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# KAEMPFEROL INHIBITS MYOSIN LIGHT CHAIN KINASE

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SUMMARY: Kaempferol, 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, was found to inhibit bovine aorta myosin light chain kinase with a Ki of 0.3-0.5 uM. It was found to be competitive with ATP and non-competitive with isolated myosin light chains. The specificity of this inhibitor was studied relative to protein kinase C and cAMP dependent protein kinase (IC50 = 15 uM and 150 uM, respectively). It appears not to interact strongly with calmodulin binding proteins, such as $Ca^{2+}$-calmodulin dependent phosphodiesterase (IC50 = 45 uM), and had little effect on actin-activated myosin subfragment-1 ATPase activity (IC50 > 100 uM) or smooth muscle phosphatase activities (IC50 > 100 uM). 

Phosphorylation of the 20,000 dalton light chain of myosin occurs in a large number of tissues and individual cells. In smooth muscle, this phosphorylation event is an obligatory step in the development of active tension (1). The myosin light chains (MLCs) are phosphorylated with high specificity by myosin light chain kinase (MLCK) (E.C. 2.7.1.117), a $Ca^{2+}$-calmodulin dependent enzyme, and are dephosphorylated by smooth muscle phosphatases. MLCs can also be phosphorylated by other kinases, notably protein kinase C (PKC) (E.C. 2.7.1.-)(2). The relative importance of these phosphorylation events in the contractile processes of a wide variety of tissues and cells is currently an active area of investigation.

An inhibitor of MLCK would be of obvious utility in such studies, and a number of compounds which inhibit this enzyme have been described. Calmodulin antagonists (3) inhibit a large number of calmodulin-dependent processes as well as inhibiting MLCK activity. A number of general kinase inhibitors have

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ABBREVIATIONS

DMSO: dimethyl sulfoxide, MLC: myosin light chain, MLCK: myosin light chain kinase, PDE: $Ca^{+2}$-calmodulin-dependent phosphodiesterase, PKA: cAMP dependent protein kinase, PKC: protein kinase C.

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been described, but most are not specific for MLCK as compared with other kinases (e.g. 4,5), or they affect other important regulatory systems which makes interpretation of their effects difficult (ML-9, for example works better on unskinned fibers than skinned fibers)(6). Peptidyl inhibitors, developed as substrate analogues (7), have been useful for in vitro studies, but have proven difficult to get into intact cells or tissues for physiological studies. Since development of a specific, nonpeptidyl inhibitor of MLCK would be useful in understanding the role of MLC phosphorylation in a number of different processes, we began a search for such an inhibitor. After evaluating about 1300 compounds for inhibition of bovine aorta MLCK activity and testing active compounds for specificity in terms of inhibition of other related enzymes, we found one of the compounds, kaempferol, a flavone found in a number of plants, to be a relatively potent inhibitor of MLCK.

## MATERIALS AND METHODS

Sample Preparation: Many of the test compunds (including kaempferol) are only slightly soluble in water. Therefore, test compounds were dissolved in 100% dimethylsulfoxide (DMSO), and were diluted into the assay mixes such that the final DMSO concentration was 1%. Controls for all assays were run in 1% DMSO. Kaempferol was obtained from Sigma.

MLCK and MLC preparation: MLCK and MLCs were prepared separately from thoracic aortae of freshly slaughtered adult domestic cattle, according to the method of Adelstein and Klee (8). On SDS polyacrylamide gels the purified MLCK is a single band at Mr = 150000, in good agreement with published values for bovine trachealis MLCK (9). At the MLCK, MLC, calmodulin and [gamma-$^{32}$P]-ATP concentrations used in the assays, the $^{32}$P phosphate incorporated was linear with time. Phosphorylatable MLC concentration was determined by the amount of radioactivity which could be incorporated in 15 minutes by 3.75 x $10^{-9}$ M MLCK.

Other proteins: Calmodulin was purchased from Boehringer Mannheim. Bovine immunoglobulin G, used at 1 mg/mL in all of the assays to prevent adsorption of the enzymes to the tubes, bovine brain $Ca^{2+}$-calmodulin dependent phosphodiesterase (3.1.4.17) and catalytic subunit of bovine heart cAMP dependent protein kinase (PKA) (E.C. 2.7.1.37) were purchased from Sigma. Purified PKC from rat brain was obtained from Lipidex. Myosin subfragment-1 and actin were prepared as reported previously (10). Partially purified smooth muscle phosphatases (E.C. 3.1.3.53) SMPI, SMPII, SMPIII and SMPIV were a generous gift from Dr. Mary D. Pato, University of Saskatchewan (11,12). Protein concentrations were determined by the method of Bradford (13) using IgG as a protein standard.

## RESULTS

The purpose of this study was to search for novel specific inhibitors of mammalian vascular MLCK which could subsequently be used in physiological studies of smooth muscle regulation and function. The primary assay was for inhibition of bovine aorta MLCK activity. The interesting compounds were then tested for specificity as defined by significantly lower IC50 for MLCK as compared with other kinases and calcium-calmodulin-dependent enzymes and

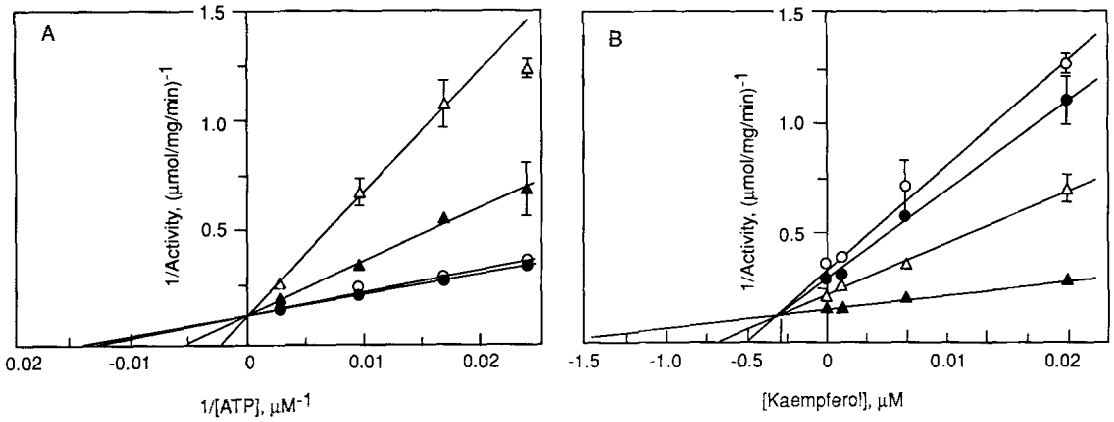


Figure 1: Bovine aorta MLCK activity as a function of [ATP]. [MLC] = 5uM; [MLCK] = 1.5 x $10^{-9}$ M; [calmodulin] = 1 x $10^{-7}$ M. [kaempferol] in DMSO as noted. Figure 1a, Lineweaver Burk plot: (●), DMSO blank; (○), 100 nM kaempferol; (▲), 500 nM kaempferol; (△), 1.5 uM kaempferol. Figure 1b, Dixon plot: (▲), 375 uM ATP; (△), 105 uM ATP; (●), 60 uM ATP; (○) 42 uM ATP.

phosphatases. From this battery of tests, kaempferol, 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, emerged as the most interesting compound. The mode of inhibition was then determined for this compound, which prompted further testing for specificity against the actin-activated myosin subfragment-1 ATPase (E.C. 3.6.1.3).

Mode of inhibition: Inhibition of bovine aorta MLCK by increasing concentrations of kaempferol was measured as a function of ATP concentration and of phosphorylatable MLC concentration. Figures 1a and 2a are Lineweaver-Burk plots which show that kaempferol is competitive with ATP and noncompetitive with the phosphorylatable MLCs in its inhibition of the

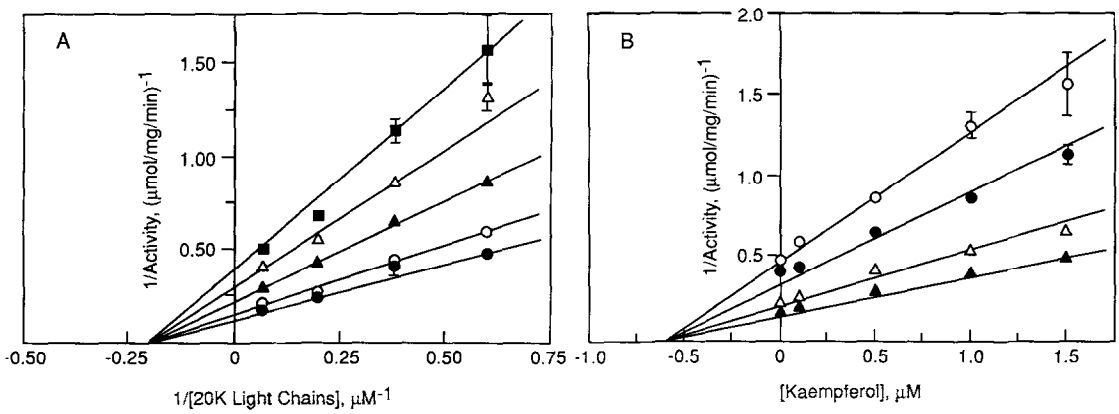


Figure 2: Bovine aorta MLCK activity as a function of [phosphorylatable MLC]. [ATP] = 100uM; [MLCK] = 1.5 x $10^{-9}$ M; [calmodulin] = 1 x $10^{-7}$ M. [kaempferol] in DMSO as noted. Figure 2a, Lineweaver Burk plot: (●), DMSO blank; (○), 100 nM kaempferol; (▲), 500 nM kaempferol; (△), 1.0 uM kaempferol; (■), 1.5 uM kaempferol. Figure 2b, Dixon plot: (▲), 14.85 uM MLCs; (△), 5.06 uM MLCs; (●), 2.63 uM MLCs; (○) 1.67 uM MLCs.

enzyme. At a fixed [MLC] = 5 uM, the apparent Vmax for bovine aorta MLCK is 4 umol phosphate incorporated/mg MLCK/min, and the Km for ATP is 75 uM, while at a fixed [ATP] = 100 uM, the Km for phosphorylatable MLCs is 5 uM and the apparent Vmax is 4.2 umol/mg/min. Since both sets of experiments were performed at subsaturating fixed substrate concentrations, the apparent Vmax determined in each experiment is lower than the true Vmax. The true Vmax is calculated to be 8 - 9.8 umol/ mg /min, when the fixed [substrate]/Km relationship is taken into account. Thus all the kinetic data are similar to values obtained for the turkey gizzard enzyme (8). Analysis of the inhibition data by Dixon plot (figures 1b and 2b) indicates that the Ki for kaempferol is 300-500 nM.

Specificity of Inhibition: Specificity of kaempferol inhibition was evaluated by determining IC50 values for several other enzymes. As shown in Table I, kaempferol inhibits bovine aorta MLCK with an IC50 of about 0.45 uM, while the IC50s for PKC and PKA are about 15 uM and >150 uM respectively. Thus, under these conditions, kaempferol is specific for MLCK as compared with other kinases tested by a factor of 30 or greater. Calmodulin antagonism was measured with a $Ca^{2+}$-calmodulin dependent phosphodiesterase (PDE). The IC50 for this enzyme was 45 uM, indicating that if kaempferol is a calmodulin

TABLE I

SPECIFICITY OF INHIBITION BY KAEMPFEROL

| Enzymes (Source, assay reference and notes) | | [Enzyme], M | IC50, uM |
|---|---|---|---|
| Myosin light chain kinase (a) | | $1.5 \times 10^{-9}$ | 0.45 |
| Protein kinase C (b) | | $6.7 \times 10^{-10}$ | 15 |
| $Ca^{2+}$-calmodulin dependent phosphodiesterase (c) | | $4.4 \times 10^{-8}$ | 45 |
| Actin-activated myosin subfragment-1 MgATPase (d) | | $1 \times 10^{-7}$ | >100 |
| Smooth muscle phosphatases (e) | (I) | $4.3 \times 10^{-10}$ | >100 |
| | (II) | $7.2 \times 10^{-10}$ | >100 |
| | (III) | unknown MW | >100 |
| | (IV) | $7.0 \times 10^{-10}$ | >100 |
| cAMP dependent protein kinase (f) | | $6.2 \times 10^{-8}$ | >150 |

a) Bovine aorta, [calmodulin] = $1 \times 10^{-7}$, Adelstein and Klee ref(8). b) Rat brain, ref(14) , bovine aorta MLCs as substrate. c) Bovine brain, [calmodulin] = $1 \times 10^{-8}$, ref(15) . Tritiated cAMP and adenosine were from New England Nuclear. d) Rabbit skeletal muscle (15°C), ref(16). Vanadate-free ATP was from Sigma, [gamma-$^{32}$P] ATP was from New England Nuclear. e) Turkey gizzard, ref(17). f) Bovine heart, catalytic subunit, ref(18).

antagonist, it is about 100-fold better as a MLCK inhibitor. Note that kaempferol could interact at the cAMP binding site of the PDE rather than the calmodulin binding site. The conclusion, however, is still valid--kaempferol does not interfere with the prototypical enzyme used to measure calmodulin antagonism as much as it does with the ATP binding site of MLCK. Inhibition of smooth muscle phosphatase activity was measured for four partially purified smooth muscle phosphatases from turkey gizzard. The IC50 is above 100 uM, the highest concentration tested. Finally, since kaempferol interacts with the ATP binding site of MLCK, rabbit skeletal muscle actin-activated myosin subfragment-1 MgATPase was also tested. The IC50 for kaempferol in this system is >100 uM, indicating that kaempferol would not be likely to inhibit acto-myosin interactions by direct binding.

## DISCUSSION

The data presented above suggest that kaempferol is a relatively specific and potent inhibitor of purified bovine aorta MLCK. The MLCK-MLC system was purified from mammalian vasculature because of its relatively direct applicability to cardiovascular physiological and pharmacological studies. For such studies, these data should be useful in determining the conditions at which kaempferol might be most potent and specific as an inhibitor of MLCK in tissues. The kaempferol concentration used in such studies would be limited by the desire for specificity relative to PKC. An indication of the specificity of the kaempferol was determined by comparison of IC50 for different enzyme systems. Although this is a relatively rapid means of looking at specificity, it is (unlike Ki) dependent upon assay conditions for each of the various enzymes tested. The concentrations of all of the enzymes are noted, and most of these concentrations are similar to the MLCK concentration.

In Figure 1a, it can be seen that if [kaempferol] is maintained near its Ki for MLCK of 0.5 uM, it will not significantly inhibit MLCK at the normal intracellular concentration of ATP (1-2 mM). However, if the ATP concentration is lowered to 0.1 or 0.2 mM, kaempferol has a much more significant inhibition. This suggests that kaempferol would have its greatest effect in skinned fibers held at low ATP concentrations by a tightly-coupled ATP regenerating system. In the case of a "leaky" fiber, with most of the membrane associated proteins remaining with the fiber, if the ATP concentration were lowered to 0.1 to 0.2 mM, MLCK would be expected to be 50-70% saturated with ATP (Km = 75 uM), and PKC would be expected to be >95% saturated with ATP (Km = 6 uM). The data indicate that kaempferol is competitive with ATP, presumably binding at or near the ATP binding site of

MLCK. If we assume that kaempferol also competes with ATP in the case of the other kinases, a lowered ATP concentration would probably not increase significantly the effect of 0.5 uM kaempferol on the other kinases.

Preliminary experiments indicate that kaempferol is able to relax methoxamine-contracted rabbit aorta rings and phasically contracting rat portal veins with IC50s of 10 uM and 3 uM, respectively (R.J. Winquist, personal communication). Although this appears to be consistent with inhibition of MLCK activity, we cannot be certain of the cellular mode of action of kaempferol. It seems doubtful that the major effect of kaempferol is to inhibit PKA activity, as this would tend to antagonize relaxation, as would inhibition of the $Na^+/K^+$ or $Ca^{+2}$ATPases. Unlike the MLCK inhibitor ML-9, kaempferol does not appear to possess calcium channel blocker activity: 50 uM ML-9 inhibits diltiazem binding by 87% while kaempferol does not significantly inhibit diltiazem or fluspirilene binding at this concentration (G.J. Kaczorowski, V.F. King, personal communication). Although kaempferol has been reported to be a free-radical scavenger and inhibits some lipoxygenases, it appears to do so only at higher concentrations. Inhibition of caldesmon phosphorylation remains a possiblity, although no consensus appears to exist as to the role caldesmon plays in the regulation of contraction. While many of these possibilities remain to be studied, the fact that kaempferol inhibits MLCK in vitro, and appears to be relatively specific in its action on this enzyme, suggests that it may be of some use in studies of regulation of smooth muscle contraction.

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