studied the effects of anti-βcom (against various β-subunits including β1, β2, β3, and β4) on I_{KMS}. Our data demonstrated the ability of anti-βcom antibody (10 µg/ml) to inhibit both I_{KACH} and I_{KMS} (Fig. 2B). This finding indicates that Gβγ may also be a functional subunit in M3-I_{KMS} signaling system.

Activation of Gq protein can result in activation of PLC that hydrolyzes the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to form 1,4,5-inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates Ca2+ release from intracellular stores. Increase in [Ca2+]i and DAG further activates the downstream PKC activity. Our data showed that PLC inhibitor U73122 (1 µM), neutralizing monoclonal PIP2-specific antibody (60 nM), a DAG analogue OAG (10 µM 1-oleoyl-2-acetyl-sn-glycerol), IP3 (100 µM), PMA (a phorbol ester activator of PKC, 100 nM), and Bis (bisindolylmaleimide, a selective PKC inhibitor, 40 nM) all failed to affect I_{KMS} (Fig. 2C). Positive control experiments were performed to assure the effectiveness of these agents. The effects of U73122, OAG, PMA, and Bis were verified with I_{Kur.d} which we have previously shown to be modulated by PKC (18). While bath application of the α1-
adrenoceptor agonist phenylephrine (10 μM) caused enhancement of \(I_{K_{r ea}}\) on-application with U73122 or Bis reversed the effects (Fig. 2C). Superfusion with either PMA or OAG directly increased \(I_{K_{r ea}}\) and the increases were reversed by Bis. The effectiveness of Pip2 was verified by experiments showing that Pip2 (10 μM) included in the pipette increased the \(K^+\) current carried by human \(\text{ether-a-go-go}\) channels (\(I_{HERG}\)) and shift the activation of \(I_{HERG}\) toward hyperpolarizing potentials, effects consistent with the previous findings (17, 27). Since our pipette solution contained 10 mM EGTA, and L-type \(Ca^{2+}\) current was inhibited by Cd 2+ present in the superfusate (internal solution), but with choline and ACh absent in the pipette in the cell-attached patch and sustained after a membrane excision into an inside-out patches, only \(I_{K\beta 4}\)-like current was activated in the cell-attached patch and sustained after a membrane excision into an inside-out patch during the 30-min recording period with little rundown (Fig. 3A). Similarly, when ACh was present in the pipette solution \(I_{K\alpha 4}\) was induced in both cell-attached and inside-out configurations. When GTPγS (10 μM, a nonhydrolyzable analogue of GTP) was present in the superfusate (internal solution), but with choline and ACh absent in the pipette in the inside-out patches, only \(I_{K\beta 4}\)-like currents were activated in seven out of eight patches tested, and there was no apparent concomitant activation of \(I_{K\alpha 4}\)-like currents (Fig. 3B). Only when the internal GTPγS was elevated to 50 μM did \(I_{K\alpha 4}\)-like currents become manifested in five out of eight patches, consistent with the previous finding that higher concentrations of GTPγS are required for \(G_{\alpha i}\) activity relative to \(G_{\alpha o}\) (28). To separate the \(I_{K\beta 4}\)-like-currents from concomitantly activated \(I_{K\alpha 4}\)-like currents, 2 mM Ba2+ was added to the superfusate to inhibit the latter. The \(I-V\) relationships of \(I_{K\beta 4}\)-like and \(I_{K\alpha 4}\)-like currents resemble those of whole-cell \(I_{K\alpha 4}\) and \(I_{K\beta 4}\) with inwardly and outwardly rectifying properties, respectively (Fig. 3B). The results from our macropatch recordings indicate that \(I_{K\beta 4}\) is activated by G protein in a membrane-delimited fashion.

To further investigate the role of α- and βγ-subunits of G proteins in \(I_{K\beta 4}\) activation, we performed experiments using the \(G_{\alpha i}\) and \(G_{\alpha o}\) proteins purified from SF9 cells infected with baculoviruses encoding the recombinant \(G_{\alpha i}\)- and \(G_{\alpha o}\)-subunits, respectively, and the purified βγ-subunit of G proteins (Chemicon International, Inc.). Application of Goi to intracellular side of the inside-out macropatch induced small \(I_{K\alpha 4}\), but failed to induce \(I_{K\beta 4}\) in all eight patches tested. By comparison, application of Goi activated an \(I_{K\beta 4}\)-like current in four out five cells, but in none of six cells tested was \(I_{K\alpha 4}\) induced by Goi (Fig. 3, C and D). On the other hand, perfusion with the βγ-subunit of G protein activated large \(I_{K\alpha 4}\) current and \(I_{K\beta 4}\) too (Fig. 3D). It is therefore possible that the signal generated upon \(M_2\) activation requires Goi for specificity, but the signal is transduced by both Goi-subunit and Gβγ dimer. Interestingly, one elegant study focusing on the \(M_2\) receptor signal transduction for \(Ca^{2+}\) release in \(Xenopus\) oocytes (29) revealed a similar mechanism to what was found in this study.

If it is true that \(I_{K\beta 4}\) activation by the \(M_2\) receptors is mediated by Goi protein, then the conserved sequence of mACH for G protein coupling must be present in the heart. Indeed, we cloned a cDNA fragment representing dog cardiac \(M_3\) receptor (GenBank™ accession number AF056305) spans a part of the third intracellular loop thought to contain a region critical for G protein binding. Particularly, our fragment covers the first 16–21 amino acids of the third intracellular domain, which determine the G protein coupling specificity (30, 31). This cardiac \(M_3\) cDNA fragment shares 81% homology to the same region of human \(M_3\) gene (20) in the amino acid level, but only 4% homology to human \(M_2\) receptor and 3.8% homology to the dog cardiac \(M_2\) receptor (GenBank™ accession number AF084483). The data provide an evidence for the presence of \(G_{\alpha 15}\) protein coupling domain of the \(M_3\) receptor in dog heart.

The K+ channel superfamly is composed of several K+ channel families (voltage-gated, inward rectifier, ligand-gated, second-messenger-gated, and G protein-gated K+ channel families). \(I_{K\alpha 4}\) is a prototype member of the G protein-gated K+ channel family identified to date and probably the only current, among the cardiac currents identified to date, belonging to a member of the G protein-coupled K+ channel subfamily, although some evidence suggests that ATP-sensitive K+ current \(I_{KATP}\) is also gated by G protein (15). A major distinction of G protein-coupled K+ channels from others is that activation of G protein is absolutely required for channels to open. Although other K+ channel families like voltage-gated K+ channels are also modulated by G protein activity, their activation is not dependent on G protein. Our present finding that \(I_{K\beta 4}\) is a \(G_{\alpha i}\)-protein-coupled K+ channel can be viewed as an addition to the G protein-coupled K+ channel family. We therefore propose that the G protein-coupled K+ channel family could be divided into two subfamilies: G protein-coupled K+ channel subfamily and \(G_{\alpha i}\)-protein-coupled K+ channel subfamily.

\(I_{K\alpha 4}\) has been shown to be down-regulated in both its function and expression (6, 32, 33), whereas \(I_{K\beta 4}\) was found to be increased (6), in atra with atrial fibrillation in both animal models and human hearts. Correspondingly, the atrial \(M_2\) receptor density decreased, whereas \(M_2\) receptor density increased (6), and \(G_{\alpha 15}\) protein level decreased by 12% (34), in atrial fibrillation. It is tempting to speculate that an increase in \(I_{K\beta 4}\) may contribute to initiation and perpetuation of atrial fibrillation. Yet future studies are needed to test this notion.

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