Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 200-206

CALCIUM-DEPENDENT INTERACTIONS OF AN IONOPHORE A23187 WITH CALMODULIN

Masaki Inagaki, Toshio Tanaka, Yasuharu Sasaki and Hiroyoshi Hidaka*

Department of Pharmacology, Mie University School of Medicine,
Edobashi, Tsu 514, Japan

Received May 22, 1985

We found that ionophore A23187 interacted reversibly with calmodulin (CaM), in a calcium-dependent fashion. It was found that A23187 interacts selectively with CaM, among calcium binding proteins (such as troponin C and S-100 protein) and other proteins. However, apparently differing from W-7, A23187 did not suppress CaM-dependent enzyme activity such as myosin light chain kinase and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. Our observations suggest that there are novel calcium-dependent regions of CaM which can be monitored using ionophore A23187 and may not be related to enzyme activation.

The polyether antibiotic ionophore A23187 is now used widely to examine the regulatory role of calcium ion in biological system. These studies have provided evidence for the involvement of Ca^{2+} in a wide spectrum of biological control mechanisms including muscle contraction, stimulus-secretion coupling, mitosis, fertilization, gluconeogenesis, glycogenolysis, and many others (1,2). On the other hand, calmodulin is ubiquitously distributed in eukaryotic cells and has been proposed as a universal intracellular receptor for calcium ion and the major physiological effector for a wide range of cellular responses evoked by the cation (3).

Studies on the mechanism and specificity of biological effects of A23187 have traditionally focused on interactions between the calcium ionophore and lipid bilayers. However, the molecular mechanisms of A23187 action on the Ca^{2+}-CaM regulatory system has not been established. We now report the evidence for the Ca^{2+}-dependent interaction of A23187 with CaM.

* To whom all correspondence and the reprint requests should be addressed.

0006-291X/85 $1.50

MATERIALS AND METHODS

A23187 was obtained from Calbiochem-Behring Corp. [γ -^{32}P]ATP was obtained from Amersham, England. The myosin light chain kinase from chicken gizzard was purified by the method of Adelstein and Klee (4). Calmodulin-deficient myosin light chain of chicken gizzard, used as substrate for the kinase assay, was prepared by the method of Perrie and Perry (5). The light chain was separated from calmodulin by DEAE-cellulose (Whatman) chromatography. Calmodulin-deficient Ca^{2+}-phosphodiesterase from bovine brain and bovine brain calmodulin were prepared as described (6). Protein was measured by the method of Lowry et al. (7) with bovine serum albumin or purified bovine brain calmodulin as standard. Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was measured using our own method (8). CaM was assayed for its ability to activate a fixed amount of CaM-deficient phosphodiesterase under standard conditions (9). One unit of CaM was defined as the amount activating 50% of the maximal phosphodiesterase activity attainable under standard experimental conditions, and was equivalent to 10ng of CaM (9). The equilibrium binding procedure between [^{3}H]W-7 and CaM followed that described by Hummel and Dreyer (10). Myosin light chain kinase activity was assayed in 0.2 ml of 20 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 20 μm [γ-^{32}P]ATP (3μCi/assay tube), 35 μM smooth muscle myosin light chain and either 0.05 mM $CaCl_2$ or 2 mM EGTA at 30°C. Unless otherwise noted, 0.2 μg calmodulin was used. Rates of phosphate incorporation were constant during the incubation period. For some experiments, incubations were terminated by addition of 20 μl of 10% sodium dodecyl sulfate with 5 μl of 100% mercaptoethanol. Samples (60 μl) were subjected to electrophoresis in 15% polyacrylamide containing 0.2% SDS. Gels were either stained with Coomassie brilliant blue or divided into 2-mm slices for liquid scintillation counting. Alternatively, incubations were terminated by the addition of 1 ml of 10% trichloroacetic acid followed by centrifugation (3000 rpm, 10 min). The pellet was suspended in 5% trichloroacetic acid and the centrifugation-resuspension cycle was repeated three times. The final pellet was dissolved in 1ml of 0.5 M NaOH for radioassay. Phosphodiesterase activity was assayed as previously described.

Smooth muscle cells, derived from medial layers of the rabbit thoracic aorta were prepared according to (11). After careful removal of the adventitia, medial rings (thickness 0.5 mm) were placed on Falcon flasks and cultured in a MEM medium supplemented with 10% calf serum. After 3 weeks, growth of primary cells around the explants ceased. Cells were then trypsinized and dispensed into flasks for subculture. The cells were incubated at 37°C in 5% CO_2 and the medium was changed every 2 days. Smooth muscle cell cultures exhibited at confluency the characteristic "hills and valleys" pattern (12). Electron microscopy was routinely performed. A23187 was applied to cell preparations at a concentration of 1 μM in phosphate-buffered saline for a period of 2 min at 37°C followed by rinsing with phosphate-buffered saline and the preparations were examined using an Olympus microscope (BH-2) equipped with an epifluorescence apparatus and UV filter was used for fluorescence microscopy. Photographs were taken using Kodak Tri-X pan film.

Fluorescence spectra were recorded using an Aminco-Bowman spectrofluorometer with a thermostat at 20 ± 1°C. Total dilution never exceeded 5% and relative fluorescence values were uniformly corrected for dilution. A23187 was dissolved in 100% dimethyl sulfoxide and added so that the concentration in the samples undergoing measurement was 0.5%. Excitation was at 350 nm or 280 nm and fluorescence intensity was monitored at 437 nm. Solutions contained 50 mM Tris-HCl (pH 7.5), 0.1 mM.

RESULTS AND DISCUSSION

The fluorescence of A23187 provides a convenient means for examining its interactions with the target molecule. Figure 1 suggests that

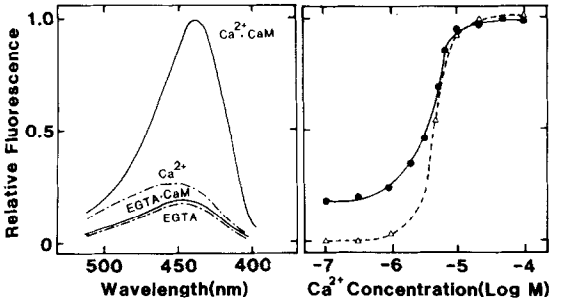

Fig. 1. Effect of calmodulin on A23187 fluorescence.
(A); Emission spectra of 3 μM A23187 in 50 mM Tris-HCl (pH 7.5) with or without 15 μM calmodulin, and 50 μM Ca^{2+}. Excitation at 350 nm.
(B); (●); Solutions contained 50 mM Tris-HCl (pH 7.5), 15 μM calmodulin, 3 μM A23187 and Ca^{2+}-EGTA buffer as indicated. Excitation at 350 nm, emission at 437 nm. (△); [^{3}H]W-7 binding to calmodulin was measured as described under Experimental Procedures.

ionophore A23187 interacts with purified CaM, in a Ca^{2+}-dependent fashion. A23187 in an aqueous solution displayed a broad emission spectrum with a maximum at 456 nm. In the presence of 0.1 mM $CaCl_2$, CaM produced significant enhancement in A23187 fluorescence intensity with a blue shift in the maximum to 437 nm (Fig. 1A). In the absence of calcium ions (2 mM ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), CaM did not lead to a significant enhancement. Calcium ions alone did not affect significantly the fluorescence of A23187 at 437 nm.

This fluorescence enhancement in nonpolar environments can serve as an indication of ionophore partitioning into Ca^{2+}-dependent hydrophobic regions of CaM (13). The effect of A23187 fluorescence was significantly increased with 1 μM calcium ion and was maximal with 10 μM Ca^{2+}. The Ca^{2+} concentration required for Ca^{2+}, CaM-dependent enzyme activation and Ca^{2+} dependent binding of CaM antagonists such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) by CaM is similar to that needed to increase A23187 fluorescence (Fig 1B). As A23187 in polar media at neutral pH has a binding affinity for calcium ion of Ka 1 mM (14), this enhancement in A23187 fluorescence may be related to Ca^{2+}-binding to CaM and not to A23187.

Table I shows the influence of calcium ion on the binding of A23187 to calcium binding proteins and several other proteins. Calcium ion had a

Table 1
Interaction of A23187 with various proteins

Proteins	Increase of relative fluorescence intensity *	
	0.1 mM $CaCl_2$	2 mM EGTA
Calmodulin	53.0	0.0
S-100 protein	4.8	0.0
Troponin C	3.5	0.0
20-K myosin light chain (chicken gizzard)	12.8	0.0
Parvalbumine	2.8	0.0
Egg Albumin	2.1	2.0
Chicken serum albumin	3.8	3.8
Lysozome	0.0	0.0
Immunoglobulin G	0.0	0.0
Chymotrypsin	0.0	0.0
Histone III-S	0.0	0.0

*Fluorescence measurements were performed by preparation of samples containing 10 μM of each proteins and 3 μM A23187 in 2 ml of 50 mM Tris-HCL (pH 7.5) and 0.1 mM $CaCl_2$ or 3 mM EGTA. Each value was the mean of duplicate determinations.

some effect on the binding of A23187 to various Ca^{2+}-binding proteins with an approximate order of fluorescence increase of CaM, myosin light chain, S-100 protein, troponin-C, parvalbumin. These results suggest that A23187 is obviously different from hydrophobic probes such as 2-p-toludinyl-naphthalene-6-sulfonate (15,16). It is most difficult to demonstrate the significant difference in interaction of various hydrophobic probes with some Ca^{2+}-binding proteins (17). Other proteins examined, including egg albumin, chicken serum albumin, lysosome, immunoglobulin G, chymotrypsin, and histone III-S, did not significantly increase the A23187 fluorescent intensity Ca^{2+}-dependence. As A23187 binds significantly to membranes used for equilibrium dialysis, binding of A23187 to CaM was measured by the increase in fluorescence intensity of A23187 in the presence of calcium ion and 15 μM CaM at pH 7.5. The apparent dissociation constant was approximately 5×10^{-6} M. We examined the effect of A23187 on two Ca^{2+}, CaM-dependent enzymes, smooth muscle myosin light chain kinase and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. A23187 did not inhibit these enzyme activities in doses up to 1×10^{-4} M. Therefore, the Ca^{2+}-dependent hydrophobic regions recognized by A23187 may differ from

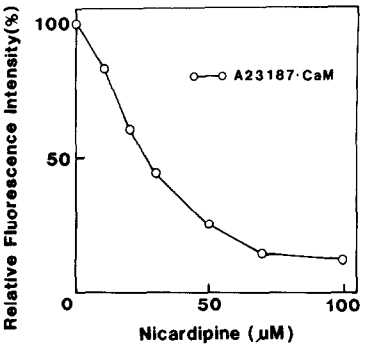

Fig. 2. Nicardipine-induced inhibition of A23187-CaM fluorescence and Dansyl-CaM fluorescence in the presence of 50 μM calcium ion. Each CaM was used at the concentration of 15 μM. Experimental conditions were described in "Experimental Procedure".

that demonstrated by hydrophobic probes and CaM antagonists related to enzyme activation (15,16,17).

A dihydropyridine calcium channel blocker, nicardipine can also interact with CaM in a Ca^{2+}-dependent manner, and cannot inhibit Ca^{2+}-CaM dependent myosin light chain kinase activity, in a dose up to 1×10^{-4} M. Figure 2 shows that nicardipine suppressed A23187 fluorescence in the presence of Ca^{2+}-CaM complex in a concentration-dependent fashion with an IC_{50} value of 25 μM. These results suggest that the ionophore A23187 and this calcium antagonist nicardipine have similar binding site(s) on the CaM molecule and that these sites are formed after the Ca^{2+}-binding to CaM. Since CaM distributes mainly in cytoplasm (3), we investigated fluoromicroscopically whether or not A23187 could penetrate cell membrane and interact with CaM. A23187 fluorescence (Fig. 3) appeared to be associated with discrete elements in the cytoplasm. These results do suggest that the ionophore A23187 penetrates the cell membrane and is distributed mainly in the cytoplasm. A23187 increased the concentration of free cytosolic calcium ion by interacting with the cell membrane and also bound to calmodulin in a Ca^{2+}-dependent fashion. Whether or not the A23187 binding to CaM is involved in molecular mechanisms of the ionophore action has to be determined. A23187 interacts not only with the cell membrane but also with CaM. A23187 and nicardipine have proved to be useful tools to monitor the novel Ca^{2+}-dependent regions of CaM.

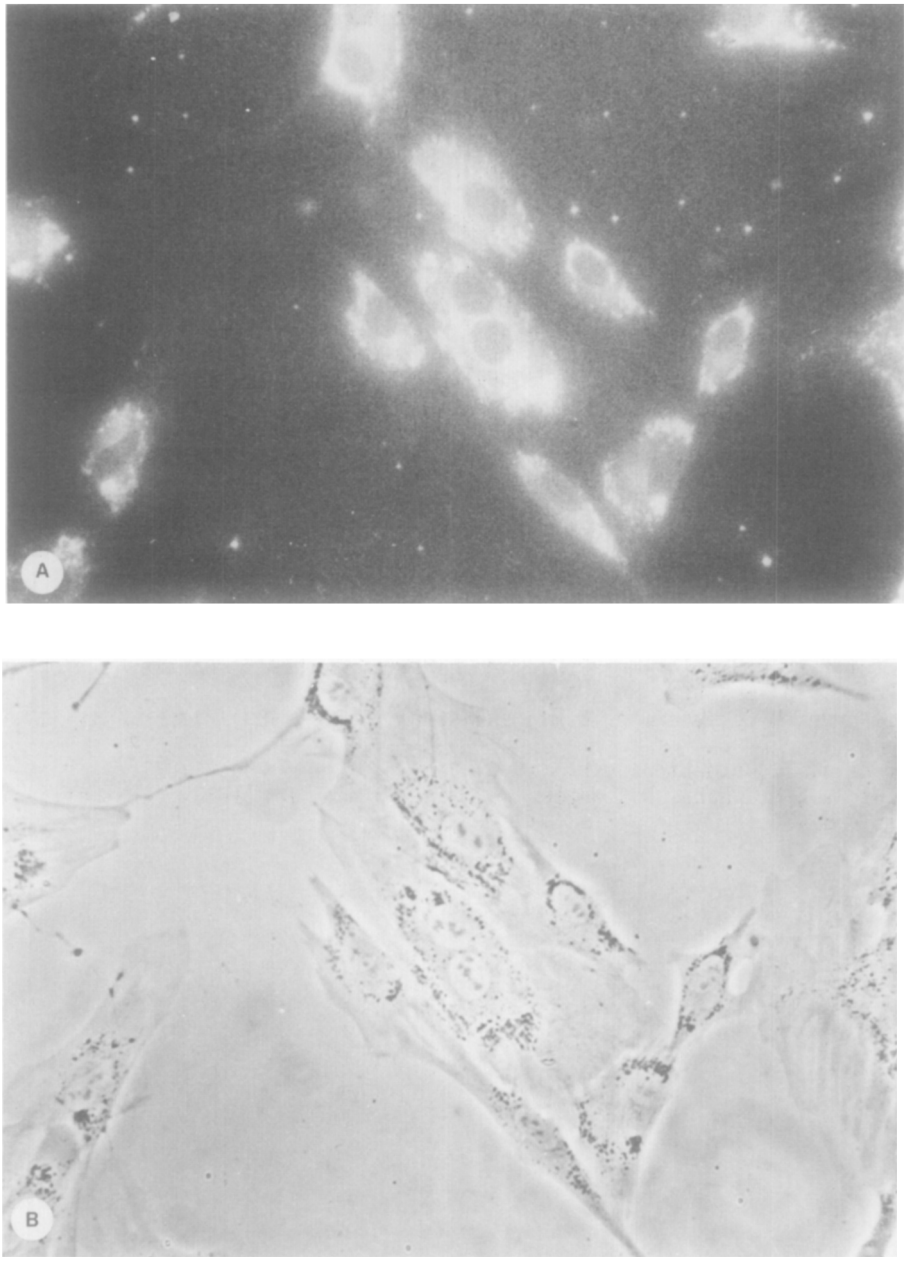

Fig. 3. Distribution of A23187 in the cultured rabbit arterial smooth muscle cell. After a 5 min incubation in the presence of 1 μM A23187, phase contrast microscopy (B) and fluorescence microscopy (A) were used for observation.

ACKNOWLEDGMENTS

We thank M. Watanabe and T. Shin for kind cooperation with the experiments and M. Ohara of Kyushu University for critical readings of the manuscript.

This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Krestinger, R.H. (1979) Adv. Cyclic Nucleotide Res. 11, 1
2. Rasmussen, H., and Goodman, D.B.P. (1977) Physiol. Rev. 57, 421
3. Klee, C.B., and Vanaman, T.C. (1982) Adv. Protein Chemistry 35, 213-, Academic Press Inc.
4. Adelstein, R.S., and Klee, C.B. (1981) J. Biol. Chem. 256, 7501
5. Perrie, W.T., and Perry, S.V. (1970) Biochem. J. 119, 31
6. Hidaka, H., Yamaki, T., Totsuka, T., and Asano, M. (1979) Mol. Pharmacol. 15, 49
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265
8. Hidaka, H., and Asano, T. (1976) J. Biol. Chem. 251, 7508
9. Hidaka, H., Yamaki, T. Naka, M., Tanaka, T., Hayashi, H., and Kobayashi, R. (1980) Mol. Pharmacol. 17, 66
10. Hummel, J.P., and Dreyer, W.J. (1962) Biochim. Biophys. Acta 63, 530
11. Blaes, N., Bourdillon, M.C., Crouzet, B., and Boissel, J.P. (1980) Cell Tissue Kinet 13, 445
12. Chamley-Campbell, J., Campbell, G.R., and Ross, R. (1979) Physiol. Rev. 59, 1
13. McClure, W.O., and Edelman, G.M. (1966) Biochemistry 5, 1908
14. Pfeiffer, D.R., Taylor, R.W., and Lardy, H.A. (1978) Annals New York Academy of Sciences 307, 402
15. LaPorte, D.C., Wierman, B.M., and Storm, D.R. (1980) Biochemistry 19, 3814
16. Tanaka, T., and Hidaka, H. (1980) J. Biol. Chem. 255, 11078
17. Inagaki, M., Naka, M., Nozawa, Y., and Hidaka, H. (1983) FEBS Lett. 151, 67-70.