Review

Side-effects of protein kinase inhibitors on ion channels

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Protein kinases are one of the largest gene families and have regulatory roles in all aspects of eukaryotic cell function. Modulation of protein kinase activity is a desirable therapeutic approach for a number of human diseases associated with aberrant kinase activity, including cancers, arthritis and cardiovascular disorders. Several strategies have been used to develop specific and selective protein kinase modulators, primarily via inhibition of phosphorylation and down-regulation of kinase gene expression. These strategies are effective at regulating intracellular signalling pathways, but are unfortunately associated with several undesirable effects, particularly those that modulate ion channel function. In fact, the side-effects have precluded these inhibitors from being both useful experimental tools and therapeutically viable. This review focuses on the ion channel side-effects of several protein kinase inhibitors and specifically on those modulating K^+, Na^+ and Ca^{2+} ion channels. It is hoped that the information provided with a detailed summary in this review will assist the future development of novel specific and selective compounds targeting protein kinases both for experimental tools and for therapeutic approaches.

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1. Introduction

Numerous small molecules or proteins interact within complex signalling networks in response to external stimuli in all living cells. Individual components of these signalling pathways are potential therapeutic targets and modulators have been developed for both clinical and experimental applications (figure 1). One of the major issues, particularly for therapeutically relevant drugs, is the lack of selectivity of these modulators for a specific protein/component of a signalling pathway, which leads to a number of undesirable side-effects in the clinic. Such side-effects are particularly noted for drugs developed to regulate protein kinases, which also impact ion channel function. Understanding the nature of these 'side-effects' is essential for interpretation of experimental results and for the development of new, more specific, drugs for clinical use.

Protein kinases represent a family of proteins therapeutically relevant to many diseases. Perhaps the most pertinent example is cancer, for which a number of small molecule inhibitors are now FDA approved. To highlight a few: Imatinib (Gleevec), in 2001, and more recently, Bosutinib, in 2012, were approved for the treatment of chronic myelogenous leukemia (Druker *et al*. 2001; Goldman and Melo 2001; Khoury *et al*. 2012), Axitinib was approved for renal cell carcinoma in 2012 (Kessler *et al*. 2012), and Lapatinib was approved in 2007 for HER^+ breast cancer (Konecny *et al*. 2006). Cardiovascular disease is a potential therapeutic target for modulators of protein kinase C (PKC), which is known to regulate

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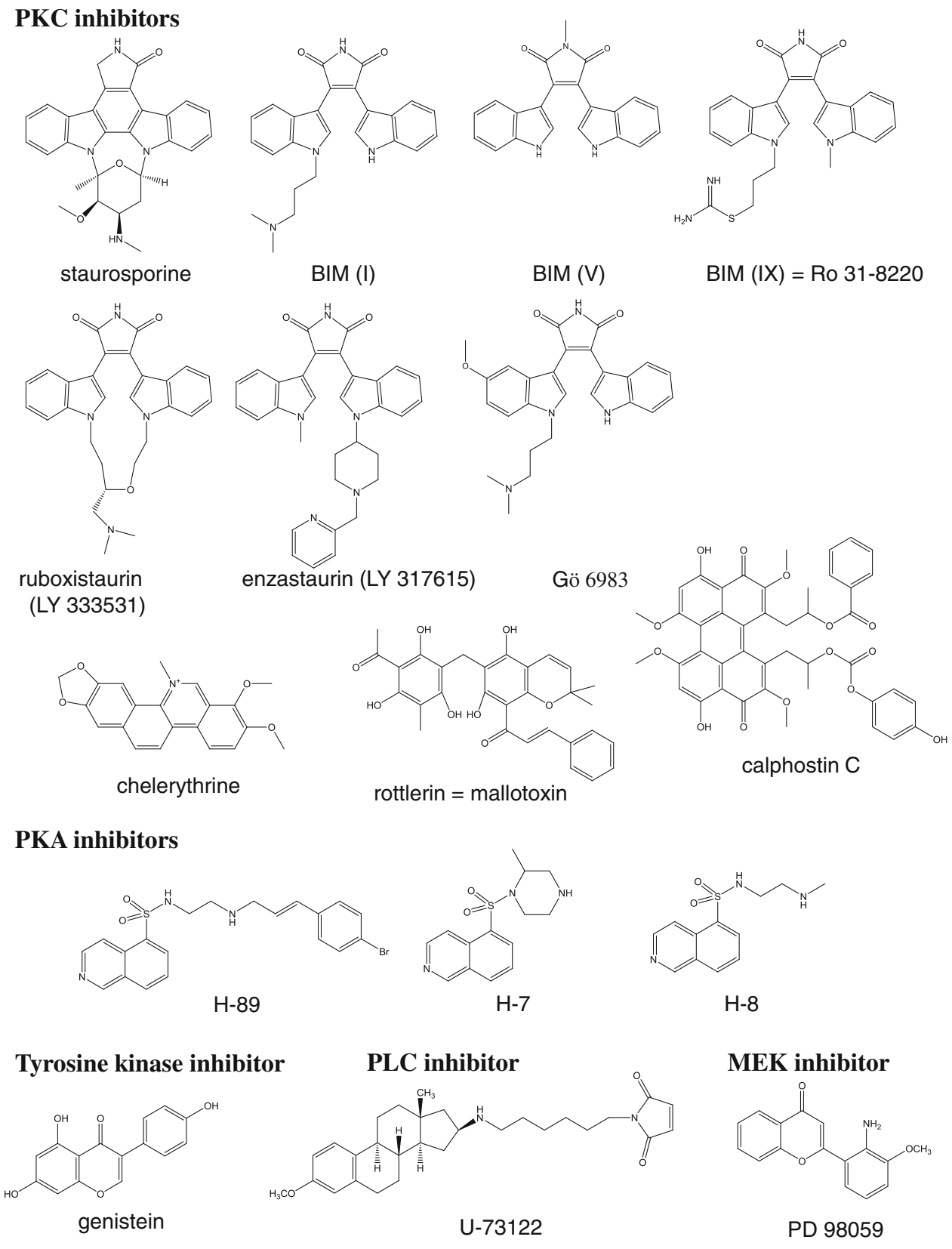

Figure 1. Chemical structures of protein kinase inhibitors.

functional responses in cardiomyocytes (Zhang *et al*. 2002; Budas *et al*. 2007). PKC has a number of isoforms, discussed later, with opposing functional roles. There are, however, known structural differences between the isoforms, enabling the development of specific modulators, although no PKC modulators are at present available in the clinic. In addition to their therapeutic potential, PKC inhibitors are also important pharmacological tools for investigation of intracellular signalling pathways.

This review provides a summary of the side-effects on ion channels of currently available drugs that target protein kinases and provides mechanistic insight into their actions (table 1). This evaluation of experimental processes and current data will contribute to the discovery of novel drugs.

2. Protein kinases: A brief overview

Protein kinases are a family of enzymes that phosphorylate proteins via transfer of a phosphate group from a nucleoside triphosphate, usually adenosine triphosphate (ATP). They are key regulators of all aspects of eukaryotic cell function and represent one of the largest functionally diverse gene families. Protein kinases were initially divided into two categories; conventional and atypical. Conventional protein kinases form the largest of the two groups and have since been divided into the following eight sub-groups based on catalytic domain sequence homology, the presence of accessory domains and known means of regulation: (1) The AGC (related to protein kinase A, G and C) group contains core intracellular signalling kinases, the cyclic-nucleotide-dependent family (PKA and PKG), the PKC family, the β-adrenergic receptor kinase (βARK), the ribosomal S6 family, and other close relatives; (2) the CaMK (calmodulin-regulated kinase) group whose activity is largely calcium/calmodulin dependent; (3) Casein Kinases (renamed cell kinase 1, CK1) phosphorylate key regulatory proteins including those involved in cell differentiation, proliferation, and circadian rhythm; (4) the CMGC family includes cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK), and CDK-like kinases; (5) the RCG (receptor guanylate cyclase) family, which includes the RCGs generating cGMP as a second messenger; (6) the STE (sterile kinase) family encompasses protein kinase homologs of yeast genes that regulate MAP kinase cascades; (7) the TK (tyrosine kinase) family, which specifically phosphorylate tyrosine residues; and (8) the tyrosine kinase-like kinases. Most of the protein kinase families phosphorylate serine/threonine residues near basic amino acids such as lysine and arginine, while members of the TK group specifically phosphorylate tyrosine residues.

The crystal structure of many protein kinases has been resolved beginning with the cAMP-dependent protein kinase PKA in 1991 (Knighton *et al.* 1991a; Knighton *et al.* 1991b). This discovery identified the catalytic core structure, a feature conserved in numerous protein kinases. The majority of protein kinases contain a binding site that recognizes the amino acid sequence of substrate proteins, the ATP binding site, and a catalytically active site (Cohen 2002; Engh and Bossemeyer 2002; Manning *et al.* 2002; Noble *et al.* 2004; Nolen *et al.* 2004). Strategies to develop inhibitors of protein kinases have utilized the information gleaned from studies of PKA, which has essentially served as a prototype for inhibitor design.

2.1 *PKC inhibitors*

PKC is important for signalling transduction in a variety of cells as its activation or inactivation regulates cellular processes such as secretion, gene expression, cell proliferation, and muscle contraction (Hug and Sarre 1993; Nishizuka 1995; Steinberg *et al*. 1995; Sugden and Bogoyevitch 1995; Jaken 1996; Newton 1997). It is not surprising that developing inhibitors of PKC has been a focus of numerous clinical and experimental studies. PKC was recently described as an elusive therapeutic target; despite a known increase in PKC isozyme activity in a number of diseases no PKC modulators have received regulatory approval (Mochly-Rosen *et al*. 2012). Unfortunately, many PKC inhibitors have unexpected effects on ion channel activity.

The first PKC inhibitor to be developed and widely used in the laboratory was staurosporine (Furusaki *et al.* 1978; Tamaoki *et al.* 1986). Staurosporine is a potent inducer of apoptosis in cells that causes a calcium-dependent translocation of PKC to membrane vesicles and prevents its protein phosphorylation activity (Wolf and Baggiolini 1988). Staurosporine acts by inhibiting the catalytic site of PKC with an IC_{50} of 0.0027 μM. Later studies demonstrated an inhibitory effect of staurosporine on other protein kinases including PKA, PKG, ribosomal S6 kinase, and epidermal growth factor receptor kinase (EGFRK) in an ATP-competitive manner, while having no effect on other kinases such as CK1, CK2, MAP kinase, and CSK (Meggio *et al.* 1995). The necessity for more specific inhibitors of PKC led to the discovery of UCN-01 and its stereoisomer UCN-02 (Takahashi *et al.* 1989). In specificity studies, UCN-01 more potently inhibited the Ca^{2+}-dependent isozymes (α, β, γ), compared with the Ca^{2+}-independent isozymes (δ, ε), whereas UCN-02 and staurosporine were less discriminatory. Interestingly, PKC-ζ was not inhibited by any of these agents (Seynaeve *et al.* 1994).

Bis-indole maleimide derivatives, BIM (I) and BIM (IX, also known as Ro 31-8220) were also developed to inhibit PKC. These compounds also act via competitive inhibition with ATP of the PKC catalytic site. BIM (I) and BIM (IX)

Table 1. Side-effects of protein kinase inhibitors on ion channels

Drug	Primary target : IC_{50} (μM)	Ion channels of side-effects	EC_{50} (μM) (cell type)	Reference
staurosporine	PKC 0.0027 (Tamaoki *et al.* 1986)	Kv Kv1.3 Nav *L*-type Ca musK	1.3 (rabbit coronary artery) 1.2 (expressed on CHO cell) 1.11 (rabbit atrium) 0.062 (rabbit atrium) used 0.001 ~ 0.1 (porcine coronary artery) 10 ~ 100 (frog atrium)	Park *et al.* 2005a Choi *et al.* 1999 Ko *et al.* 2006 Ko *et al.* 2005 Kageyama *et al.* 1991 Lo and Breitwieser 1994
BIM (I) = GF109203X	PKC 0.01 (Toullec *et al.* 1991)	Kv Kv1.5 musK Nav	0.27 (rabbit coronary artery) 0.23 (rat mesenteric artery) 0.38 (expressed on CHO cell) 98.69 (mouse atrium) 10 ~ 100 (rat synaptosome)	Park *et al.* 2005b Kim *et al.* 2004 Choi *et al.* 2000 Cho *et al.* 2001 Lingameneni *et al.* 2000
BIM (IX) = Ro 31-8220	PKC 0.7 (Murphy and Westwick 1992)	Nav	1.1 (rat synaptosome)	Lingameneni *et al.* 2000
Gö 6983	PKC 0.007 ~ 0.060 (Gschwendt *et al.* 1996)	*L*-type Ca	9 (mouse urinary bladder) 20 (expressed on HEK293 cell)	Welling *et al.* 2005 Welling *et al.* 2005
Calphostin C	PKC 0.05 (Kobayashi *et al.* 1989)	*L*-type Ca	0.001 ~ 1 (frog ventricle)	Hartzell *et al.* 1996
chelerythrine	PKC 0.66 (Herbert *et al.* 1990)	musK K_{sus} K_{to} *L*-type Ca	0.5~5 (PC 12 cell) 0.49 (mouse atrium) 20 (rat ventricle) 43 (rat ventricle) > 50 (rat ventricle)	Shi and Wang 1999 Cho *et al.* 2001 Voutilainen-Myllylä *et al.* 2003 Voutilainen-Myllylä *et al.* 2003 Hartzell *et al.* 1996
Rottlerin = mallotoxin	PKCδ 3 ~ 6 (Gschwendt *et al.* 1994)	BK_{Ca} (act)[1] Ca influx (act)[1]	< 0.5 (expressed on HEK293 cell) 1.7 (GH3) used 1 (HCN-1A) used 10 (mouse lens)	Zakharov *et al.* 2005 Wu *et al.* 2007 Wu *et al.* 2007 Xu 2007
H-89	PKA 0.135 (Lochner and Moolman 2006)	Kv1.3 Kv K_{ATP} Kir BK_{Ca} (act)[1] Na^+ transport	1.70 (expressed on CHO cell) 1.02 (rabbit coronary artery) 1.19 (rabbit coronary artery) 3.78 (rabbit coronary artery) 0.52 (rabbit coronary artery) used 5 (rat lung epithelium)	Choi *et al.* 2001 Son *et al.* 2006 Park *et al.* 2006 Park *et al.* 2006 Park *et al.* 2007 Niisato *et al.* 1999
genistein	tyrosine kinase 2.59 (Akiyama *et al.* 1987)	Kv1.4 Kv4.3 Kv K_{dr} Kir2.3 Kir *L*-type Ca *T*-type Ca Nav	used 50 (44.9%, expressed on CHO cell) 124.78 (expressed on CHO cell) 7.51 (rabbit coronary artery) ~ 30 (guinea pig ventricle) 16.9 (expressed on Xenopus oocyte) 19.3 (expressed on HEK293 cell) 54/27 (5/10 min application, rat osteoclast) 17.5 (guinea pig ventricle) ~ 22.7 (mouse spermatogenic cell) 9.1 (rat SCG neuron) 60 (rat brain neuron, culture)	Zhang and Wang *et al.* 2000 Kim *et al.* 2011 Ko *et al.* 2009 Washizuka *et al.* 1998 Zhao *et al.* 2008 Zhao *et al.* 2008 Okamoto *et al.* 2001 Chiang *et al.* 1996 Tao *et al.* 2009 Jia *et al.* 2008 Paillart *et al.* 1997
U-73122	PLC 2 ~ 4 (Feiβt *et al.* 2005)	Kir3.1/3.2 BK_{Ca}	0.54 (expressed on HEK293 cell) 2.3 (expressed on HEK293 cell)	Klose *et al.* 2008 Klose *et al.* 2008
PD 98059	MEK 2 ~ 10 (Alessi *et al.* 1995; Dudley *et al.* 1995)	Cav	used 10 and 50 (Urechis oocyte) 10 ~ 40 (rat cerebral artery)	Gould and Stephano 2000 Lagaud *et al.* 1999

[1] Drugs induced the channel activation.

have IC_{50} values of 0.01 and 0.7 μM for PKC inhibition, respectively (Toullec *et al.* 1991; Murphy and Westwick 1992) and are more selective for PKC over PKA and PKG than is staurosporine. PKC selectivity was further improved with the discovery of ruboxistaurin (LY333531) and enzastaurin (LY317615) (Jirousek *et al.* 1996; Graff *et al.* 2005). The effects of these compounds on ion channels in a protein kinase-independent manner have not yet been demonstrated.

Aside from their poor selectivity, staurosporine and BIM (I) have direct inhibitory effects on voltage-dependent K (Kv) channels. Our laboratory demonstrated inhibition of native Kv channel currents (I_{Kv}) in coronary arterial smooth muscle cells (SMCs). I_{Kv} inhibition occurred via open channel blockade with staurosporine and BIM (I) with IC_{50} values of 1.3 and 0.27 μM, respectively (Park *et al.* 2005a, 2005b), which corresponds to similar findings by other groups: BIM (I) inhibits I_{Kv} in mesenteric arterial SMCs with an IC_{50} of 0.23 μM (Kim *et al.* 2004). Staurosporine was reported to inhibit Kv1.3 (IC_{50} = 1.2 μM), and BIM (I) inhibited Kv1.5 (IC_{50} = 0.38 μM), by acting in the channel open-state, when over-expressed in Chinese hamster ovary (CHO) cells (Choi *et al.* 1999; Choi *et al.* 2000). Indolcarbazole is the key structure of staurosporine and BIM (I) responsible for inhibiting PKC at the catalytic functional site, which leads one to question why would these compounds functionally inhibit I_{Kv}. A key feature of Kv channel inhibitors that block in the open channel state is the presence of protonated ammonium ions (French and Shoukimas 1981; Hoshi *et al.* 1990; Heginbotham and MacKinnon 1992; Snyders and Yeola 1995; del Camino *et al.* 2000). These act by binding to the pore region of the channel, which stabilizes the channel in its inactive conformation. Groundbreaking work by Mackinnon and colleagues elucidated the crystal structure of the Kv channel in 1998 (Doyle *et al.* 1998) and subsequent studies have focused on the structural changes involved in the ion pore selectivity filter. Residues I470 and V474 were identified as the binding site for quaternary ammonium ions (Zhou *et al.* 2001). Since both staurosporine and BIM (I) have protonated tertiary ammonium ions at physiological pH, those structures have been proposed to be responsible for the direct/open state inhibition of Kv channels (Park *et al.* 2005a).

Inhibitory effects of both staurosporine and BIM (I) on muscarinic K^+ channels have also been reported (Lo and Breitwieser 1994; Cho *et al.* 2001). Staurosporine decreased the channel open probability without affecting current amplitude, which implies that staurosporine affected the gating properties without changing the permeation pathway (Lo and Breitwieser 1994). For BIM (I), although a detailed mechanism of inhibition was not assessed, it was suggested that an upstream G-protein imposed inhibitory effects on the muscarinic K^+ channel with an IC_{50} value of 98.69 μM (Cho *et al.* 2001).

Na^+ channels are also affected by staurosporine, BIM (I), and Ro 31-8220 (Lingameneni *et al.* 2000; Ko *et al.* 2006). The Na^+ channel in isolated atrial myocytes was inhibited by staurosporine in a dose-dependent manner, with an IC_{50} value of 1.11 μM. Staurosporine also shifted the steady-state activation and inactivation to more negative potentials and slowed the decay rate of Na^+ channel inactivation (i.e. extended the inactivation time). The recovery kinetics and the use-dependency of the Na^+ channel, however, were not altered by staurosporine (Ko *et al.* 2006). An identical phenomenon resulted from the application of lipid-soluble alkaloids and insecticides, such as batrachotoxin and pyrethroids (Narahashi 1996; Catterall 2000; Goldin 2001). BIM (I) inhibits voltage-dependent Na^+ channels in the synaptosome of the cerebral cortex (Lingameneni *et al.* 2000), but the detailed mechanisms remain to be determined. Inhibitory effects of Ro 31-8220 on Na^+ channel-dependent glutamate release have been reported (Lingameneni *et al.* 2000). Evidence suggests a direct inhibition rather than via PKC, primarily because it is the activation, and not the inhibition, of PKC that is known to enhance Na^+ channel inactivation (Godoy and Cukierman 1994). Moreover, BIM (V), an inactive form of BIM (I) or Ro 31-8220, also reduced Na^+ channel activity (Lingameneni *et al.* 2000).

Staurosporine and Gö 6983, another bis-indole maleimide derivative (Gschwendt *et al.* 1996), interact with Ca^{2+} channels. In arterial SMCs, staurosporine was proposed to directly inhibit of the Ca^{2+} channels, although the mechanism remains to be elucidated (Kageyama *et al.* 1991). Staurosporine also inhibited the *L*-type Ca^{2+} channel in atrial myocytes with an IC_{50} of 0.062 μM, independent of its actions on PKC activity. The voltage-dependent inactivation curve for Ca^{2+} channel currents was not altered, however, the steady-state activation curve was shifted toward more positive potentials (Ko *et al.* 2005). The inhibitory effect of Gö 6983 was tested in cells over-expressing the *L*-type Ca^{2+} channel, resulting in Gö 6983-mediated inhibition of Ca^{2+} current with an IC_{50} of 20 μM in human embryonic kidney (HEK) 293 cells, and 9 μM in urinary bladder smooth muscle cells. While no voltage- or frequency-dependent effects were observed, an accelerated inactivation of the current suggested at least a partial interaction in the open channel state (Welling *et al.* 2005). Like most of the inhibitors discussed in this review, interpretation of the findings of studies of these inhibitors is questionable because they are used at much higher concentrations to exert phosphoinositide 3-kinase (PI3-K) and PKC inhibitory effects on *L*-type Ca^{2+} channels.

Calphostin C is widely used for PKC inhibition, and requires light for its activity (Kobayashi *et al.* 1989). Similar to other PKC inhibitors, calphostin C directly and potently inhibits Ca^{2+} channels, again independent of phosphorylation

and cAMP levels. In cardiomyocytes calphostin C inhibits basal Ca^{2+} currents (I_{Ca}), 8-bromo-cAMP-induced I_{Ca} not degraded by phosphodiesterases, phosphorylation-independent I_{Ca} activation (BAY K 8644), and I_{Ca} in the presence of phosphatase inhibitors. There is 70% homology between the α-subunit of the *L*-type Ca^{2+} channel and the phorbol ester binding site where calphostin C interacts with PKC, suggesting an interaction between calphostin C and *L*-type Ca^{2+} channels (Hartzell and Rinderknecht 1996).

Chelerythrine is a benzophenanthridine alkaloid compound proposed to have PKC-specific inhibitory activity with an IC_{50} of 0.66 μM (Herbert *et al.* 1990). However, direct effects of chelerythrine on ion channels have been reported. Two independent groups reported an inhibitory effect of chelerythrine on K^+ channels (Shi and Wang 1999; Cho *et al.* 2001). In mouse atrial myocytes, acetylcholine-activated K^+ channels were inhibited by chelerythrine in a dose-dependent manner with an IC_{50} of 0.49 μM. The proposed mechanism indicated that chelerythrine acts indirectly on G-proteins and G-protein-activated channel coupling or directly on the ion channel, based on the data showing that chelerythrine inhibited the ion channel in the inside-out patch configuration (Cho *et al.* 2001). Furthermore, neither the PKC inhibitor calphostin C nor the PKC activator phorbol 12,13-dibutyrate had any effect on acetylcholine-activated K^+ channels, supporting PKC-independent regulation of ion channel activity. Chelerythrine-dependent inhibition of acetylcholine-activated K^+ channels in PC12 cells of neuronal origin has also been reported. The inhibition was reversible, concentration-dependent, pretreatment time-dependent, and voltage-dependent (Shi and Wang 1999). Again, although the mechanism remains to be elucidated, the inhibition of acetylcholine-activated K^+ channels by chelerythrine was independent of PKC activity, as intracellular dialysis with PKCI 19-31, a specific pseudo-substrate PKC inhibitor, had no effect on the inhibition by chelerythrine (Shi and Wang 1999).

In cardiomyocytes, chelerythrine prolonged the cardiac action potential duration without altering the resting membrane potential (Voutilainen-Myllylä *et al.* 2003) by inhibiting the transient outward K^+ (K_{to}) channel (IC_{50} = 43 μM) and the sustained K^+ (K_{sus}) channel (IC_{50} = 20 μM) independent of PKC activity. Although the steady-state activation and inactivation curves of both channels remained unaffected by chelerythrine, the K_{to} channel had a larger use-dependency after the application of chelerythrine than did the K_{sus} channel. The authors suggested that the K_{to} channel was inhibited by chelerythrine in an open state and the K_{sus} channel was inhibited in a closed state, which involved an additional mechanism (Voutilainen-Myllylä *et al.* 2003).

Rottlerin, also known as mallotoxin, was developed as a PKCδ-specific inhibitor with an IC_{50} value of 3–6 μM (Gschwendt *et al.* 1994). Two independent reports suggested that rottlerin increases big-conductance Ca^{2+}-activated K^+ (BK_{Ca}) currents (Zakharov *et al.* 2005; Wu *et al.* 2007). Rottlerin induced significant activation of the BK_{Ca} channel encoded by the *mSlo1* gene in the HEK 293 cells. This activation is independent of intracellular Ca^{2+} concentration and associated with the β-subunit, which has phosphorylation sites. The minimum effective concentration of rottlerin was 0.1 μM, and the EC_{50} < 0.5 μM, which is much lower than the half-maximal concentration required for PKCδ inhibition (Zakharov *et al.* 2005). Furthermore, rottlerin shifted the half-maximal activation potential by -118 mV with no significant change in use-dependency. Therefore, the authors suggested that activation of the BK_{Ca} channel by rottlerin is independent of PKC activity and may modulate the voltage sensitivity of the channel (Zakharov *et al.* 2005). However, the mechanisms by which rottlerin binds to the channel remain to be clarified, since the patch-clamp data infer that rottlerin binds within the external domain of the channel (Zakharov *et al.* 2005). A second group showed that rottlerin activated the BK_{Ca} channel in both pituitary tumour (GH_3) and human cortical HCN-1A cells with an EC_{50} of 1.7 μM (Wu *et al.* 2007). Similar to the findings of Zakharov *et al.*, rottlerin shifted the activation curve to more negative potentials and intracellular [Ca^{2+}] was not involved in the activation of BK_{Ca}. Wu and colleagues suggested that positively charged residues in the extracellular N-terminus of the BK_{Ca} α-subunit (e.g. R30, H36, H39, H63, K66) are candidate sites for interaction with rottlerin (Wu *et al.* 2007).

Interestingly, rottlerin can enhance Ca^{2+} influx in mouse lens epithelial cells (Xu 2007). This influx was not caused by phosphorylation because the ATP concentration remained unchanged during the experiment. Moreover, the effect of rottlerin was reversed by both the depletion of extracellular Ca^{2+} using the calcium chelator EGTA or by the addition of verapamil, a Ca^{2+} channel inhibitor, suggesting that the increase in Ca^{2+} influx by rottlerin occurred through activation of *L*-type Ca^{2+} channels in a PKCδ-independent manner (Xu 2007).

2.2 *PKA inhibitors*

Protein kinase A (PKA), like PKC, is a key protein kinase, although PKA is perhaps the most common and versatile kinase involved in eukaryotic intracellular signalling pathways (Taskén and Aandahl 2004). Researchers have spent decades developing molecular tools to regulate PKA activity, and a number of activators and inhibitors have been used successfully in many laboratories. Among the PKA inhibitors, H-89 is the most common and widely utilized (Chijiwa *et al.* 1990). H-89 is an isoquinoline derivative, acting as a competitive antagonist in the ATP binding site of

the catalytic subunit of PKA (Engh *et al.* 1996). H-89 is an effective inhibitor of PKA, with an IC_{50} of 135 nM and a maximal effective concentration of 1 μM (Lochner and Moolman 2006). However, several reports indicate that H-89 has additional effects on a variety of ion channels, some of which are discussed below.

H-89 inhibited Kv1.3 channels over-expressed in CHO cells in a concentration-dependent manner with an IC_{50} value of 1.7 μM. H-89 also accelerated the decay rate of Kv1.3 channel inactivation, in the excised inside-out configuration (Choi *et al.* 2001). Intracellular application of either PKA activators or PKA inhibitors had no effect on Kv1.3 currents and did not influence current inhibition by H-89, suggesting that H-89 inhibited Kv1.3 at the extracellular site in a PKA-independent manner (Choi *et al.* 2001). In rabbit coronary arterial SMCs, H-89 inhibited I_{Kv} with an IC_{50} of 1.02 μM (Son *et al.* 2006). The inhibitory effect of H-89 did not affect inactivation of the current, nor the voltage-dependent activation and inactivation curves. The recovery kinetics also remained unchanged. Other PKA inhibitors were unable to alter the inhibitory effect of H-89 on the Kv channel, and H-85, an inactive analog of H-89, showed similar effects on Kv current. Therefore, it was concluded that H-89 inhibits arterial Kv channels via a dose-dependent and PKA-independent pathway (Son *et al.* 2006).

H-89 also inhibits ATP-dependent potassium (K_{ATP}) channels and inwardly rectifying potassium (Kir) channels in rabbit coronary arterial SMCs. The effect of H-89 on both of these channels is dose-dependent, with IC_{50} value of 1.19 μM for K_{ATP} and 3.78 μM for Kir channels (Park *et al.* 2006). H-85 demonstrated similar effects on both of these channels. As with the Kv1.3 channel, other PKA inhibitors did not alter the effects of H-89, supporting a PKA-independent mechanism of action. Although the three-dimensional structures of these channels have not yet been elucidated, it was suggested that the amino acids Leu, Lys, Val and Ala could mediate the interaction with H-89 in the pore cavity of the channel, similar to the known inhibition site of H-89 in PKA (Park *et al.* 2006). In the same cell type, H-89 also enhanced BK_{Ca} channel activity in a dose-dependent manner (EC_{50} = 0.52 μM), with an increase in the open probability but no effect on the single-channel amplitude. Other PKA inhibitors and protein phosphatase inhibitors had no significant effect on the H-89-induced increase of the BK_{Ca} current. Like its actions on other potassium channels, the effect of H-89 on the BK_{Ca} channel is unlikely to be mediated by a PKA-related signalling mechanism. Indeed, H-85 also exhibits a similar effect on BK_{Ca} channels (Park *et al.* 2007).

In alveolar type II epithelial cells from the rat fetal lung, H-89 increases amiloride-sensitive Na^+ transport. In general, Na^+ transport in epithelial cells is stimulated by PKA activation via a cAMP-dependent mechanism. Interestingly, H-compound inhibitors of PKA, such as H-8, H-89, and H-7, actually stimulated Na^+ transport in a concentration-dependent manner rather than the expected inhibitory effect. Niisato and colleagues suggested that the 5-isoquinolinesulfone, present in H-compounds but not in other PKA inhibitors, is important in the stimulation of Na^+ transport via voltage dependent Na^+ channels (Niisato *et al.* 1999).

2.3 *Tyrosine kinase inhibitor*

Tyrosine kinases are particularly important for cell proliferation and transformation. Their activity is associated with receptors for several growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin and insulin-like growth factor I (IGF-I) (Ushiro and Cohen 1980; Ek *et al.* 1982; Kasuga *et al.* 1982; Nishimura *et al.* 1982; Petruzzelli *et al.* 1982; Jacobs *et al.* 1983; Rubin *et al.* 1983). Developing an inhibitor for tyrosine kinases is essential as not only an experimental tool but also as a potential anti-cancer agent. Genistein, an isoflavone compound, has high specificity for tyrosine kinase inhibition, and daidzein is used as its inactive analogue (Akiyama *et al.* 1987). However, these agents exert side-effects on a variety of ion channels.

Genistein reversibly inhibits Kv1.4 channels over-expressed in CHO cells by reducing the amplitude of the Kv1.4 channel current and slowing the activation kinetics, without altering the deactivation process (Zhang and Wang 2000). The voltage-dependence of the steady-state inactivation of the Kv1.4 channel was not changed; however, genistein shifted the activation towards more positive potentials. Daidzein, as well as several other tyrosine kinase inhibitors, did not affect Kv1.4 channel current (Zhang and Wang 2000). Thus, genistein is likely to have tyrosine kinase-independent effects on the Kv1.4 channel.

Kv4.3 channels are transient K^+ channels responsible for neuronal A-type currents and cardiac transient outward currents. Kv4.3 channels over-expressed in CHO cells are also inhibited by genistein (IC_{50} = 124.78 μM). Despite having no effects on the kinetics of activation or inactivation or the voltage-dependency of inhibition, genistein shifted the steady-state inactivation curve to more hyperpolarized potentials in a concentration- and use-dependent manner with a K_i of 1.17 μM, suggesting that genistein interacts with the Kv4.3 channel in the closed-inactivated state (Kim *et al.* 2011). This effect was replicated in freshly isolated SMCs from rabbit coronary arteries – genistein inhibited Kv channels with an IC_{50} of 7.51 μM. Although the authors did not address the mechanism underlying the inhibition of Kv

channels, the similarities to inhibition under ATP-free conditions and with daidzein led to the conclusion that Kv channel inhibition by genistein was partly tyrosine kinase-independent (Ko *et al.* 2009).

The delayed-rectifier K^+ (K_{dr}) currents are also inhibited by genistein in guinea pig ventricular myocytes (Washizuka *et al.* 1998). Genistein inhibited both the basal and the cAMP-enhanced K_{dr} current (IC_{50} = ~30 μM). Other tyrosine kinase inhibitors, such as lavendustin and tyrphostin-51, had no significant effect on the K_{dr} current; daidzein, however, inhibited K_{dr} currents to a lesser extent than genistein itself (Washizuka *et al.* 1998). Detailed analysis revealed that genistein inhibited the slow component of the currents, and the authors concluded that genistein also inhibited K_{dr} channels independent of tyrosine kinase activity (Washizuka *et al.* 1998).

Inwardly rectifying potassium channels (Kir) are particularly important in the regulation of membrane potential in cardiomyocytes and neuronal cells. When expressed in Xenopus oocytes genistein inhibits the Kir2.3 currents with an IC_{50} value of 16.9 μM (Zhao *et al.* 2008). When expressed in HEK 293 cells a similar IC_{50} for genistein was obtained (19.3 μM). Other Kir channels, such as Kir2.1 or Kir3.4, were relatively unaffected by genistein, indicating that this inhibitor may be used to selectively investigate Kir2.3 channels (Zhao *et al.* 2008). Inhibitory effects of genistein on Kir channels have been reported in rat osteoclasts (Okamoto *et al.* 2001). The IC_{50} values were dependent upon the application time, for example, 54 μM after 5 min, and 27 μM after 10 min. Unlike the studies discussed above, inhibition was partially restored after washout of genistein. Daidzein also showed weak inhibition of the Kir channel (Okamoto *et al.* 2001). Because other tyrosine kinase inhibitors (tyrphostin 23), intracellular kinase inhibitors, and the potent protein tyrosine phosphatase inhibitor vanadate did not affect Kir channel activity, it appears that the inhibitory effect of genistein was independent of tyrosine kinase, PKA and PKC (Okamoto *et al.* 2001; Zhao *et al.* 2008).

Calcium channels are also affected by genistein. Genistein reversibly and dose-dependently inhibited *L*-type Ca^{2+} channels with an IC_{50} value of 17.5 μM in guinea pig ventricular myocytes (Chiang *et al.* 1996). Daidzein showed a similar inhibitory effect on *L*-type Ca^{2+} channel and BAY-K 6944 augmented currents were also inhibited, indicating a tyrosine kinase-independent regulation (Chiang *et al.* 1996). Similar observations were made in other tissues, including pancreatic islet cells and vascular SMCs (Wijetunge *et al.* 1992; Jonas *et al.* 1995). Genistein can also inhibit *T*-type Ca^{2+} channels in mouse spermatogenic cells (Tao *et al.* 2009). Application of genistein and daidzein reversibly decreased the *T*-type Ca^{2+} current in a concentration-dependent manner (IC_{50} = ~22.7 μM), while application of other tyrosine kinase inhibitors, tyrosine phosphatase inhibitors, and ATP analogues had no significant effect on the *T*-type Ca^{2+} channel. This suggested that the inhibitory effect of genistein on the *T*-type Ca^{2+} channel is independent of tyrosine kinase activity (Tao *et al.* 2009). Furthermore, when *T*-type Ca^{2+} channels were expressed in HEK 293 cells, similar observations were made. Genistein inhibited Cav3.1 and Cav3.2 channels by shifting the voltage-dependent inactivation curve toward more hyperpolarized potentials; however, the Cav3.3 channel was not affected (Tao *et al.* 2009).

Na^+ channels are also modulated by genistein via both tyrosine kinase-dependent and kinase-independent pathways. In rat superior cervical ganglia (SCG) neurons, genistein inhibited the Na^+ channel in a concentration dependent manner with an IC_{50} value of 9.1 μM, by shifting the voltage-dependency of activation to more positive potentials (Jia *et al.* 2008). Other tyrosine kinase inhibitors, tyrphostin 23 and PP2 showed small, but significant, inhibitory effects on Na^+ channels (Jia *et al.* 2008). Both genistein and daidzein also inhibited $^{22}Na^+$ influx through a voltage-sensitive Na^+ channel in rat brain neurons with IC_{50} values of 60 and 195 μM, respectively (Paillart *et al.* 1997). Other tyrosine kinase inhibitors had no effect on Na^+ channels at concentrations known to inhibit kinase activity. Genistein shifted the voltage-dependent activation curve toward more positive potentials and the voltage-dependent inactivation curve toward more negative potentials. By application of isotopes of Na^+ channel inhibitors, [^{3}H] saxitoxin (STX) and [^{3}H] batrachotosinin A 20-α-benzoate (BTX), the binding site for genistein was identified. The results indicated that [^{3}H] BTX did compete with genistein, while [^{3}H] STX did not, suggesting a similarity in the binding of both genistein and [^{3}H] BTX in the Na^+ channel that indicated a direct and tyrosine-kinase-independent mechanism (Paillart *et al.* 1997).

2.4 *PLC inhibitor*

Phospholipase C (PLC) is involved in many cellular functions particularly those related to intracellular Ca^{2+} mobilization (Bleasdale *et al.* 1989; Bleasdale *et al.* 1990; Smith *et al.* 1990). When PLC is activated in response to various extracellular stimuli, it hydrolyses phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which subsequently releases Ca^{2+} from intracellular stores (Rhee 2001). U-73122 is an aminosteroid compound known to directly block PLC activity *in vivo* and *in vitro* (Bleasdale *et al.* 1990; Smith *et al.* 1990; Hou *et al.* 2004; Feiβt *et al.* 2005). Like the other compounds discussed in this review, it is reported that U73122 alters ion channel function independently of its PLC activity.

When the heteromultimeric Kir3.1/3.2 channel and homomeric Kir1.1 and Kir2.1 channels are over-expressed in HEK 293 cells, U73122 and U73343, the inactive form of U73122, both have inhibitory effects on Kir3.1/3.2, but not on Kir1.1 and Kir2.1 (Klose and Huth 2008). BK_{Ca} channels over-expressed in HEK 293 cells were also inhibited by U73122 and U73343 independently to PLC (Klose and Huth 2008). The alignment of the amino acid sequences of Kir3 and BK_{Ca} channels, and mutation studies revealed that both compounds interacted with the homologous domain in the long C-terminus of the protein (Klose and Huth 2008).

2.5 *MEK inhibitor*

External stimuli, such as growth factors, hormones, osmotic shock and stress, induce physiological changes in live cells through several signalling transduction pathways, including those involving mitogen-activated protein kinases (MAPKs) (Ray and Sturgill 1987; Ahn *et al.* 1990a; Ahn and Krebs 1990b; Pang *et al.* 1993; Freshney *et al.* 1994; Ohmichi *et al.* 1994; Pombo *et al.* 1994; Rouse *et al.* 1994). The regulation of MAPK-activated proteins by MAPK controls the expression of various proteins which regulate cell proliferation and differentiation (Cowley *et al.* 1994). MAPK kinase (MEK) is upstream of MAPK, and PD98059 is a compound that inhibits MEK activity with reported effects on ion channels (Alessandrini *et al.* 1992; Zheng and Guan 1993; Alessi *et al.* 1995; Dudley *et al.* 1995; Lazar *et al.* 1995; Pang *et al.* 1995; Favata *et al.* 1998). In urchin oocytes (*Urechiscaupo*), PD98059 showed inhibitory effects on voltage-gated Ca^{2+} channels independently of MAPK activity at lower concentrations than required for MEK inhibition (Gould and Stephano 2000). Furthermore, PD98059 non-specifically inhibited voltage-sensitive Ca^{2+} entry in a study of myogenic tone measurements in rat cerebral arteries (Lagaud *et al.* 1999). This study was unable to distinguish between the inhibition of MEK/MAPK activity and a potential non-specific inhibition of voltage gated Ca^{2+} entry. While Gould and Stephano did not observe a significant effect of the MEK inhibitor U0126 on the urchin oocyte I_{Ca}, Yuan and colleagues did document significant effects on I_{Kv} when studying a variety of isoforms in CHO cells (Yuan *et al.* 2006). They observed a significant acceleration of the inactivation kinetics of fast inactivating A-type current carried by Kv4.2 channels and also noted a change in the kinetics of the K_{dr} channel, Kv1.1 to a fast inactivating A-type current. To determine whether the effects were MEK dependent, the authors used two approaches; (1) U0125, a less potent MEK inhibitor, and (2) a mutant Kv4.2 channel with mutated ERK phosphorylation sites. Both approaches suggested significant effects of U0126 on I_{Kv} indicating a direct effect of U0126 on the K^+ channels independent of MEK inhibition (Yuan *et al.* 2006).

3. Concluding remarks

In this review, we have summarized the protein-kinase-independent side-effects on ion channel function of a variety of protein kinase inhibitors. While there have been several efforts to develop more specific and selective drugs regulating protein kinases in cells, most compounds exert non-specific effects on ion channels. Some of these 'side-effects' have serious implications both from an experimental standpoint and should be seriously considered for therapeutic application. Moreover, it is possible that some of these side-effects may have beneficial therapeutic effects and not compromise the efficacy of the drug. Caution must be paid when such protein kinase modulators are assessed in experimental and clinical studies, and experimental data must be interpreted carefully with due consideration of the potential side-effects on ion channels.

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