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Invited Mini Review

Direct effect of protein kinase C inhibitors on cardiovascular ion channels

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Protein kinase C (PKC) is a central enzyme that modulates numerous biological functions. For this reason, specific PKC inhibitors/activators are required to study PKC-related signaling mechanisms. To date, although many PKC inhibitors have been developed, they are limited by poor selectivity and nonspecificity. In this review, we focus on the nonspecific actions of PKC inhibitors on cardiovascular ion channels in addition to their PKC-inhibiting functions. The aim of this paper is to urge caution when using PKC inhibitors to block PKC function. This information may help to better understand PKC-related physiological/biochemical studies. [BMB reports 2011; 44(9): 559-565]

INTRODUCTION

Protein kinase C (PKC), a serine/threonine protein kinase, plays a crucial role in signaling transduction mechanisms in many cell types (1, 2). One direct method to investigate the functional role of PKC in signal transduction is to block PKC activity by PKC inhibitor application. Thus, specific PKC inhibitors are indispensable tools for studying biochemistry and molecular biology. To date, several PKC inhibitors have been developed and classified based on their site of action. For example, staurosporine and bisindolylmaleimide (BIM) (I), widely used chemicals in the laboratory, inhibit PKC activity by competitively binding at the PKC ATP-binding site (3, 4). Besides staurosporine and BIM (I), several types of PKC inhibitors block in their own specific manner with PKC interaction. However, their usefulness as PKC inhibitors is limited by nonspecific actions at ion channels. In this review, we characterize the direct and nonspecific actions of PKC inhibitors on cardiovascular ion channels and address PKC inhibitor binding at ion channels. Considering the relevance of PKC in cellular/molecular functions, our information greatly contributes to allow correct interpretation of biochemical data when using PKC inhibitors.

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PKC INHIBITORS

Protein kinases regulate target proteins by phosphorylation of the serine/threonine moiety using ATP as the phosphate source. Therefore, all protein kinases contain both a catalytic functional site and substrate binding site that recognize specific sequences, including serine/threonine.

Many PKC inhibitors are classified according to their inhibition sites. Indeed, PKC inhibitors interact with PKC at catalytic functional sites (ATP binding site), effector sites (regulatory C1 domain site), and protein substrate site (5).

The first reported and widely used drug in the laboratory is staurosporine (6), which inhibits the PKC catalytic functional site. Staurosporine contains indolcarbazole as a key structure (Fig. 1., highlighted). However, staurosporine has low selectivity for PKC and influences other protein kinases (7). The next generation of PKC inhibitors are bis-indole maleimides (e.g., BIM (I); Fig. 1), which are designed to improve PKC selectivity. Similar to staurosporine, bis-indole maleimide targets the catalytic functional site (competes with the ATP-binding site in the PKC hydrophobic pocket). Considering that other protein kinases, such as protein kinase A (PKA) and protein kinase G (PKG), contain an ATP-binding site, bis-indole maleimide also has relatively low selectivity for PKC inhibition. However, continuous trials to improve PKC selectivity using bis-indole maleimide derivatives have led to more specific inhibitors such as ruboxistaurin (LY333531) (8) and enzastaurin (LY317615) (9).

Calphostin C is also widely used for PKC inhibition because it has relatively high selectivity (Fig. 1) (10). It contains a phobol ester/DAG-binding group and interacts with the effector site, regulatory C1 domain of PKC (11). However, calphostin C requires light for activation (12), which is inconvenient for experimental drug application.

PKC pseudosubstrate inhibitory oligopeptide (e.g., 19-36, 19-31), which is derived from the PKC regulatory C1 domain, inhibits PKC activity with high affinity, as the oligopeptide is

recognized by specific amino acid sequences. It consists of arginine-rich and lysine-rich sequences with non-phosphorylatable alanine instead of serine in the protein substrates (5, 13). However, the peptide is membrane impermeable, and thus a myristoyl group should be added to penetrate the cell membrane.

Chelerythrine is a benzophenanthridine alkaloid derived from plants (Fig. 1) (14). This chemical inhibits the PKC protein substrate binding site in the micromolar range, but its selectivity for PKC is dubious (15).

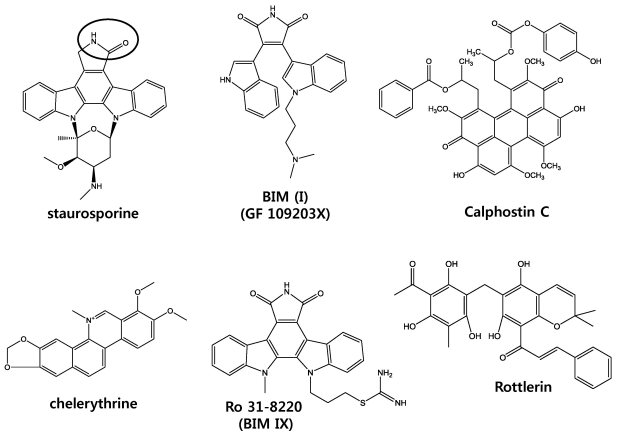

Fig. 1. Structure of PKC inhibitors.

BIOLOGICAL ACTIONS OF PKC INHIBITORS ON ION CHANNELS

The PKC enzyme is recognized as a core molecule related to various signal transduction pathways. Intensive studies on the biochemical/physiological role of PKC over several decades have revealed that PKC activation closely linked to cell growth, cell cycle, differentiation, hormone release, reactive oxygen species (ROS) generation, and ion channels modulation (16-19). Thus, PKC inhibitors are indispensable for studies on PKC-related signaling pathways. Ion channel modulation by PKC activation is relatively well characterized. For example, PKC activation leads to the activation of *L*-type Ca^{2+} channels, Cl^- channels, and nonselective cation channels in vascular smooth muscle (20-22). Generally, K^+ channels in vascular smooth muscle, including voltage-dependent K^+ (Kv), ATP-sensitive K^+ (K_{ATP}), and inward rectifier K^+ (Kir), are inhibited by PKC activation (18, 23, 24). However, several PKC inhibitors are known to directly affect the ion channels independently of PKC-related signaling pathways, making it difficult to right interpretation of the experimental results. Considering that functional studies of PKC mainly depend on PKC activators/inhibitors, clear identification of direct effect of PKC inhibitors on ion channels is very important for the field of biochemistry and/or molecular biology looking to elucidate PKC-related signaling mechanisms. Therefore, in this review, we summarize the direct effect of PKC inhibitors on ion channels (mainly focusing on cardiovascular ion channels) as described below.

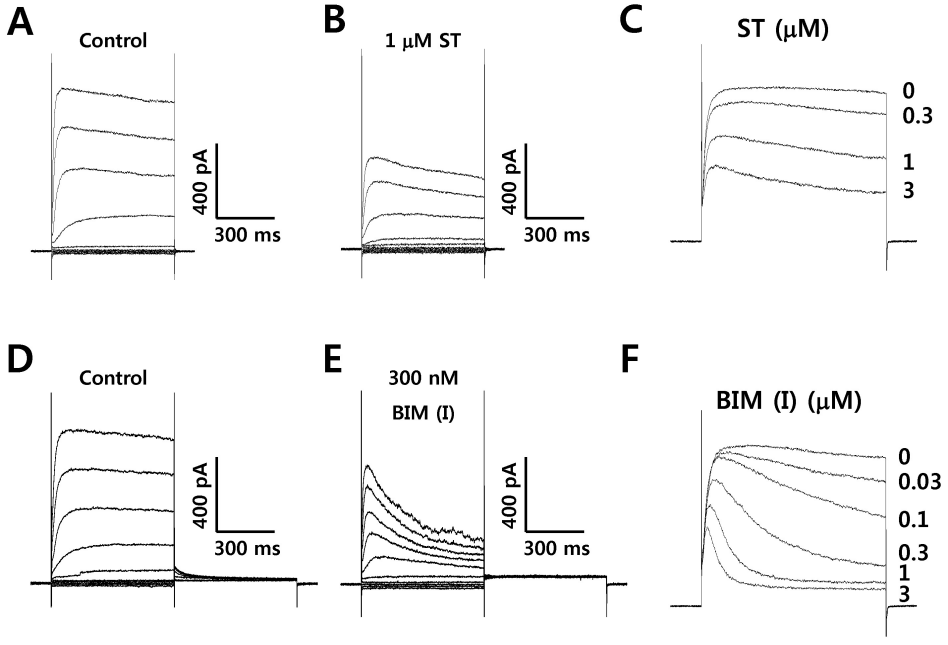

Fig. 2. Effects of staurosporine and BIM (I) on Kv channels in coronary arterial smooth muscle cells. Superimposed currents were elicited by depolarizing step pulses from -120 to +60 mV in steps of 20 mV in the absence (A) and presence (B) of 1 μM staurosporine (ST). (C) Concentration dependence of ST-induced block of Kv currents. Data from Park et al. (20). Representative Kv currents without (D) and with (E) BIM (I). (F) Concentration dependent inhibition of Kv currents by BIM (I). Data from Park *et al*. (40).

DIRECT EFFECTS OF STAUROSPORINE ON ION CHANNELS

Over several decades, staurosporine has been widely used in many laboratories because it is membrane-permeable and inhibits PKC activity at nanomolar concentrations (IC_{50} value: 3 nM) (3). However, higher staurosporine concentrations have additional inhibitory effects on other protein kinases such as PKA, PKG, tyrosine kinases, and MLCK with IC_{50}s up to 20 nM (7). Furthermore, several reports demonstrate the direct effect of staurosporine on ion channels independently of PKC inhibition as described below.

Effects on cardiovascular ion channels

The direct effect of staurosporine on voltage-dependent K^+ (Kv) channels in vascular smooth muscle cells was published by our group (Fig. 2A-C) (25). In that paper, we proposed that the inhibitory mechanism of Kv channels is an open state block because staurosporine accelerates the decay of Kv currents in a concentration-dependent manner with an IC_{50} value of 1.2 μM (Table 1). In general, Kv channel blockers have protonated ammonium atoms for the inhibition of Kv channels in the open state (26-30). Similar to general Kv channel blockers, staurosporine also has a protonated tertiary ammonium ion in the physiological pH range (pKa $\approx$ 10), which is a key structure for the direct/open state inhibition of Kv channels. Moreover, the 5-aromatic-ring motif in staurosporine could contribute to the stabilization of the protonated ammonium ion. The 5-aromatic-ring motif also increases hydrophobic interactions between drugs and channels, which increases the channel-drug-binding probability (25). Staurosporine also affects steady-state Kv channel kinetics. The steady-state activation curve of Kv channel was shifted to a positive potential after application of staurosporine. From these findings, our group suggested that staurosporine also affects the voltage sensitivity of Kv channel in arterial smooth muscle cells (25).

The direct effect of staurosporine on Ca^{2+} channels was reported in coronary arterial smooth muscle cells and atrial myocytes (31, 32). The study of calcium dynamics in coronary arterial smooth muscle implies that staurosporine directly inhibits Ca^{2+} channels (31), but the detailed mechanism is unclear. In atrial myocytes, staurosporine inhibits *L*-type Ca^{2+} channels in a PKC-independent manner with an IC_{50} of 0.062 μM (Table 1) (32). In the present study, staurosporine did not significantly alter the voltage-dependence of Ca^{2+} channel, but the steady-state activation curve was shifted toward a more positive potential.

Staurosporine also has a direct effect on voltage-dependent Na^+ channels in atrial myocytes with an IC_{50} value of 1.107 μM (Table 1) (33). Although recovery kinetics and use-dependency of Na^+ channel do not change due to staurosporine, the steady-state activation and inactivation curves are shifted to the negative potential. Moreover, staurosporine slows decay of Na^+ current inactivation (extends the inactivation time). A similar phenomenon is observed with many lipid-soluble alkaloids and insecticides. In fact, lipid-soluble alkaloids such as batrachotoxin and pyrethroid have a similar mechanism for shifting the voltage dependence of activation curve toward a negative potential and/or slowing the inactivation process (34-36).

A direct effect of staurosporine on muscarinic K^+ channels was also reported in bullfrog atrial myocytes (37). The inhibition of muscarinic K^+ current activated by GTP or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) is rapid and reversible. The inside-out configuration under non-phosphorylating condition shows that the open probability of the GTPγS-induced activation of muscarinic K^+ current decreases without changing the single-channel current amplitude. From those results, the authors concluded that staurosporine affects gating properties without altering the permeation pathway.

Effects on other ion channels

Besides cardiovascular ion channels, staurosporine was reported to inhibit the Kv1.3 channel expressed in Chinese hamster ovary (CHO) cells (IC_{50} = 1.2 μM) (38). Staurosporine accelerates the inactivation rate of Kv1.3 channel, which implies that it interacts with the open state of the channel. However, the steady-state inactivation was not changed by staurosporine, suggesting that staurosporine does not bind to the inactivated state of Kv1.3 channel. Staurosporine reduced the tail current amplitude and slowed the decay kinetics, resulting in a crossover of the tail current. This phenomenon is evidence for open-state inhibition (39).

DIRECT EFFECTS OF BIM (I) ON ION CHANNELS

BIM (I) (GF 109203X) is a widely used PKC inhibitor because of its high selectivity for PKC inhibition compared to staurosporine. Furthermore, the inactive form of BIM (I), BIM (V), has also been developed as a negative control, which is advantageous for experiments of PKC-related signaling mechanisms. However, nonspecific actions of BIM (I) on cardiovascular ion channels also have been reported as described below.

Effects on cardiovascular ion channels

In coronary and mesenteric arterial smooth muscle cells, BIM (I) has reported to inhibit the Kv channels directly (Fig. 2D-F) with an IC_{50} value of 0.27 and 0.23 μM, respectively (Table 1) (40, 41). Similar to staurosporine, this inhibition occurs in the open state of Kv channel in a voltage-, time-, and use-dependent manner. In fact, BIM (I) is a weak base (pKa = 8.52). Therefore, it exists in a protonated form in the physiological pH range, which increases the possibility of direct interaction of BIM (I) with Kv channels as described above. The open block of BIM (I) is supported by results that steady-state activation and inactivation curves are not altered by BIM (I) in both arterial smooth muscle cells.

In ventricular myocytes, BIM (I) inhibits the sustained outward K^+ channel independently of PKC inhibition with an IC_{50}

value of 0.051 μM (Table 1) (42). Clues for the direct inhibition of sustained outward K^+ channels by BIM (I) include that BIM (V), an inactive analog of BIM (I), also inhibits the sustained outward K^+ channels, and other PKC inhibitors fail to produce any effect on outward K^+ channels. Although the detailed inhibitory mechanisms are obscure, the authors suggested a closed channel block rather than an open channel block because of the lack of use-dependency.

BIM (I) also affects acetylcholine-activated K^+ (K_{Ach}) channels by a PKC- independent mechanism (Table 1) (43). Although the inhibitory mechanism of BIM (I) has not been thoroughly investigated, BIM (I) inhibits the K_{Ach} channel at the muscarinic receptor, which is upstream of the G-protein.

Human ether-a-go-go-related gene (hERG) channels have been identified mainly in the heart and brain (44). The direct effect of BIM (I) on hERG channels was also reported. The IC_{50} values of the BIM (I) for hERG channel inhibition are 13.2 μM and 1.0 μM (Table 1) when the channels are expressed in *Xenopus oocytes* and HEK293 cells, respectively (45). Staurosporine and BIM (V), which have a similar structure to BIM (I), also inhibit the hERG channel, suggesting that the inhibitory mechanism of BIM (I) on hERG channel might be closely related to their common structure (bis-indole maleimide). The inhibitory action of BIM (I) on hERG channels occurs in the inactivated state. BIM (I) has no significant effect on the steady-state activation curve of hERG channel, but the half-maximal potential of steady-state inactivation was shifted by -20 mV. Moreover no use-dependency of hERG channel inhibition is demonstrated by BIM (I). The detailed binding mechanisms between BIM (I) and hERG channels are known; point mutation at Y652A and F656A of hERG S6 domain partially reduce the inhibitory effect of BIM (I) suggesting that BIM (I) interact with the hERG channel S6 region (46).

Effects on other ion channels

The open block mechanism by BIM (I) on Kv1.5 channels expressed in CHO cells was demonstrated by Choi et al. (Table 1) (47). In the present study, BIM (I) showed a voltage dependency of Kv1.5 inhibition. Under physiological conditions, BIM (I) is mainly positively charged (pKa=8.52), which facilitates channel inhibition by increasing the depolarization stimulus between a positively charged BIM (I) and membrane depolarizing potential. Therefore, blockade produced by BIM (I) increases steeply in the voltage range of channel activation. Additional experiments revealed that the inhibitory mechanism is an open state block, which is supported by the results from tail currents, use-dependency, and acceleration of current decay.

BIM (I) is also known to inhibit voltage-dependent Na^+ channels in rat cerebral cortex synaptosome (48), but the detailed mechanisms remain to be elucidated.

DIRECT EFFECTS OF OTHER PKC INHIBITORS ON ION CHANNELS

Effects of chelerythrine on ion channels

Chelerythrine, a benzophenanthridine alkaloid, is a specific PKC inhibitor with an IC_{50} value of 0.66 μM (14). However,

Table 1. Effects of PKC inhibitors on cardiovascular ion channels. Kv channel, voltage-dependent K^+ channel; hERG channel, human ether-a-go-go-related gene channel; K_{sus} channel, sustained K^+ channel; K_{Ach} channel, acetylcholine-activated K^+ channel; K_{to} channel, transient outward K^+ channel; BK_{Ca} channel, big conductance Ca^{2+}-activated K^+ channel

Drug	Target channel	IC_{50} (μM)	Tissues, Cells
Staurosporine	Kv channel	1.3	Rabbit, coronary artery
	Ca^{2+} channel	0.001-0.1	Porcine, coronary artery
	Ca^{2+} channel	0.062	Rabbit, atrial myocytes
	Na^+ channel	1.107	Rabbit, atrial myocytes
	muscarinic K^+ channel	10-100	Bullfrog, atrial myocytes
	Kv1.3 channel	1.2	CHO cell
BIM (I) (GF 109203X)	Kv channel	0.27	Rabbit, coronary artery
	Kv channel	0.23	Rat, mesenteric artery
	K_{sus} channel	0.051	Rat, ventricular myocytes
	K_{Ach} channel	0.099	Mice, atrial myocytes
	hERG channel	13.2	Xenopus laevis oocytes
	hERG channel	1.0	HEK 293 cell
	Kv1.5 channel	0.38	CHO cell
	Na^+ channel	10-100	Rat, cerebral cortex (synaptosome)
Chelerythrine	Kv channel	K_{to}=43 K_{sus}=20	Rat, ventricular myocytes
	K_{Ach} channel	0.49	Mice, atrial myocytes
	K_{Ach} channel	5-10	PC 12 cells
BIM IX (Ro31-8220)	Na^+ channel	1.1	Rat, cerebral cortex (synaptosome)
Rottlerin (Mallotoxin)	BK_{Ca} channel (activation)	0.5 used	HEK 293 cell
	BK_{Ca} channel (activation)	1.7 (EC_{50})	GH3, HCN-1A cell

several reports have demonstrated the direct effect of chelerythrine on ion channels in a PKC-independent manner as described below.

Chelerythrine was reported to prolong cardiac action potential duration without changing the resting membrane potential (42). The prolonged action potential duration by chelerythrine is mainly attributable to the inhibition of transient outward K^+ (K_{to}) channel and sustained K^+ (K_{sus}) channel in a PKC-independent manner with an IC_{50} value of 43 μM and 20 μM, respectively (Table 1). The inhibitory mechanism of the two ion channels is different. For example, comparing the big change in use-dependency in K_{to} channels, the effect of chelerythrine on K_{sus} channels shows relative small, but statistically significant, use-dependency. The fact that chelerythrine does not change the steady-state activation and inactivation in either channel suggests that chelerythrine inhibits K_{to} channels in an open state and inhibits K_{sus} channels in a closed state by a different mechanism.

Two different groups reported that chelerythrine inhibits muscarinic K^+ channels independent of PKC activity (43, 49). In mouse atrial myocytes, chelerythrine inhibits acetylcholine-activated K^+ channels in a concentration-dependent manner with an IC_{50} value of 0.49 μM (Table 1) (43). The authors suggested that chelerythrine also inhibits acetylcholine- activated K^+ channels in an inside-out patch configuration. From this result, they concluded that chelerythrine acts either on the coupling between G-protein and G-protein-activated channels or on the ion channel itself.

In PC12 cells of neuronal origin, chelerythrine also inhibits acetylcholine-induced currents (49). This inhibition is completely independent of PKC activation, as PKC activation reduces the acetylcholine-induced currents in neuronal cells. Chelerythrine-induced inhibition is reversible, voltage-independent, concentration-dependent, and pretreatment time-dependent. Although the detailed mechanism is unknown, this inhibition may be mediated by a new pharmacological mechanism.

Effects of Ro 31-8220 and rottlerin on ion channels

Ro 31-8220 [BIM (IX)] is a derivative of bis-indol maleimide, and a known specific inhibitor of PKC (IC_{50} = 0.5 μM) (50). The direct effect of Ro 31-8220 on Na^+ channel-dependent glutamate release was reported (48). In that paper, the authors proposed that inhibition is direct rather than through signaling transduction as PKC activation (not inhibition) reduces Na^+ channel activity. Furthermore, BIM (V), an inactive form of BIM (I) or BIM (IX), reduces Na+ channel activity. Considering that staurosporine and BIM (I) also inhibit Na^+ channels (33, 48), the inhibitory effect of Ro 31-8220 on Na^+ channels is attributable to the common structure of BIM.

Rottlerin, also known as mallotoxin, is a PKCδ-specific inhibitor with an IC_{50} value of 3-6 μM (51). The nonspecificity of rottlerin on big conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels was demonstrated in two studies (52, 53). When the *mSlo1* gene is expressed in HEK 293 cells, rottlerin induces significant activation of BK_{Ca} channels (52). BK_{Ca} channel activation is independent of intracellular [Ca^{2+}] and of the β subunit (phosphorylation site). The minimum effective concentration of rottlerin on BK_{Ca} channel is 0.1 μM, which is definitely lower than the IC_{50} for PKCδ inhibition. From that result, the authors suggested that BK_{Ca} channel activation by rottlerin is independent of PKC activity. Rottlerin shifts the half-maximal potential of activation curve by -118 mV and has no significant change in use-dependency, suggesting that the inhibitory action of rottlerin on BK_{Ca} channels occurs in the inactivated state. The binding mechanisms are unclear, but patch clamp data suggest that acting site of rottlerin could be external (52).

In pituitary tumor (GH3) cells and human cortical HCN-1A cells, rottlerin also have reported to activate BK_{Ca} channels independently of PKC activity (53) with an EC_{50} value of 1.7 μM (Table 1). Similar to the above paper, BK_{Ca} channel activation by rottlerin does not involve intracellular [Ca^{2+}] and the activation curve is shifted to left. The detailed binding mechanisms are proposed in this paper. The α subunit of BK_{Ca} channel is similar to Kv channels except for an additional membrane-spanning region, S0 (54, 55). As rottlerin has no significant effect on Kv channels (52), the S0 segment near the extracellular N terminus could be the key structure for the activation effect of rottlerin on BK_{Ca} channels. The author suggested that the positive charged residues in the extracellular N-terminus of BK_{Ca} channels (e.g. R30, H36, H39, H63, K66) could be a candidate for rottlerin interaction.

CONCLUDING REMARKS

The physiological/biochemical role of PKC has attracted many researchers due to the possibility of clinical usage. However, many recently developed PKC inhibitors fail in the clinic because they induce cellular toxicity or produce side effects by acting on other targets. In basic science, PKC inhibitors are indispensable to studies on cellular signaling pathways, but they have nonspecific actions toward untended targets. Although our review paper focuses on the direct effect of PKC inhibitors on cardiovascular ion channels, our information can help to correctly interpret the results of experiments when PKC inhibitors are used.

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