Muscarinic M2 Receptor Stimulation of Cav1.2b Requires Phosphatidylinositol 3-Kinase, Protein Kinase C, and c-Src

B. Callaghan, S.D. Koh, K.D. Keef

Abstract—This study investigated regulation of L-type calcium channels (Cav1.2b) by acetylcholine (ACh) in rabbit portal vein myocytes. Whole-cell currents were recorded using 5 mmol/L barium as charge carrier. ACh (10 μmol/L) increased peak currents by 40%. This effect was not reversed by the selective muscarinic M3 receptor antagonist 4-DAMP (100 nmol/L) but was blocked by the M2 receptor antagonist methoctramine (5 μmol/L). The classical and novel protein kinase C (PKC) antagonist calphostin C (50 nmol/L) abolished ACh responses, whereas the classical PKC antagonist Gö6976 (200 nmol/L) had no effect. ACh responses were also abolished by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (20 μmol/L), by the c-Src inhibitor PP2 (10 μmol/L) (but not the inactive analogue PP3), and by dialyzing cells with an antibody to the G-protein subunit Gβγ. Cells dialyzed with c-Src had significantly greater currents than control cells. Current enhancement persisted in the presence of LY294002, suggesting that c-Src is downstream of PI3K. Phorbol 12,13-dibutyrate (PDBu, 0.1 μmol/L) increased currents by 74%. This effect was abolished by calphostin C and reduced by Gö6976. The PDBu response was also reduced by PP2, and the PP2-insensitive component was blocked by Gö6976. In summary, these data suggest that ACh enhances Cav1.2b currents via M2 receptors that couple sequentially to Gβγ, PI3K, a novel PKC, and c-Src. PDBu stimulates the novel PKC/c-Src pathway along with a second pathway that is independent of c-Src and involves a classical PKC. (Circ Res. 2004;94:626-633.)

Key Words: smooth muscle ■ L-type calcium channels ■ kinase ■ patch clamp

Cav1.2b channels (L-type Ca2+ channels) are voltage-dependent channels that open in response to membrane depolarization to allow the entry of Ca2+ into the cell. They are ubiquitously expressed in vascular smooth muscle and play a fundamental role in the regulation of tone. Agonists such as norepinephrine can increase Cav1.2b channel activity through cell depolarization. However, studies over the past 15 years have suggested that a variety of agonists can also more directly affect Cav1.2b by increasing the amount of current generated at a given voltage.1,2 The mechanisms involved in this kind of channel regulation are still not well understood and are the topic of this study.

We have previously shown that Cav1.2b currents in rabbit portal vein myocytes are increased when adrenergic β1a receptors are stimulated with isoproterenol. This action involves both G-protein subunits, ie, effects of Gαs are mediated by protein kinase A (PKA) and effects of Gβγ are mediated by protein kinase C (PKC).3,4 The Gβγ subunit and PKC are also required for AT1 receptor stimulation of Cav1.2b currents in rat portal vein myocytes.5,6 In these studies, the immediate downstream mediator of Gβγ was found to be phosphatidylinositol-3 kinase (PI3K).7 Indeed, dialysis of cells with activated PI3Kγ also stimulated Cav1.2b currents, and this effect was sensitive to PKC blockade. Thus, Gβγ coupled to two different receptors as well as two different Gβγ subunits dialyzed into cells all stimulate Cav1.2b currents in a PKC-dependent manner, suggesting that the Gβγ/PI3K/PKC pathway may represent a ubiquitous mechanism by which agonists stimulate Cav1.2b.

Muscarinic M2 receptors (coupled to the G-protein Gi)8 have also been shown to activate a Gβγ/PI3K/PKC pathway in Xenopus oocytes and tracheal smooth muscle cells.9 In contrast, a recent study by Jin et al10 has suggested that M2 receptor stimulation of Cav1.2b in rabbit colonic myocytes involves the nonreceptor tyrosine kinase c-Src. c-Src is abundantly expressed in vascular smooth muscle11 and recently has been linked to agonist-induced inhibition of potassium channels.12 PKC can increase c-Src activity,13–16 and c-Src has been shown to stimulate Cav1.2b currents in vascular smooth muscle cells.17,18 In light of these observations, we hypothesized that c-Src is the downstream mediator of Cav1.2b channel stimulation in the Gβγ/PI3K/PKC pathway. To explore this hypothesis, we evaluated the role of Gβγ, PI3K, PKC, and c-Src in M2-receptor stimulation of Cav1.2b currents in rabbit portal vein myocytes. Various blockers were used to examine the actions of acetylcholine (ACh), phorbol 12,13-dibutyrate (PDBu), and c-Src on Cav1.2b currents. Our results show for the first time that M2
receptor stimulation of Cav1.2b currents is coupled to the GβγPI3K/PKC pathway, lending support to the ubiquitous nature of this agonist-induced pathway. They additionally demonstrate that an important downstream mediator is c-Src, which either directly stimulates the channel or activates an additional second messenger to enhance Cav1.2b activity.

Materials and Methods

Isolation of Rabbit Portal Vein Myocytes

Myocytes were isolated using previously described methods.4 Male albino rabbits (1.5 to 2.0 kg; Western Oregon Rabbitry, Philomath, Ore) were killed with an intravenous overdose of sodium pentobarbital (50 mg/kg). Smooth muscle cells were isolated from the portal vein. The animal use protocol was reviewed and approved by the Animal Care and Use Committee of the University of Nevada.

Electrophysiology

The patch-clamp experiments were performed as previously described.1 Inward currents were measured using an Axopatch-1D patch-clamp amplifier, digitized with a 16-bit analog to digital converter (Model DIGIDATA 1320A, Axon Instruments), and controlled by pClamp8 (Axon Instruments). Ba2+ currents (Iba) in myocytes were measured using both the dialyzed whole cell and the perforated patch configurations. The bath solution used to record Iba was composed of (in mmol/L) NaCl 120, TEACl 10, BaCl2 5, MgCl2 0.5, glucose 5.5, CsCl 5, and HEPES 10, pH 7.40, with NaOH. Both TEACl and CsCl were used to block potassium currents. For perforated patch experiments, the composition of the pipette solution was (in mmol/L) cesium aspartate 120, TEACl 20, EGTA 1, and HEPES 20, adjusted pH 7.2 with CsOH. Amphotericin B (90 mg/mL) was dissolved with dimethylsulfoxide (DMSO), sonicated, and diluted to give a final concentration of 270 μg/mL in the pipette solution. For the dialyzing whole-cell experiments, the composition of the pipette solution was (in mmol/L) CsCl 120, TEACl 20, glucose 5.5, MgCl2 2, ATP 5, EGTA 5, and HEPES 10, pH 7.2 with CsOH.

Drugs

Collagenase type I, protease type XXVII, BSA, ACh, amphotericin B, PDBu, calphostin C, 4-diphenylacetoxy-N-methylpiperide (4-DAMP), and methoctramine were purchased from Sigma. PP2, LY294002, chelerythrine Cl, and G6976 were from CalBiochem. Purified c-Src kinase dephosphorylated at the terminal inhibitory tyrosine was obtained from Upstate Biotechnology. Gβγ antibody (T20) was obtained from Santa Cruz Biotechnology Inc. Drugs insoluble in water were first dissolved in DMSO and then additionally diluted so that the final concentration of DMSO was <0.2%. DMSO alone at 0.2% had no effect on Iba.

Data Analysis

All experimental values are presented as mean ± SEM, and n refers to the number of cells tested. Differences between the values from different groups were compared using Student’s paired and unpaired t tests and 2-way ANOVA, where appropriate. P < 0.05 was considered significantly different. The IC50 for PP2 was determined by fitting the data with a nonlinear least-squares regression program (GraphPAD).

Results

ACh Enhances Cav1.2b Currents via M2 Receptors and the Gβγ Subunit

Ba2+ currents (Iba) through L-type Ca2+ channels (Cav1.2b) were recorded in freshly isolated rabbit portal vein myocytes using the whole-cell voltage-clamp technique. Currents were elicited by stepping voltage from a holding potential of −70 to 0 mV for 370 ms at 30-second intervals. These currents are blocked by nicardipine.19 Peak steady-state current amplitudes were obtained after 4 to 5 minutes (166 ± 11.5 pA, n=36). Addition of ACh (10 μmol/L) caused a significant increase in peak Iba to 140 ± 5% of the basal level (n=14) (Figures 1A and 1B). The increase in currents occurred without a shift in the voltage dependence of activation or inactivation (data not shown).

The predominant muscarinic receptors mediating contraction in smooth muscle are M2 and M3.20,21 and previous studies have suggested that the actions of ACh on Cav1.2b are attributable to M2 receptor stimulation.10 To ascertain whether this is the case in the portal vein, we examined the effect of the selective M3 antagonist 4-DAMP (100 nmol/L) and the selective M2 antagonist methoctramine (5 μmol/L). 4-DAMP gave rise to a small but significant increase in the ACh-induced response (140% versus 154%, n=9), whereas methoctramine significantly reduced the response (Figures 1C and 1D). These data suggest that ACh enhances Cav1.2b currents via the M2 receptor. Unless otherwise specified, the remaining experiments with ACh were carried out in the presence of 4-DAMP (100 nmol/L).

We have hypothesized that M2 receptors stimulate Cav1.2b via Gβγ. To provide direct evidence for the role of Gβγ in the actions of ACh, we tested a blocking antibody to the Gβ subunit. In previous studies, we have shown that this antibody distinguished between the Gβγ and Gαi effects associated with adrenergic β2 receptors in portal vein myocytes.3 Dialysis of cells with Gβ antibody (10 μg/mL) entirely blocked the stimulatory effects of ACh (n=7; Figures 1E and 1F).

Cav1.2b Is Regulated by Both Novel and Classical PKCs

Previous studies have shown involvement of PKC in Gβγ stimulation of Cav1.2b currents3,6; therefore, we tested the effects of several PKC inhibitors on the ACh response. Calphostin C (50 nmol/L), which blocks both classical and novel PKCs, reversed the effect of ACh (n=6) (Figures 2A through 2C). However, G6976 (200 nmol/L), which blocks only classical PKCs,22 had no significant effect on the ACh response (n=6) (Figures 2D through 2F). These results suggest that ACh stimulates Iba via a novel PKC isoform.

To further investigate which isoforms of PKC lead to current stimulation, we tested PDBu, which activates both classical and novel PKCs.23 Application of PDBu (100 nmol/L) consistently increased peak Iba (n=20; Figure 3) without a shift in the voltage dependence of activation or inactivation (data not shown). A steady-state response was reached after 3 to 6 minutes and remained stable thereafter (ie, up to 20 minutes). The response to PDBu was entirely blocked by calphostin C (n=11) (Figures 3A through 3C). In contrast, G6976 (200 nmol/L) significantly reduced but did not abolish the PDBu response (n=8; Figures 3D through 3F). These data suggest that both novel and classical PKCs are linked to simulation of Iba in portal vein myocytes.

M2 Stimulation of Cav1.2b Involves PI3K

Studies by others suggest that the immediate downstream mediator of Gβγ is PI3Kγ.6,7,24 To examine the role of PI3K
in the ACh-induced response, we tested the PI3K inhibitor LY294002 (20 μmol/L). LY294002 completely reversed current stimulation induced by ACh (Figures 4A through 4C).

To determine whether PI3K is upstream or downstream of PKC, PDBu (100 nmol/L) was applied to cells preexposed to LY294002 (20 μmol/L). LY294002 alone had no significant

Figure 1. ACh stimulates Cav1.2b currents via M2 receptors and the Gβγ subunit. Sample traces (A) and plot of peak I_{Ba} as a function of time (B) under various experimental conditions in one cell. Numbers on plot indicate which points were used for sample traces. C, Plot of the I-V relationship for Cav1.2b currents in the presence (●) and absence (○) of ACh (10 μmol/L) (n=14). D, Bar graph of summed results. ACh significantly increased I_{Ba}. Blocking M3 receptors with 4-DAMP (100 nmol/L) led to a small but significant additional increase in I_{Ba} (M3), whereas current stimulation was reversed by blocking M2 receptors with methoctramine (5 μmol/L) (M2). Bars plot mean peak I_{Ba}=SEM (n=9). E and F, Dialysis of cells with Gβγ antibody blocks ACh stimulation of Cav1.2b. E, Plot of peak I_{Ba} as a function of time in 2 cells from the same experiment using dialyzing patch pipettes. For one cell, Gβγ antibody was included in the pipette (●); for the other, it was not (○). ACh (10 μmol/L) was applied to both cells 5 minutes after attaining the whole-cell configuration. This increased current in the control cell but not in the cell dialyzed with Gβγ antibody (10 μg/mL). F, Bar graph plotting I_{Ba} under various conditions. ACh (n=6) significantly increased current above baseline levels in control cells (n=6) but not in cells dialyzed with Gβγ antibody (n=7). Bars represent mean peak I_{Ba}=SEM. *P<0.05, **P<0.01, ***P<0.001, significantly different from the control value under the same experimental conditions.

Figure 2. ACh stimulation of Cav1.2b is dependent on a novel PKC. Sample traces (A and D) and plot of peak I_{Ba} as a function of time (B and E) under various experimental conditions. Numbers on plot indicate which points were used for sample traces. A through C, Effect of calphostin C on ACh-induced current stimulation. C, Bar graph of summed results. ACh (10 μmol/L) significantly increased I_{Ba}, and calphostin C (50 nmol/L) reversed this effect (n=6). D through F, Effect of Gö6976 on ACh-induced current stimulation. F, Bar graph of summed results. ACh (10 μmol/L) significantly increased I_{Ba} but this stimulation was unchanged by Gö6976 (200 nmol/L, n=6). Bars represent mean peak I_{Ba}=SEM. *P<0.05, **P<0.01, ***P<0.001, significantly different from the control value under the same experimental conditions.
effect on $I_{Ba}$. Current stimulation with PDBu in the presence of LY294002 was not significantly different from the response to PDBu in the absence of LY294002 (Figures 4D through 4F), suggesting that PK3K is not downstream of PKC activation.

**M2 Stimulation of Cav1.2b Involves c-Src**

We next investigated the hypothesis that the downstream mediator of the M2/Gβγ/Pi3K/PKC pathway is c-Src. Cav1.2b in native cells seems to be basally phosphorylated by c-Src, because blockers of c-Src are known to reduce basal $I_{Ba}$. PDBu significantly increased $I_{Ba}$ and this stimulation was significantly reduced but not abolished by G6976 (200 nmol/L, n=8). Bars represent mean peak $I_{Ba} \pm$ SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, significantly different from the control value under the same experimental conditions.
Cav1.2b currents. This was also the case for rabbit portal vein myocytes, because superfusion with the c-Src inhibitor PP2 (10 μmol/L) led to a 46% (n=5) reduction in peak current amplitude (Figure 5). We therefore applied PP2 first to test its effect on ACh. In the presence of PP2, the response to ACh was entirely abolished. Removal of PP2 in the continued presence of ACh revealed the stimulatory effects of ACh (Figures 5B and 5C; n=6). The inactive analogue PP3 had no effect on the ACh response (n=4). These results suggest that c-Src plays a role in the stimulatory effects of ACh on Cav1.2b. Because the potency of PP2 for different Src family members varies, we also examined the concentration dependence of block. Half block of the ACh response was 0.4 μmol/L PP2 (Figure 5D).

To additionally investigate the relationship of novel PKCs to c-Src, we examined the effect of PP2 on the PDBu response. The response to PDBu in the presence of PP2 was significantly reduced but not abolished (Figure 6). The component of the PDBu response that persisted in the presence of PP2 was attributable to a classical PKC, because it was entirely abolished by Gö6976. Removal of both PP2 and Gö6976 while retaining PDBu revealed additional PDBu stimulation (n=8) (Figure 6). These data support the hypothesis that a novel PKC leads to c-Src–dependent stimulation of Cav1.2b. They additionally suggest that a classical PKC can stimulate the channel via a c-Src–independent pathway.

c-Src Enhances Cav1.2b, and This Effect Is Independent of PI3K
We have proposed that the downstream mediator of the Gβγ/PI3K/PKC pathway is c-Src. To test whether c-Src can directly stimulate Cav1.2b, we applied activated c-Src to cells by including it in the pipette solution (7.5 U) and dialyzing it into the cell using the whole-cell patch configuration. In the absence of c-Src, a small amount of current run up occurred immediately after access, and a steady state was reached 2 to 3 minutes later. Thereafter, currents remained stable for ~8 minutes before run down began. When c-Src was included in the patch pipette, the currents recorded were significantly larger than those of control cells, but there was no significant shift in the I–V relationship (Figure 7). c-Src can lead to activation of PI3K. To investigate this possibility, we repeated some experiments in the presence of the PI3K inhibitor LY294002. Cells were bathed with LY294002 (20 μmol/L) for at least 5 minutes before the whole-cell patch configuration was attained. Thereafter, currents were recorded in cells dialyzed with c-Src. LY294002 did not significantly reduce c-Src stimulation (n=4) (Figure 7B).

Discussion
The present study investigated the actions of ACh on Cav1.2b currents in rabbit portal vein myocytes. ACh significantly increased currents via stimulation of muscarinic M2 receptors. Our results suggest that this response is coupled to a second messenger pathway that includes Gβγ, PI3K, and a novel PKC and that the downstream mediator of the Gβγ/PI3K/PKC pathway is c-Src. A diagram of the proposed pathway is shown in Figure 8.

Previous studies of rabbit colonic myocytes suggest that ACh stimulates Cav1.2b currents via M2 receptors. The present study concurs with this conclusion, because ACh responses were blocked with the M2 receptor antagonist methoctramine but not the M3 receptor antagonist 4-DAMP. M2 receptors are generally coupled to the G protein Gs. In rabbit portal vein myocytes, the stimulatory effects of ACh seem to be mediated by the Gβγ subunit of Gs, because the response was abolished by dialyzing cells with a Gβ blocking antibody. A known target of Gβγ is PI3K, and there is evidence from rat portal vein studies that Gβγ stimulates Cav1.2b via PI3K. We found that the response to ACh was abolished by the selective PI3K inhibitor LY294002, suggesting that PI3K participates in the M2/Gβγ pathway in rabbit portal vein as well. In contrast, the stimulatory effect of PDBu was not blocked by LY294002, suggesting that PKC is downstream of PI3K rather than the reverse.

At present it is unclear how PI3Kγ activates PKC. PtdIns(3,4,5)P3, the lipid product of PI3Kγ, targets several different second messengers, including PKC. Furthermore,
PI3Kγ itself has serine-kinase activity,31 and this could lead to PKC activation. The ACh/M2/GBP/PI3K pathway regulating chloride channels in oocytes was linked to PtdIns(3,4,5)P3-dependent activation of PKCζ, an atypical PKC that is insensitive to both Ca2+ and DAG.9 However, this does not seem to be the case for rabbit portal vein myocytes, because current stimulation with either ACh (present study) or dialysis of cells with GBP was abolished (n=8). Bars represent mean peak I0.5 SEM. *P<0.05, **P<0.01, ***P<0.001, significantly different from the control value under the same experimental conditions.

Our results suggest that the downstream mediator of PKC is c-Src. C-Src is a nonreceptor tyrosine kinase that is abundantly expressed in vascular smooth muscle cells.11 The response to ACh was abolished with the c-Src inhibitor PP2, suggesting a role for this kinase in the GBP/PI3K/PKC pathway. We and others17,18 have also found that dialysis of cells with activated c-Src enhances Cav1.2b currents in vascular smooth muscle cells. PKC activation precedes c-Src activation, because current stimulation with PDBu is also reduced after c-Src inhibition with PP2. PKC has also been shown to activate c-Src in other smooth muscles and other cell types.13–16 In addition, G-protein activation has been linked sequentially to PKC and c-Src in other cell types.40–42
Finally, c-Src seems to be the downstream mediator of agonist-induced inhibition of K+ channels in coronary artery.23

At present, it is unclear how PKC activates c-Src. It is possible that other nonreceptor tyrosine kinases (ie, Pyk2 and FAK) are interposed between PKC and c-Src. Alternatively, recent studies suggest that PKC may enhance c-Src activity by stimulating a protein tyrosine phosphatase that dephosphorylates the inhibitory c-terminal tyrosine of c-Src.43 Dephosphorylation of this tyrosine will enhance c-Src activity by allowing the protein to unfold to expose its catalytic domain and undergo autophosphorylation.44 Additional studies will be needed to identify the details leading from PKC to c-Src activation.

Both classical and novel PKCs appear capable of Cav1.2b current stimulation, because the PDBu response is abolished by calphostin C and reduced by the classical PKC inhibitor Go6976. In contrast, the ACh response is insensitive to Go6976. As discussed above, we have proposed that stimulation of Cav1.2b by ACh is specifically coupled to activation of c-Src by a novel PKC. In a similar manner, the novel PKC component of the PDBu response is blocked by PP2, whereas the classical PKC component persists. Thus, a separate pathway must exist by which classical PKCs stimulate Cav1.2b independent of c-Src.

The IC50 for PP2 block of the ACh response is 0.4 μmol/L. PP2 and PP1 are almost identical in structure, and in vitro studies suggest that both are potent blockers of Lck and Fyn (IC50, ~5 nmol/L) than of c-Src (IC50, ~170 nmol/L).45 Other IC50 values reported for block of Src activity range from 0.07 to 0.5 μmol/L,11 to 2 μmol/L.25 Thus, the potency we observed for PP2 is compatible with an action on c-Src, but the possible contribution of other Src family members cannot be eliminated.

The mechanism by which c-Src enhances Cav1.2b currents is still unclear and may vary between tissues. Recent studies in the rat model suggest that c-Src phosphorylates a particular tyrosine on the c-terminus of Cav1.2.17,46 However, the rabbit smooth muscle isoform of Cav1.2b cannot be regulated by this mechanism, because this phosphorylation site is absent. Several other sites with moderate and high phosphorylation potential are present at the c-terminus of the rabbit sequence. Furthermore, there is evidence that c-Src regulates Cav1.2b in other rabbit smooth muscles, ie, colon19,47 and ear artery.18,25,48 These data suggest that c-Src may still directly regulate rabbit Cav1.2b without the tyrosine phosphorylation site identified in the rat. Interestingly, the human isoform of Cav1.2b also lacks the tyrosine phosphorylation site identified in the rat, but it does contain five potential phosphorylation sequences at the c-terminus.

In summary, this study has provided evidence for a second messenger pathway initiated by M2 receptors that couples sequentially from Gβγ to PI3K, a novel PKC, and the tyrosine kinase c-Src. Taken together with other studies, these results suggest that this pathway may have broad relevance to the control of Cav1.2b currents mediated by a variety of different receptor/G-protein complexes in smooth muscle as well as to the regulation of other ionic conductances.

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References


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