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Review Article

Interaction between Calcineurin and Ca^{2+}/Calmodulin Kinase-II in Modulating Cellular Functions

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Roles of calcineurin (CaN), a Ca^{2+}/calmodulin- (CaM-) dependent protein phosphatase, and Ca^{2+}/CaM-dependent protein kinase-II (CaMKII) in modulating K^+ channel activity and the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) have been investigated in renal tubule epithelial cells. The channel current through the cell membrane was recorded with the patch-clamp technique, and $[Ca^{2+}]_i$ was monitored using fura-2 imaging. We found that a CaN-inhibitor, cyclosporin A (CyA), lowered the K^+ channel activity and elevated $[Ca^{2+}]_i$, suggesting that CyA closes K^+ channels and opens Ca^{2+}-release channels of the cytosolic Ca^{2+}-store. Moreover, both of these responses were blocked by KN-62, an inhibitor of CaMKII. It is suggested that the CyA-mediated response results from the activation of CaMKII. Indeed, Western blot analysis revealed that CyA increased phospho-CaMKII, an active form of CaMKII. These findings suggest that CaN-dependent dephosphorylation inhibits CaMKII-mediated phosphorylation, and the inhibition of CaN increases phospho-CaMKII, which results in the stimulation of CaMKII-dependent cellular actions.

1. Introduction

Protein kinases and phosphatases regulate major parts of functional proteins through the phosphorylation and dephosphorylation of individual proteins. The characterization and roles of many kinds of protein kinases and phosphatases have been widely investigated in a variety of cells.

Ion channels are well-known functional proteins and their activity can be estimated by monitoring ion currents using the patch-clamp technique. Since the activity of several kinds of channel protein is regulated by protein kinase-mediated phosphorylation and phosphatase-mediated dephosphorylation [1, 2], the investigation of regulatory mechanisms for ion channels often provides important findings regarding the functional significance of protein kinases and phosphatases. To date, it has been demonstrated that some serine/threonine protein kinases, such as protein kinase A (PKA), protein kinase G (PKG), and protein kinase C (PKC), affect ion channel activity. In renal tubule cells, PKA [3–5] and PKG stimulates, [6, 7] and PKC inhibits [3, 5] the activity of inwardly rectifying K^+ channels. In such cases, it was suggested that the PKA- or PKG-mediated phosphorylation site was different from the PKC-mediated phosphorylation site, since the effects of PKA and PKG on the channel functions were different from those of PKC. Although the PKG-mediated site is still unknown, molecular analyses have revealed that PKA-mediated and PKC-mediated sites exist in channel proteins [8, 9]. As for the effect of protein phosphatase on the activity of K^+ channels, protein phosphatase-1 (PP-1) [10] and protein phosphatase-2A (PP-2A) [10, 11] were reported to inhibit channel activity in renal tubule cells, suggesting that PKA-mediated phosphorylation was dephosphorylated by PP-1 and/or PP-2A.

It was also reported that the inhibitory effect on channel activity of PKC was mimicked by Ca^{2+}/CaM kinase II (CaMKII) in renal tubule cells [12]. However, the phosphatase which induces dephosphorylation of PKC or the CaMKII site remains unknown. Recently, we discovered that calcineurin (CaN), a Ca^{2+}/CaM-dependent protein phosphatase, had an opposite effect to CaMKII on channel activity in human renal tubule cells [12].

Based on the recent reports including our data [13], we have reviewed the functional significance and mutual effects of CaN and CaMKII on channel activity, as well as on

modulation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We will further discuss the regulatory mechanisms controlling channel activity and $[Ca^{2+}]_i$ by phosphorylation and dephosphorylation in cultured renal tubule cells, which we have used in recent studies [7, 10, 13].

2. Mutual Effects of Calcineurin (CaN) and CaMKII on K^+ Channel Activity in Human Tubule Cells

CaN is a Ca^{2+}-dependent protein phosphatase [14], often called protein phosphatase-2B (PP-2B) [15], and is known to be involved in some functions of various cells [15–17]. Since CaN induces the dephosphorylation of several phosphorylated protein serine/threonine residues [14, 17, 18], it is likely that phosphorylation induced by Ca^{2+}-dependent protein kinase, such as PKC or CaMKII, is dephosphorylated by CaN.

It was demonstrated that cyclosporin A (CyA), an inhibitor of CaN, suppressed the activity of the inwardly rectifying K^+ channels in renal tubule cells [19, 20]. We also found that inhibitors of CaN, CyA and FK520, both of which are well-known immunosuppressive agents [21], suppress K^+ channel activity in cell-attached patches of tubule cells under normal conditions [19, 20]. Since inhibitors of CaN are effective under normal conditions, CaN would be functionally activated even in a normal $[Ca^{2+}]_i$. However, the mechanism of CyA-induced channel inhibition is still unknown. To examine the involvement of Ca^{2+}-dependent protein kinase in CyA-induced K^+ channel suppression, we applied a PKC inhibitor, GF109203X [22], and a CaMKII inhibitor, KN-62 [23], and observed their effects on CyA-induced channel suppression. As the results, the CyA-induced channel suppression was not affected by GF109203X, but significantly attenuated by KN-62 [13], suggesting that the CyA-induced channel inhibition is mediated mainly by CaMKII. Although PKC may have an inhibitory effect on the inwardly rectifying K^+ channels in renal tubule cells [3, 5], our results suggest that the major candidate evoking CyA-induced channel inhibition is CaMKII [13]. Indeed, Western blot analysis revealed that CyA increased phospho-CaMKII, an active form of CaMKII [13].

The direct effects of CaN and CaMKII on channel activity were also examined in inside-out patches. Before investigation of the effects of CaN and CaMKII, we analyzed the direct effects of cytoplasmic Ca^{2+} and CaM on channel activity, since it was reported that the activity of some channels was directly affected by Ca^{2+} [24] or CaM [16]. We confirmed that Ca^{2+} (1 μM) or CaM (0.6 μM) barely affected the K^+ channels from the inside of the cell membrane, at least under our experimental conditions [13]. Moreover, the application of CaN in the presence of Ca^{2+} and CaM also induced no appreciable change in channel activity [13]. These findings suggest that CaN has no inhibitory effect on channel activity. In contrast, CaMKII markedly suppressed the channel activity. Then, we tested the direct effect of CaN on channel activity in inside-out patches in the presence of Ca^{2+}, CaM, and CaMKII. The suppressed channel activity due to CaMKII was restored following the application of CaN [13]. These results suggest that CaMKII-mediated phosphorylation has an inhibitory effect on channel activity, and CaN reactivates channels by the dephosphorylation of CaMKII-mediated phosphorylation sites. However, the phosphorylation and dephosphorylation sites of kinases and phosphatases are still unclear. It is also possible that CaN induces the dephosphorylation of phospho-CaMKII, resulting in the inhibition of CaMKII.

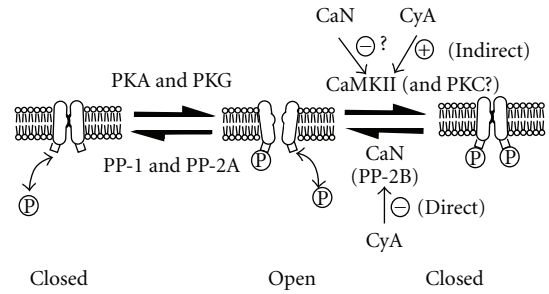

Figure 1: A schematic representation of the model for the regulation of the inwardly rectifying K^+ channels in renal tubule cells by phosphorylation and dephosphorylation processes. CaN is often called protein phosphatase-2B (PP-2B). The circled "P" indicates phosphate. Circled "+" and "–" indicate stimulation and inhibition, respectively. Small squares attached to the lower part of the channel are putative phosphorylation sites. See text for details.

A schematic representation of the mechanism of K^+ channel regulation by phosphorylation and dephosphorylation is shown in Figure 1. Previously, it was reported that PKA- and PKG-mediated phosphorylation induced channel opening (an active state) in proximal tubule cells [4–6] and that the open channels were closed by PP-1 and PP-2A [10], suggesting that PKA- or PKG-mediated phosphorylation was dephosphorylated by PP-1 or PP-2A. In addition to the above data, our findings strongly suggest that CaMKII phosphorylates other sites, resulting in the closed state of channels. On the other hand, CaN induces channel opening by the dephosphorylation of the CaMKII-mediated phosphorylation site or may inhibit CaMKII by the dephosphorylation of phospho-CaMKII. CyA directly inhibits CaN and indirectly increases the active type of CaMKII, phospho-CaMKII, through inhibition of the dephosphorylation process by CyA.

3. Role of CaN and CaMKII in Modulation of $[Ca^{2+}]_i$ in Human Tubule Cells

Our data on $[Ca^{2+}]_i$ measurement showed that both CyA and FK520, inhibitors of CaN, elevated $[Ca^{2+}]_i$ [13], similar to the data reported previously [25, 26]. Thus, it is suggested that CaN has an inhibitory effect on Ca^{2+}-release from the intracellular Ca^{2+}-store. As mentioned above, CyA-induced K^+channel suppression results from stimulation of CaMKII-dependent processes. Thus, CaMKII may be a key factor for CaN inhibitor-mediated $[Ca^{2+}]_i$ elevation in the tubule cells. The following experiments with inhibitors of both CaMKII and CaN revealed that CyA-induced $[Ca^{2+}]_i$ elevation was blocked by KN-62 [13], suggesting the importance

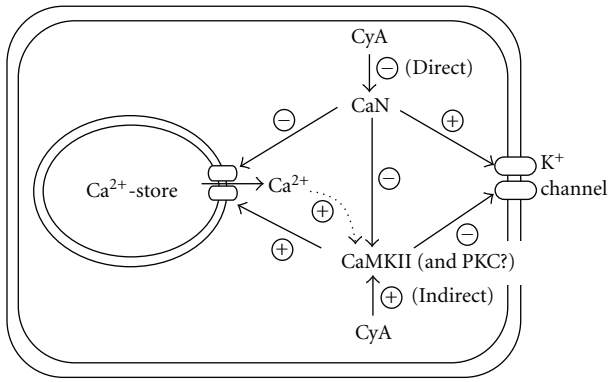

Figure 2: A simplified model of the involvement of CaN and CaMKII in the modulation of K^+ channel activity and Ca^{2+}-release channels in intracellular Ca^{2+}-stores in renal tubule cells. Circled "+" and "–" indicate stimulation and inhibition, respectively. Actions of CyA on Ca^{2+}-release channel and CaMKII are also shown. Ca^{2+} released from the Ca^{2+}-store may further enhance the activity of CaMKII, as shown by the dotted line.

of CaMKII in the elevation of $[Ca^{2+}]_i$. Similar data were demonstrated in a previous report showing that CaMKII stimulates Ca^{2+} release from Ca^{2+}-stores in skeletal muscle [27].

Figure 2 shows a simplified putative model of the mechanisms regarding Ca^{2+}-release channels in intracellular Ca^{2+}-stores and K^+ channels in the cell membrane by CaN and CaMKII. CaN activates the K^+ channel in the cell membrane and inhibits the Ca^{2+}-release channels of intracellular Ca^{2+}-stores. However, CaMKII inhibits K^+ channels and activates Ca^{2+}-release channels. Although there is a report of the role of CaN in the stimulation of Ca^{2+} flux via the 1,4,5-triphosphate receptor [28], our data strongly suggest that the inhibition of CaN stimulates Ca^{2+}-release channels [13]. Moreover as mentioned above, the inhibition of CaN-mediated dephosphorylation by CyA increases phospho-CaMKII, an active form of CaMKII. Thus, it is suggested that $[Ca^{2+}]_i$ elevation by CyA is induced mainly by increased phospho-CaMKII through activation of the Ca^{2+}-release channels of the intracellular Ca^{2+}-store. It is also conceivable that CaN exhibits an inhibitory effect on CaMKII, probably by the dephosphorylation of the phospho-CaMKII. The elevated $[Ca^{2+}]_i$ may further stimulate the activity of CaMKII, which would enhance the suppression of K^+ channel activity, as shown by the dotted curve in Figure 2. Moreover, the elevated $[Ca^{2+}]_i$ may stimulate not only CaMKII but also PKC.

4. Functional Relationship between CaN and CaMKII

Previous reports have shown that CaN plays several key roles in cellular functions. As for Ca^{2+}-release from intracellular stores, CaN is directly involved in its regulation [26], or CaN inhibitors lead to a higher probability of the ryanodine receptor (RyR)/Ca^{2+}-release channels being open [25]. On the other hand, CaMKII reportedly stimulates the Ca^{2+}-release in skeletal muscle cells [27]. Thus, it is likely that the CaMKII-mediated stimulation of Ca^{2+}-release was similar to the enhanced Ca^{2+}-release induced by the inhibition of CaN, as observed in our study [13]. These reports support our experimental data that CaN and CaMKII have opposite effects on channel activity. However, it has been reported that target phosphorylation sites dephosphorylated by CaN are mainly mediated by PKA [29]. Indeed, the functional coupling of CaN and PKA was shown to modulate Ca^{2+}-release in ventricular myocytes [29]. It has also been demonstrated that Na^+/K^+ ATPase at the basolateral membrane of kidney tubular epithelia was inhibited by CaN [30] and stimulated by PKA [31]. On the other hand, a cardiac Na^+/Ca^{2+} exchanger was reported to be regulated by CaN and PKC [32]. A cellular process dependent on mitogen-activated protein kinase was reported to be negatively regulated by CaN [33]. Thus, protein kinases opposed to CaN-mediated processes would act not in unity. Only a few reports suggested that CaMKII-mediated processes were abolished by CaN [34, 35]. Our data indicate the possibility that CaMKII-mediated phosphorylation is blocked by CaN [13], although it is still unclear whether the CaMKII-mediated phosphorylation site is identical to the CaN-mediated dephosphorylation site. It is also conceivable that the target dephosphorylation site for CaN is the phosphorylation site of CaMKII. In such a case, CaN would directly inhibit CaMKII activity by the dephosphorylation of phospho-CaMKII.

Our data suggest that CaN-mediated protein dephosphorylation would be dominant compared to CaMKII-mediated phosphorylation under normal conditions, since CaN inhibitors were found to significantly suppress K^+ channel activity, but an inhibitor of CaMKII alone did not [13]. Although the involvement of CaN in the modulation of channel activity is variable [36–39], our data suggest that the maintenance of channel opening under normal conditions would require the CaN-mediated action to exceed the CaMKII-mediated action in the tubule cells used in our study. This notion was supported by a previous report that the CaN content in proximal tubule cells is high among the several nephron segments [40]. However, it is still unknown whether the site of CaN-mediated dephosphorylation is identical to that of CaMKII-mediated phosphorylation in the modulation of channel activity. Moreover, there remains a possibility that the elevation of $[Ca^{2+}]_i$ may be dependent on the entry of extracellular Ca^{2+} across the cell membrane [41]. Recently we also demonstrated Ca^{2+} entry across the cell membrane in renal tubule cells [42], although a CyA-induced $[Ca^{2+}]_i$ elevation is considered to mainly be mediated by Ca^{2+}-release channels in the membranes of intracellular Ca^{2+}-stores. Further studies are necessary to clarify the precise mechanisms for the molecular regulation of channel activity as well as $[Ca^{2+}]_i$ by protein kinases and phosphatase, such as CaMKII and CaN.

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