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REVIEW ARTICLE

Role of calcium channels in cellular antituberculosis effects: Potential of voltage-gated calcium-channel blockers in tuberculosis therapy

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The immunity of human immune cells and their ability to inhibit *Mycobacterium tuberculosis* (MTB) are key factors in the anti-MTB effect. However, MTB modulates the levels and activity of key intracellular second messengers, such as calcium, to evade protective immune responses. Recent studies suggest that inhibiting L-type calcium channel in immune cells using either antibodies or small interfering RNA increases calcium influx, upregulates the expression of proinflammation genes, and reduces MTB burden. First, we will review the key factors in calcium-signaling pathway that may affect the immunity of immune cells to MTB infection. Second, we will focus on the role of calcium channels in regulating cellular immunity to MTB. Finally, we will discuss the possibility of using calcium-channel blockers as anti-MTB chemotherapy drugs to enhance chemotherapy effects, shorten treatment period, and overcome drug resistance.

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB). Although MTB may invade various organs, it mainly affects the lungs, causing pulmonary TB. In 2012, more than 20 million people worldwide were infected with MTB, including 8.6 million

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new cases and 1.3 million deaths.[1] The major challenges in the prevention and treatment of TB are the large amount of floating population and hidden infections, the slow progress in treating multidrug-resistant TB (MDR-TB), and the co-infected immune-compromised population.[2] At present, chemotherapy alone is not enough to cope with these challenges. Therefore, there is a necessity to develop new treatment methods, including the use of an integrated approach to develop new drugs, immunotherapy, and gene therapy.

It was found recently that during MTB infection, increase in calcium influx or release of calcium from intracellular calcium pool activates the intracellular calcium-signaling pathway, thereby activating the gene expression of anti-infection and immune-protective proteins in immune cells, especially macrophages.[3,4] This increase in calcium signaling enhances the phagocytic activity and the anti-MTB ability of immune cells, ultimately enhancing the anti-MTB ability of the whole immune system. As the key player in maintaining the intracellular calcium level, calcium channels have crucial roles in regulating the calcium-signaling pathway. Blocking of L-type calcium channel with verapamil, which is an L-type calcium channel-specific blocker, enhances the intracellular calcium level and triggers the downstream calcium-signaling pathway, ultimately activating the anti-infection gene expression.[3] We herein review the findings on immune cell calcium signaling during MTB infection, discuss the mechanism behind the anti-MTB effect of verapamil, assess the possibility of using verapamil as a candidate in combined chemotherapy, and propose future research directions.

Intracellular calcium-signaling pathway and its physiological significance

As a key intracellular second messenger, calcium has crucial physiological roles in muscle contraction, synaptic transmission and plasticity, cell motility, fertilization, cell growth, cell proliferation, and gene expression. It is also involved in regulating the enzyme activity, the ion-channel permeability, and cytoskeleton components.[5] The resting intracellular calcium concentration is usually maintained at 10—100 nmol/L. To maintain such low intracellular calcium levels, calcium is actively transported out of the cell or into the endoplasmic reticulum (ER), sarcoplasmic reticulum, and mitochondria. When calcium signaling is activated, calcium enters into the cell through cell-surface calcium channels and activates the ER calcium channels, which release more calcium from the intracellular calcium store. The specific signal may trigger the sudden increase of intracellular calcium levels to 500—1000 nmol/L.[6] The most common calcium-signaling pathway is the phospholipase C (PLC) pathway. Many cell-surface receptors, including G protein-coupled receptors and receptor tyrosine kinases, can activate the PLC pathway. The PLC hydrolyzes membrane phospholipids (phosphatidylinositol 4,5-bisphosphate) to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG), which are two classic second messengers.[6] DAG activates protein kinase C (PKC), whereas IP_3 spreads to the ER and binds to the IP_3 receptor (IP_3R). The IP_3R is an ER calcium channel that is responsible for calcium release from the ER. Gene expression can then be activated by calcium mainly through the following: the ternary complex factor pathway, the serum response factor pathway, and the cyclic adenosine monophosphate (AMP) response element pathway. Gene expression is activated through different calcium-signaling pathways in different cell types.[7]

The possible calcium-signaling pathway in immune cells

The exact calcium-signaling pathway in immune cells is not clear. However, it may regulate cell function in the following manner (Fig. 1): the extracellular calcium enters into the cells through the cell-membrane calcium channel, including selective calcium channels, such as voltage-gated calcium channels (VGCCs), or nonselective calcium channels, such as purine receptor (P2X7), cyclic nucleotide-gated ion channels (CNGs), and canonical transient receptor potential channels (TRPCs). VGCC is activated by changes in membrane potential, P2X7 by adenosine triphosphate (ATP), CNG by cAMP, and TRPC by PLC or DAG. On the one hand, calcium entering into the cell can directly activate calmodulin (CaM) and PKC to produce a series of cascading effects.[8] Calcium activates downstream kinases through the mitogen-activated protein kinase (MAPK) pathway, and MAPK regulates the phosphorylation of several transcription factors, including *C-myc* gene, the MAPK-interacting kinase (Mnk), and cAMP response element binding protein (CREB).[9] MAPK regulates gene transcription by modulating the level and activity of transcription factors, which is very important for cell growth, differentiation, and apoptosis. On the other hand, calcium enters into cells and activates the calcium channels on the ER surface, such as the ryanodine receptor (RyR), to induce more calcium release from the ER (calcium-induced calcium release or CICR).[10] Meanwhile, Ras and Src participate in the synthesis of IP_3 in the presence of PLC, and activate the IP_3R on ER surface to release calcium. IP_3 activates the calcium-signaling pathway mainly through PLC and phosphoinositide 3-kinase, which activate a series of infection and cell differentiation-related transcription factors, such as nuclear factor of activated T cells and CREB.[6]

The role of ion channels in anti-TB therapy

The role of purine receptors in TB pathology and cellular immunity

Understanding the mechanism of TB pathology and cellular immunity from the ion-channel perspective is a new field of study. The current studies focus on the role of purine receptor P2X7 in TB infection and immunity, as well as on the anti-TB therapy targeting P2X7. Human P2X7 is a membrane ligand-gated ion channel widely distributed in immune cells.[11] It has two transmembrane domains and is activated by extracellular ATP.[12,13] In macrophages and myeloid cells, activation of P2X7 receptor by ATP induces K^+ efflux and Ca^{2+} influx, and triggers the processing and secretion of cytokines interleukin-1β (IL-1β), IL-18, and IL-12.[11] IL-12 synergizes with IL-18 or IL-1β for the production of interferon-γ from

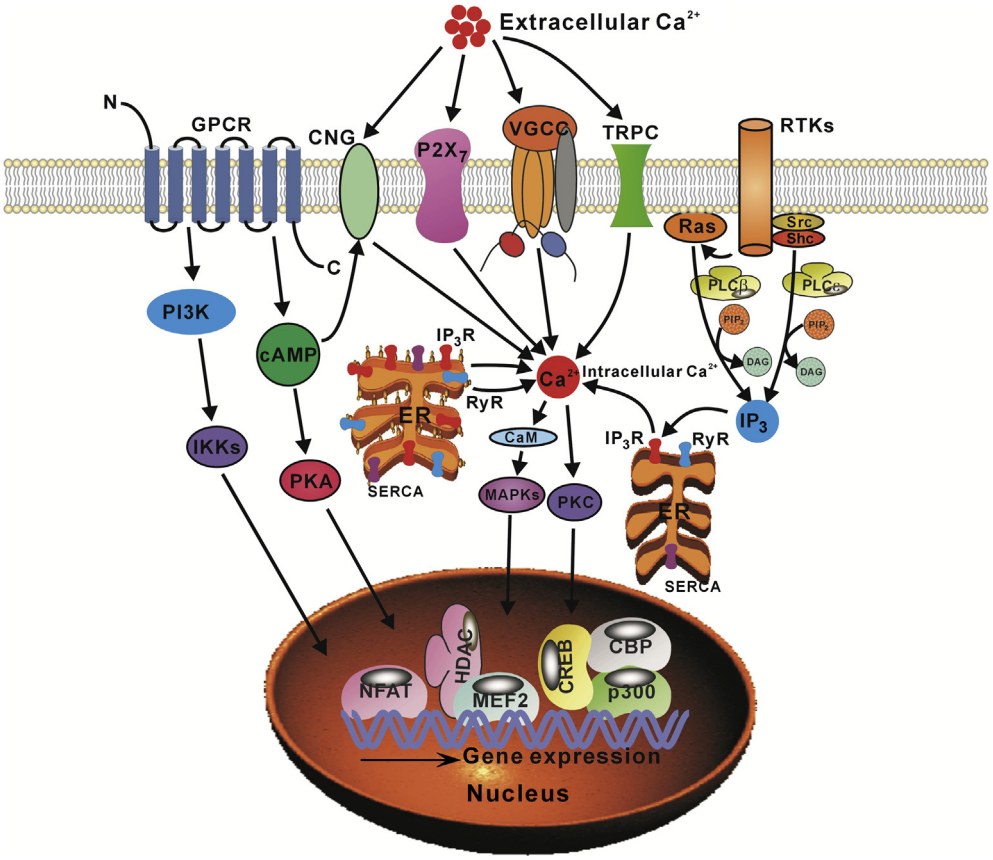

Figure 1. Possible calcium-signaling pathways in immune cells. Extracellular calcium enters the cell through membrane calcium channels, including selective calcium channels, such as VGCC, or nonselective calcium channel, such as P2X7, CNG, and TRPC. Calcium activates CaM and PKC, and then further triggers downstream protein kinases (such as MAPKs) and initiates gene expression. Calcium also activates ER surface calcium channels, such as RyR, to achieve CICR. Meanwhile, IP_3 is also produced in the presence of Ras, Src, and PLC and activates the ER IP_3R to release calcium. Calcium signaling regulates the expression of a range of gene expression related to anti-infection and cell differentiation, such as NFAT and CREB. CaM = calmodulin; cAMP = cyclic adenosine monophosphate; CBP = CREB binding protein; CICR = calcium-induced calcium release; CNG = cyclic nucleotide-gated ion channel; CREB = cyclic adenosine monophosphate response element binding protein; DAG = diacylglycerol; ER = endoplasmic reticulum; GPCR = *G protein-coupled receptor;* HDAC = histone deacetylase; IKK = I kappa B kinase; IP_3 = inositol triphosphate; IP_3R = IP_3 receptor; MAPK = mitogen-activated protein kinase; MEF2 = myocyte enhancer factors 2; NFAT = nuclear factor of activated T cells; PI3K = phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP_2 = phosphatidylinositol-4,5-bisphosphate; PKC = protein kinase C; PLC = phospholipase C; RTK = receptor tyrosine kinase; SERCA = sarco/endoplasmic reticulum Ca^{2+}-adenosine triphosphatase; TRCP = transient receptor potential channels; VGCC = voltage-gated calcium channel.

human T cells.[14] A series of downstream processes is initiated within minutes following the activation of P2X7, including the dissociation of certain surface molecules, the release of microparticles/exosome, membrane shrinkage, and apoptosis. Activation of P2X7 requires high concentrations of ATP (>100 μmol/L). However, it is not clear where such high concentrations of ATP are present, and they may possibly exist in a confined space such as bone.[11]

MTB can survive in immune cells and infect humans due its ability to escape from immune defense by several ways, including reactive oxygen intermediate, reactive nitrogen intermediates, and lysosomal enzyme destruction by monocytes/macrophages (M/M). The latter is now considered a key step in MTB survival, including the ability to prevent the fusion of lysosome and MTB-infected phagosome, that is, the formation of active phagocytic lysosomes.[15] The death of MTB-infected M/M is often considered the cause of MTB spread, and M/M activation is widely regarded as an important step of MTB killing. However, the activated M/M can survive for a long time and offer the potential habitat for MTB, or even support the growth of MTB. Because necrosis leads to the release of intracellular substances, whereas apoptosis does not, apoptosis, but not necrosis of host cells, is regarded as an effective way to prevent the spread and growth of MTB, and is considered to be a protective response of the body to MTB infection.[16–19]

Studies have shown that a high dose of MTB exposure can cause M/M apoptosis, whereas a low dose of MTB exposure could not cause M/M apoptosis. However, the mechanism for this phenomenon is not clear.[20,21] The latest research showed that the activation of P2X7 was coupled with the apoptosis of a number of immune cells, including human thymus cells, monocyte-derived macrophages,[16,22] and mouse microglial cells.[23] The study by Lammas et al[24]

confirmed that the apoptosis of MTB-infected macrophages was activated by ATP and was accompanied by MTB elimination. Similarly, another study proved that ATP stimulation reduces the viability of MTB in MTB-infected macrophages.[25] In macrophages infected with a high dose of MTB, increase in both P2X7 expression and ATP release was observed, suggesting the presence of a P2X7-mediated pathway. Furthermore, stimulation of ATP in MTB-infected cells results in reduction of P2X7-dependent MTB replication, and this effect is closely coupled with ATP-induced apoptosis.[26] These studies strongly suggest the significant role of P2X7 receptor in TB pathology. However, some studies also showed that there was no difference in bacillary burdens of MTB in $P2X7^{-/-}$ versus wild-type animals, suggesting that the P2X7 receptor is not required in the control of pulmonary MTB infection.[27] Therefore, further study is still needed to clarify the anti-TB role of P2X7.

The role of VGCC in TB pathology, cellular immunity, and TB therapy

Apart from P2X7, another transmembrane ion channel, the VGCC, has shown great potential as a target for anti-TB therapy.[3,4,28] VGCC is composed of one molecule each of the α, β, $\alpha_2\delta$, and γ subunits. Calcium entering the cell through the channel is involved in a number of important physiological functions, including muscle contraction, synaptic signaling, neuroendocrine regulation, cell growth, apoptosis, and gene expression.[29,30] VGCC can affect the expression of anti-infection genes downstream of calcium-signaling pathway, and in turn, can affect the immunity of immune cells and their phagocytic ability to MTB.[3]

VGCC is becoming a hot spot in investigating the *in vitro* and *in vivo* regulation of protective immunity to MTB. Several studies have demonstrated that VGCC is a promising target for anti-TB therapy. VGCC blockers have become a potential new candidate for TB chemotherapy.[3,4,28] It has been found that calcium influx can be increased by blocking the L-type or R-type VGCC with specific antibodies or inhibiting the VGCC expression using small interfering RNA in dendritic cells (DCs). This increase in calcium influx is IP_3 dependent and closely related to CICR, resulting in enhanced proinflammatory gene expression.[3] In addition, blocking VGCC in DCs can activate T lymphocytes and strengthen the ability of macrophages to kill MTB. Similarly, in the infected macrophages and monocytes, blocking of VGCC increases the expression of proinflammatory genes and enhances the MTB inhibitory effect.[3] More interestingly, the peripheral blood mononuclear cells from TB patients exhibit a higher VGCC expression level than those from healthy controls, and this expression level is significantly reduced after chemotherapy.[3] This suggests that VGCC could become a potential indicator for TB progression and prognosis. Finally, *in vivo* experiments on MTB-infected mice showed that MTB load was decreased following the blockage of VGCC with a specific antibody, whereas the intracellular calcium level was increased.[3,4] These observations indicate that poor calcium influx and calcium signaling in DCs in response to mycobacterial stimulation could be a result of increased expression of L-type and R-type VGCC. Blocking of VGCC during MTB infection inhibited the VGCC function and reversed the effect of excessive VGCC expression, and subsequently triggered the calcium influx from the intracellular calcium store and the downstream calcium signaling.[3] Therefore, L-type and R-type VGCC may regulate the cellular protective immunity to MTB by manipulating the intracellular calcium.

Recent research has applied this theory in TB experimental chemotherapy. First, animal experiments in mice showed that TB conventional chemotherapy (isoniazid + rifampin + pyrazinamide, HRZ) plus L-type calcium-channel blocker verapamil (Isoniazid + rifampin + pyrazinamide + verapamil, HRZV) accelerated the clearance of MTB in mice. The HRZV regimen group exhibited quantitative colony-forming unit (CFU) counts that were significantly lower than the HRZ group from the 2^{nd} weeks to the 24^{th} week of treatment in C3HeB/FeJ mice. The difference in CFU counts between the HRZV group and the HRZ group continued to widen over the course of treatment in C3HeB/FeJ mice. By contrast, the difference in CFU counts between the HRZV group and the HRZ group in C3H/HeJ mice was not significant until the 12^{th} week of treatment.[4] These observations indicate that the HRZV regimen accelerated the clearance of MTB in both C3HeB/FeJ and C3H/HeJ mice, although verapamil was less effective in C3H/HeJ mice. Second, HRZV combination significantly reduced the TB relapse rate and the course of treatment compared with conventional chemotherapy (HRZ).[4,28] In both C3HeB/FeJ mice and C3H/HeJ mice, the HRZV regimen exhibited much lower 3-month drug-free relapse rate than the HRZ regimen after 16, 20, and 24 weeks of treatment, indicating that much less time is needed to ensure relapse-free treatment using the HRZV method. Third, it was also reported that verapamil restored the susceptibility to rifampicin in mice infected with MDR-MTB.[31] Tuberculous mice infected with MDR-MTB were treated with verapamil plus rifampicin, and the results showed that the combination of rifampicin and verapamil significantly decreased the pulmonary bacillary loads (CFU) at every time point, particularly after 60 days of treatment, compared with the animals treated with rifampicin alone, verapamil alone, or control animals treated with a saline solution. This observation suggests that the presence of verapamil in the combination is necessary for restoring the susceptibility of MDR-MTB to rifampicin. Verapamil possibly works by inhibiting the resistance of MTB to the first-line anti-TB drugs, thereby enhancing the effect of anti-TB drugs and accelerating the clearance of MTB.[28,32,33] At the cellular level, the effects of verapamil may be explained in two aspects. First, at the MTB level, verapamil directly inhibits the efflux pump on the MTB cell surface, thereby increasing the concentration and acting time of anti-TB drugs in MTB cells.[4,28] Second, at the host immune cell level, blockage of L-type calcium channel by verapamil had an impact on intracellular calcium signaling, which strengthened the immunity of immune cells.[31] The inhibition effects of verapamil on drug resistance are important at both levels.

One important observation is that the MTB resistance to anti-TB drugs persists in the caseous necrosis tissues. This is because drug resistance persists after the death of macrophages, and the resistance spreads out of the macrophages along with the drug-resistant MTB. The drug resistance is transferred to other healthy immune cells by MTB infection, which leads to continued drug resistance.[4] The application of

verapamil suppresses the drug resistance, thereby enhancing the efficacy of the existing first-line anti-TB drugs. In addition, the use of verapamil restores MTB susceptibility to rifampin, and the combined chemotherapy (HRZV) significantly reduces lung bacilli loads in BALB/c mice infected with an MDR strain.[4,28,30] These animal experiment data suggest that the combination of verapamil with conventional anti-TB drugs enhances the efficacy of chemotherapy and increases the susceptibility of MTB to chemotherapy. It also shortens the course of treatment, lowers the drug-resistance rates, and reduces the side effects. The aforementioned findings not only provide a strong basis for future human research using a calcium-channel blocker in the combined chemotherapy regimen, but also provide strong evidence for calcium-channel blockers as a type of new drug for TB therapy. Calcium-channel blockers, such as verapamil, have been used for many years clinically with obvious safety and advantage. We believe its participation in TB therapy will benefit TB patients.

Future directions in anti-TB research related to VGCC

MDR-TB in immune-compromised population is a major challenge in TB prevention and treatment. The combination of the first-line anti-TB drugs with verapamil has provided a potential solution for this challenge. Verapamil regulates VGCC to trigger the intracellular calcium signaling and enhance the cellular immunity to MTB. VGCC expression may also be a marker for TB progress and therapeutic effect. VGCC modulation is therefore a crucial factor in studying the mechanism of TB infection and developing new therapeutic methods. The following aspects should be explored further to understand the roles of VGCC in TB pathology and future drug development.

Targeting VGCC to enhance cellular immunity and reduce MTB load

As an intracellular second messenger, calcium regulates important physiological functions and gene expression. Factors affecting calcium-signaling pathway may affect calcium regulation. We have reviewed that calcium-channel blockers inhibit the membrane VGCC, subsequently activating the expression of a series of cytokines (especially IL-12) and transcription factors, and triggering the calcium release from ER through the IP_3R and RyR calcium channels. These effects upregulate a series of downstream gene expression, especially genes related to anti-infection, and enhance the immunity of immune cells to MTB and reduce MTB load, and ultimately strengthen the immune system. However, how and to what extent calcium achieves these effects and how the calcium signaling is regulated in MTB infection still need further investigation.

Explore the potential of VGCC blocker to enhance the anti-MTB chemotherapeutic effect

The current TB chemotherapy regimen is challenged by drug resistance, and therefore the development of new drugs or new treatment method has become imperative. Combination of the conventional TB chemotherapy regimen with VGCC blockers may provide a new method of chemotherapy to help solve the problem of drug resistance, enhance the therapeutic effect of conventional chemotherapy, shorten the course of treatment, and improve the tolerance of patients. However, VGCC blockers may produce cardiovascular side effects (e.g., tachycardia, low blood pressure, and arrhythmia), nervous system side effects (e.g., headache and dizziness), and gastrointestinal side effects (e.g., constipation and vomiting). Therefore, further study is still required to provide more details on VGCC blocker efficacy when it is used together with traditional chemotherapy drugs in TB therapy. More studies in animals are also needed to confirm its effects before it moves to human experiments.

Explore the relationship between the changes in the immune cell VGCC expression and the progress and prognosis of TB

It was found in recent years that the expression level of ion channel is upregulated in many diseases, especially those related to the immune system, such as cancer. In cancer, the upregulation is closely related to cancer cell proliferation, invasion, and metastasis and could be a sign of malignancy. Similarly, VGCC upregulation is observed in MTB-infected immune cells, which could be a cellular anti-infection response related to stress stimulation and reflects a self-protection mechanism. Therefore, it would be interesting to investigate the relationship between VGCC expression and TB infection and to explore the possibility of using VGCC expression level as a biomarker for TB progress, severity, and prognosis.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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