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 Gō6976. The PDBu response was also reduced by PP2, and the PP2-insensitive component was blocked by Gō6976. 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
receptor stimulation of Cav1.2b currents is coupled to the Gβγ/Pi3K/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.5 Male albino rabbits (1.5 to 2.0 kg; Western Oregon Rabbitry, Philomath, Ore) were killed with an intravenous overdose of sodium pentobarbitol (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.3 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 (Ina) in myocytes were measured using both the dialyzed whole cell and the perforated patch configurations. The bath solution used to record Ina 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) CsCl 120, CsOH 40, MgCl2 10, ATP 10, EGTA 10, HEPES 10, pH 7.2 with CsOH.

Drugs
Collagenase type I, protease type XXVII, BSA, ACH, amphoterin B, PDBu, calphostin C, 4-diphenylacetoxy-N-methylpiperide (4-DAMP), and methochemtane were purchased from Sigma. PP2, LY294002, chelerythrine Cl, and Go6976 were from CalBiochem. Purified c-Src kinase dephosphorylated at the terminal inhibitory tyrosine was obtained from Upstate Biotechnology. Gβγ antibody distinguishes between the Gβγ 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, Go6976 (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 Ina 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 Ina (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, Go6976 (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 Ina 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

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**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 basal 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.

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**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

Figure 3. PDBu enhances Cav1.2b currents via both a classical and 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 PDBu-induced current stimulation. C, Bar graph of summed results. PDBu (100 nmol/L) significantly increased $I_{Ba}$, blocking PKC with calphostin C (50 nmol/L) reversed current stimulation ($n=11$). D through F, Effect of Gö6976 on PDBu-induced current stimulation. F, Bar graph of summed results. PDBu (100 nmol/L) significantly increased $I_{Ba}$, and this stimulation was significantly reduced but not abolished by Gö6976 (200 nmol/L, $n=8$). 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 4. Response to ACh but not PDBu is dependent on PI3K. 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 LY294002 on ACh stimulation. C, Bar graph of summed results. ACh (10 μmol/L) significantly increased $I_{Ba}$, and this stimulation was reversed by LY294002 (20 μmol/L, $n=6$). D through F, Effect of LY294002 on PDBu-induced current stimulation. F, Bar graph of summed results. PDBu (100 nmol/L) significantly increased $I_{Ba}$ in the presence of LY294002 ($n=4$). The effect of PDBu in the absence of LY294002 was not different ($n=4$). 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.
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 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.

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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, and this could lead to PKC activation. The ACh/M2/Gβγ/Pi3K pathway regulating chloride channels in oocytes was linked to PtdIns(3,4,5)P3-dependent activation of PKCγ, an atypical PKC that is insensitive to both Ca2+ and DAG. 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 Gβγγ2 is blocked with calphostin C. Calphostin C inhibits both classical and novel PKCs but not atypical PKC. Furthermore, the effects of ACh (present study) and Gβγγ2 are not blocked when selective antagonist of classical PKCs are used, leading us to the conclusion that a novel PKC is involved. PKCe has been identified in rabbit portal vein myocytes, so it is possible that PKCe is the novel PKC isoform activated by the Gβγ/Pi3K pathway. PI3K-γ and PtdIns(3,4,5)P3 have been linked to activation of PKCe in other studies. Interestingly, the regulation of Kv currents by angiotensin II in rabbit portal vein myocytes is also suggested to involve PKCe. Finally, another important downstream target of PtdIns(3,4,5)P3 is phosphoinositide-dependent kinase 1 (PDK1). PKCe immunoprecipitates with PDK1, and some studies have suggested that PI3K isoforms may be controlled by PDK1 via PDK1. Taken together, our results clearly suggest a link between Gβγ, PI3K, and a novel PKC in the actions of ACh on Cav1.2b currents. However, additional studies are required to understand the details of this cascade.

Wang et al also investigated the M2 receptor pathway in Xenopus oocytes and native tracheal cells. Their results suggest that the Gβγ/Pi3K/PKC pathway couples to the regulation of ion channels in both models and that this signaling cascade may have broad relevance to neurotransmitter signaling. Our study provides additional support for this proposal and, when considered in conjunction with studies of adrenergic β2 receptors and AT1 receptors, suggests that agonist stimulation of Cav1.2b via the Gβγ/Pi3K/PKC pathway represents a general scheme that may encompass a variety of different receptor/G-protein complexes.

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. The response to ACh was abolished with the c-Src inhibitor PP2, suggesting a role for this kinase in the Gβγ/Pi3K/PKC pathway. We and others 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. In addition, G-protein activation has been linked sequentially to PKC and c-Src in other cells types.
Finally, c-Src seems to be the downstream mediator of agonist-induced inhibition of K+ channels in coronary artery.12

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 Gö6976. In contrast, the ACh response is insensitive to Gö6976. 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 more 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 can not 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, colon10,47 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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