J. Membrane Biol. 96, 121–128 (1987)

The Journal of
Membrane Biology

Evidence for the Involvement of Calmodulin in the Operation of Ca-Activated K Channels in Mouse Fibroblasts

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Summary. The oscillation of membrane potential in fibroblastic L cells is known to result from periodic stimulation of Ca^{2+}-activated K^+ channels due to the oscillatory increase in the intracellular Ca^{2+} concentration. These repeated hyperpolarizations were inhibited by putative calmodulin antagonists, trifluoperazine (TFP), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and promethazine (PMZ), and the concentrations required for half-maximal inhibition were 25, 30 and 300 μM, respectively. These doses were lower than those for reducing the membrane resistance due to nonspecific cell damages. Another calmodulin antagonist, chlorpromazine (CPZ), was also effective, but CPZ-sulfoxide was not. Intracellular pressure injections of calmodulin-interacting divalent cations, Ca^{2+}, Sr^{2+}, Mn^{2+} and Ni^{2+}, elicited slow hyperpolarizations, whereas Mg^{2+} and Ba^{2+}, which are known to be essentially inert for calmodulin, failed to evoke any responses. The injection of purified calmodulin also brought about a similar hyperpolarization. Quinine, an inhibitor of Ca^{2+}-activated K^+ channels, abolished both Ca^{2+}- and calmodulin-induced hyperpolarizations. TFP prevented Ca^{2+}-induced hyperpolarizations. The TFP effect was partially reversed by the calmodulin injection. It is concluded that calmodulin is involved in the operation of Ca^{2+}-activated K^+ channels in fibroblasts.

Key Words calmodulin · Ca-activated K channel · hyperpolarizing response · potential oscillation · fibroblast

Introduction

Mouse fibroblastic L cells exhibit spontaneous, repeated hyperpolarizations, displaying membrane potential oscillations (Okada et al., 1977), and respond with slow hyperpolarizations to mechanical or electrical stimuli (Nelson, Peacock & Minna, 1972), as well as to chemical stimuli via receptor systems (Okada et al., 1984; Oiki, Ueda & Okada, 1985). These hyperpolarizing responses have been known to be caused by increases in the cytosolic free Ca^{2+} concentration (Oiki et al., 1985; Ueda, Oiki & Okada, 1986), which in turn stimulate Ca^{2+}-activated K^+ channels (Okada, Tsuchiya & Inouye, 1979; Okada, Tsuchiya & Yada, 1982).

It has been known that many Ca^{2+}-dependent intracellular processes are mediated by calmodulin, a ubiquitous calcium-binding protein (Cheung, 1970; Kakiuchi, Yamazaki & Nakajima, 1970). Thus, this protein might also be involved in the operation of Ca^{2+}-activated K^+ channels. In fact, this possibility has been tested, using purified calmodulin. The involvement of calmodulin has thus been suggested in the membranes isolated from erythrocytes (Pape & Kristensen, 1984) and adipocytes (Pershadsingh & McDonald, 1985) as well as in *Paramecium* (Hinrichsen et al., 1986), but not in snail neurons (Levitan & Levitan, 1986). The present study provides direct evidence at the cell level, with the intracellular calmodulin injection, for the involvement of calmodulin in the operation of Ca^{2+}-activated K^+ channels in L cells. This is also supplemented by compatible pharmacological data.

Preliminary accounts of some of these results have been given in abstract form (Okada, Ueda & Yada, 1981) and a review article (Okada et al., 1986).

Materials and Methods

Mouse fibroblastic L cells were cultured in the Fischer medium supplemented with 10% bovine serum without adding any antibiotics. A monolayer of multinucleate giant L cells produced by cell fusion with polyethylene glycol 6000 (Nakarai Chemical Co.) was subjected to electrophysiological studies. The cell volume was estimated to be 10 to 30 pl by their morphology under a light microscope or a scanning electron microscope (Tsuchiya et al., 1981). The technique of intracellular recordings and electronic instruments employed in the present study were the same as those described in a previous paper (Okada et al., 1977). The input (membrane) resistance was monitored by passing a brief constant current (0.3 nA) through the recording microelectrode using a bridge circuit. To stimulate the cell, a large outward current (15 nA) was applied. Intracellular injections (0.05 to 1 pl) of calmodulin or divalent cations were made through micropipettes (tip bore, about 1 μm) filled with 10 mg/ml calmodulin, 0.8

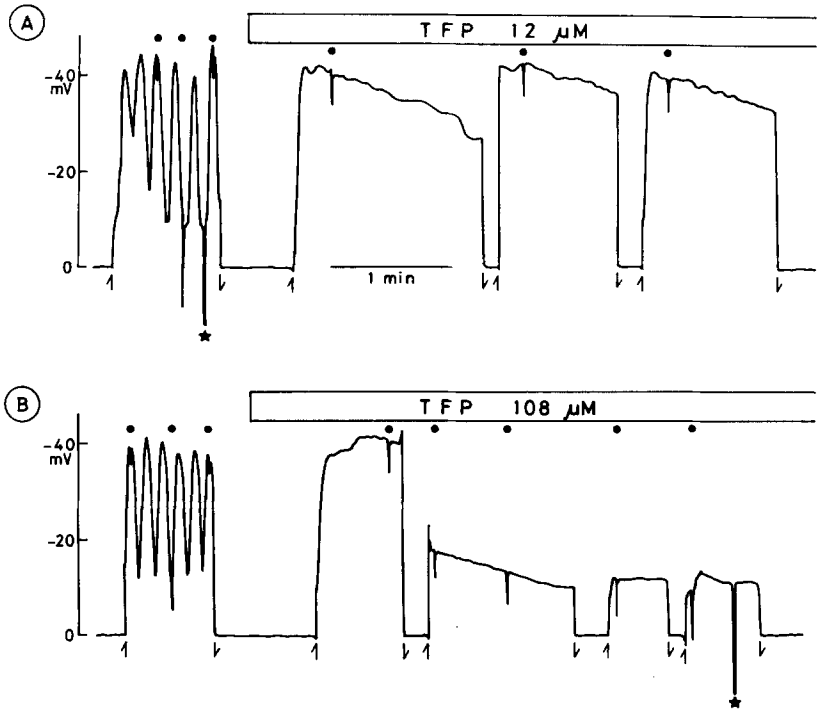

Fig. 1. Effects of TFP on the membrane potential profiles successively recorded from neighboring giant L cells. Upward and downward arrows indicate penetration and withdrawal of the recording electrode, respectively. Dots indicate the applications of small outward currents (0.3 nA) to monitor membrane resistances. To stimulate the cell, large outward currents (15 nA) were applied at the time indicated with asterisks

to 100 mM $CaCl_2$ (plus 0.1 M KCl) or 100 mM chloride salts of other divalent cations by applying a brief pulse (0.5 to 2 sec, using a solenoid-operated three-way valve) of gas pressure (2 to 4 kg/cm). The injected quantity was estimated with an ocular micrometer from the shift of the phase boundary, taking the micropipette taper and the diameter of the fluid column into consideration. Siliconization of the micropipettes with vapor of tri-*n*-butylchlorosilane (Rink & Tsien, 1980) was found to facilitate the pressure injection of the protein (but not electrolytes).

The bathing medium was a Tris-buffered saline (TBS) containing (mM): 143 NaCl, 4.2 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20 mannitol and 10 Tris-HCl (pH 7.2). Purified calmodulin dissolved in a nominally Ca^{2+}-free solution composed of 100 mM NaCl, 0.5 mM $MgSO_4$ and 5 mM Tris-HCl (pH 7.5) was a generous gift from the late Professor S. Kakiuchi and Dr. K. Sobue (Osaka University). They had kindly removed chelating agents from the protein at the final stage of the preparation. In some experiments, calmodulin denatured by leaving the protein at room temperature for six months was used. Trifluoperazine dimaleate (TFP) and promethazine hydrochloride (PMZ) were gifts from Yoshitomi Pharmaceutical Co. Chlorpromazine hydrochloride (CPZ) was purchased from Sigma Chemical Co. and CPZ-sulfoxide hydrochloride was a gift from Smith-Kline & French Laboratories. They were dissolved directly in TBS and the pH was adjusted to 7.2. N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7: a generous gift from Professor H. Hidaka, Mie University) was dissolved in TBS by acidification with 1 N HCl to pH 2.0 and stirring in the dark; the final pH was then adjusted to 7.0 with 1 N NaOH. These calmodulin antagonists were applied to the cells by replacing a control bathing solution (TBS) with TBS containing the drugs. Quinine hydrochloride (Nakarai) was applied by adding an aliquot of the solution (1/100 volume) into the bathing solution.

All data presented in the text are expressed as the mean ± SE (*n*: number of the observations).

Results

Almost all the L cells impaled with a recording microelectrode exhibited spontaneous oscillations in the membrane potential (Fig. 1), as found previously (Okada et al., 1977). The most depolarized level is defined as the resting level, because the membrane potential remained at this level after subsiding the oscillation and the cell at this level can be activated to produce a hyperpolarization in response to an electrical stimulus (15 nA). Both spontaneous and stimulus-induced hyperpolarizations were associated with decreases in the membrane resistance (Fig. 1). A relatively low dose of a calmodulin antagonist, TFP, gave rise to a sustained hyperpolarization. During TFP-induced hyperpolarization, the membrane resistance was consistently lower than the resting membrane resistance (Fig. 1*A*). It is known that spontaneous and stimulus-induced hyperpolarizations result from the operation of Ca^{2+}-activated K^+ channels (Okada et al., 1979; 1982), that the Ca pump is involved in extrusion of elevated cytosolic Ca during oscillatory hyperpolarizations in L cells (Okada et al., 1982) and that the Ca pump is regulated by calmodulin in a variety of cell species (Larsen & Vincenzi, 1979; Pershadsingh, Landt & McDonald, 1980; Pershadsingh, McDaniel et al., 1980; Tuana et al., 1981; White & Raymor, 1982). Therefore, it is likely that the sustained hyperpolarization induced by TFP may be

due to inhibition of the Ca pump, which retards clearance of elevated cytosolic Ca^{2+}, thereby stimulating Ca^{2+}-activated K^+ channels. After the exposure to a relatively high dose of TFP, the cell membrane was initially (≤3 min) hyperpolarized and then depolarized to the resting level without exhibiting potential oscillations (Fig. 1*B*). Electrical stimuli also failed to elicit hyperpolarizing responses during the depolarization phase (Fig. 1*B*, asterisk). This depolarizing effect of TFP can be attributed to the inhibition of Ca^{2+}-activated K^+ channels but not to nonspecific cell damage, because the membrane resistance progressively increasing during the depolarizing phase (Fig. 1*B*) and the potential oscillation as well as the stimulus-induced hyperpolarizing response were restored when the drug was washed out within 15 min (*data not shown*). Similar dose- and time-dependent effects were also observed with another antipsychotic phenothiazine, PMZ, which is a weak inhibitor of calcium-calmodulin (Levin & Weiss, 1976), and with a sulfonamide derivative, W-7 (Hidaka, Naka & Yamaki, 1979), which is structurally unrelated to phenothiazines. Figure 2 shows the dose-dependency of the effects of these calmodulin antagonists (3- to 15-min exposure) on the resting membrane potential (filled circles), input resistance (open triangles) and the peak value of spontaneous hyperpolarization (filled squares). Drug-induced hyperpolarizations and depolarizations were accompanied by decreases and increases in the membrane resistance, respectively, except for very high doses of TFP and W-7 (Fig. 2, triangles). Decreases in the membrane resistance observed at very high doses of TFP and W-7 (but not PMZ up to 0.5 mM) are probably due to nonspecific cell damage. However, the drug-induced suppression of spontaneous hyperpolarizations was observed at doses lower than those for nonspecific effects. The drug concentrations required for the half-maximal inhibition for Ca^{2+}-activated K^+ channels (IC_{50}) can be estimated by the degree of inhibition of the peak hyperpolarization (Fig. 2, squares): the values were approximately 25, 30 and 300 μM for TFP, W-7 and PMZ, respectively. The observed order of potencies of these drugs agreed with that of their potencies in inhibiting Ca^{2+}- and calmodulin-dependent activation of phosphodiesterase and platelet myosin B (Morimoto et al., 1982). A relatively high dose of CPZ also showed qualitatively similar effects on the potential profile (Fig. 3*A*), although its dose-dependency was not examined. In contrast, an exceedingly poor calmodulin antagonist, CPZ-sulfoxide, which shares several membrane perturbing actions of phenothiazines and is equipotent with CPZ in the nonspecific effects (Weiss et al., 1980), did not affect the potential profile even at much higher doses

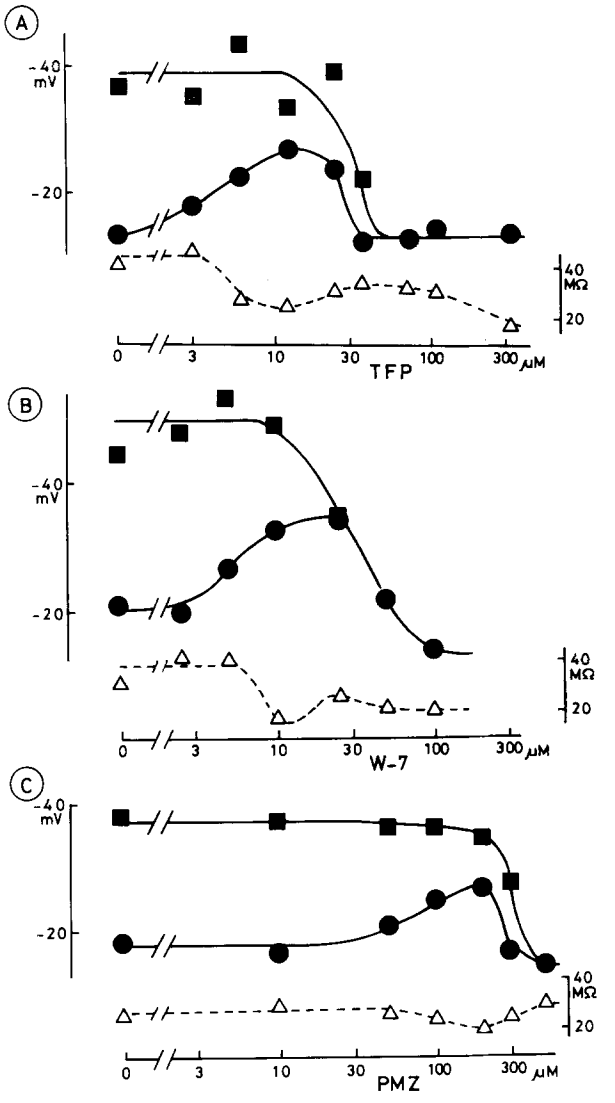

Fig. 2. Dose-dependent effects of calmodulin antagonists, TFP (*A*), W-7 (*B*) and PMZ (*C*), on the resting membrane potential (circles) and resistance (triangles) as well as on the peak hyperpolarization (squares). The drug effects were observed 3 to 15 min after application. Each value represents the mean of 4 to 46 observations. The standard errors were 0.5 to 4.3 mV or less than 7.5 MΩ

(Fig. 3*B*). This suggests that phenothiazines exert their effects by inhibiting the calmodulin rather than by their hydrophobic properties. All the present pharmacological results are consistent with the hypothesis that calmodulin is involved in the operation of Ca^{2+}-activated K^+ channels in L cells.

As observed upon electrophoretic Ca^{2+} injection (Okada et al., 1979; 1982), a slow hyperpolarization was evoked by pressure injection of Ca^{2+} (up to 10 to 100 μM) (Fig. 4*A*, left). The mean amplitude of Ca^{2+}-induced hyperpolarization was 14.8 ± 1.3 (n = 4) mV. During the hyperpolarization, the

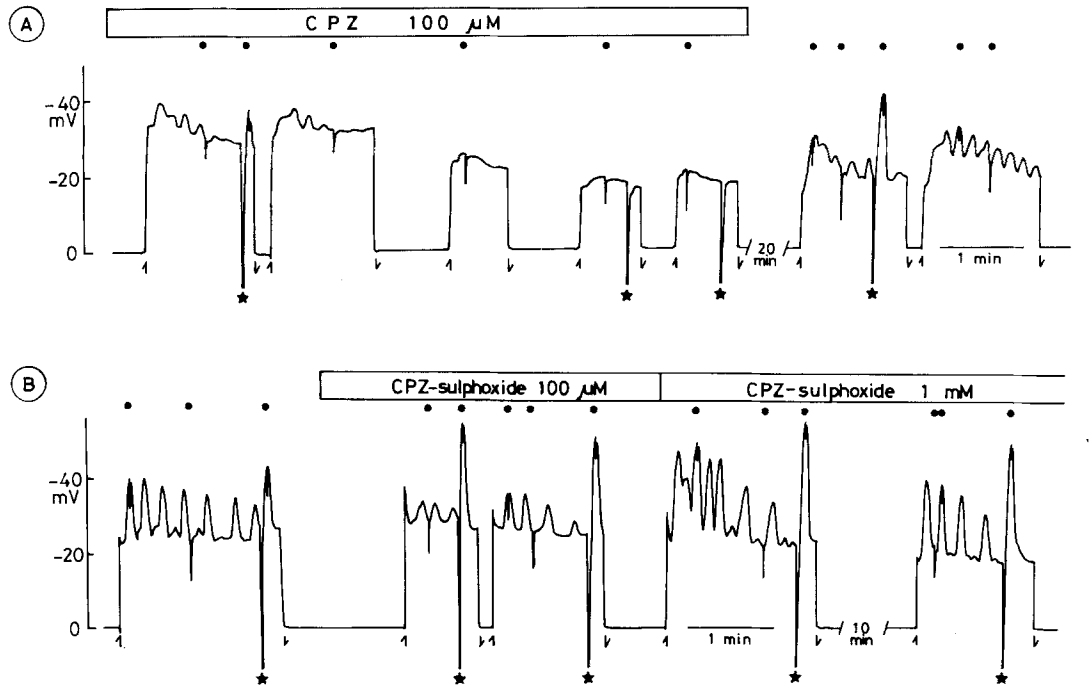

Fig. 3. Effects of CPZ and CPZ-sulfoxide on the membrane potential profiles successively recorded from neighboring giant L cells. Arrows, dots and asterisks are the same as in Fig. 1

membrane resistance was consistently diminished. Single-channel studies have confirmed that quinine is an inhibitor of Ca^{2+}-activated K^+ channels in fibroblasts (Gray et al., 1986), though not in an insulin-secreting cell line (Findlay et al., 1985). Consistently, quinine abolished the hyperpolarization induced by the Ca^{2+} injection into fibroblastic L cells (Fig. 4*B*, left). Similarly, a calmodulin antagonist (TFP 72 μM) prevented the hyperpolarization in response to the pressure Ca^{2+} injection (Fig. 4*C*, left).

Intracellular injections of calmodulin are expected to increase the concentration of calcium-calmodulin complex by the law of mass action. As shown in Fig. 4*A* (right: CaM), the cell also exhibited hyperpolarizations following the pressure injection of purified calmodulin. This response was again associated with a decrease in membrane resistance (Fig. 4*A*, right: dots). The mean amplitude of calmodulin-induced hyperpolarizations was 14.2 ± 1.1 (n = 7) mV. The pressure injection of calmodulin mixed with Ca^{2+} produced a somewhat greater hyperpolarization (17.8 ± 2.1 (n = 5) mV). Injections of denatured calmodulin did not produce any hyperpolarizing responses (*data not shown*). The results may rule out the possibility that calmodulin-induced hyperpolarizations were caused by some free Ca^{2+} ions contaminating in purified calmodulin. The hyperpolarization induced by the calmodulin injection was abolished by quinine (Fig. 4*B*, right). In contrast, TFP reduced but did not abolish the response to the calmodulin injection (Fig. 4*C*, right). Presumably, injected calmodulin overwhelmed the TFP effect, thereby producing a small hyperpolarization (4.3 ± 0.3 (n = 4) mV). In the light of these observations, it can be concluded that calmodulin mediates the operation of Ca^{2+}-activated K^+ channels in L cells.

Fig. 5 (*A*, *D*, *E*) summarizes the effects of different divalent cations. Pressure injections of Sr^{2+}, Mn^{2+} or Ni^{2+} produced a hyperpolarization with a decrease in the membrane resistance. In contrast, pressure injections of Ba^{2+} and Mg^{2+} failed to induce hyperpolarizations and rather inhibited spontaneous potential oscillations and hyperpolarizing responses to electrical stimuli (Fig. 5*F*, *G*). It is known that Sr^{2+}, Mn^{2+} and Ni^{2+} can be substituted for Ca^{2+} in interaction with calmodulin to form the complexes, whereas Mg^{2+} and Ba^{2+} cannot (Lin, Liu & Cheung, 1974; Levin & Weiss, 1977). Thus, these results are also consistent with the hypothesis that calmodulin mediates the activation of Ca^{2+}-dependent K^+ conductance in L cells. In fact, the Sr^{2+}-induced hyperpolarization was inhibited not only by quinine but also by TFP (Fig. 5*B*, *C*).

Discussion

Evidence has accumulated that calmodulin, a ubiquitous calcium binding protein, plays a pivotal role in regulating a variety of calcium-dependent processes in eukaryotic cells. The principal question in the present study was whether the protein is also involved in the activation of Ca^{2+}-dependent K^+ conductance. This possibility has actually been suggested by studies with putative calmodulin antagonists in a number of cell species (Lackington & Orrego, 1981; Okada et al., 1981; Caroni & Carafoli, 1982; Wen, Famulski & Carafoli, 1984; Yingst & Hoffman, 1984). The present pharmacological studies also supported this possibility (Figs. 1–3). While the specificity of these calmodulin antagonists is established under appropriate experimental conditions using soluble proteins (Levin & Weiss, 1979), all the calmodulin antagonists are also known to have nonspecific side effects, including hydrophobic actions, causing perturbation of cell membranes (Seeman, 1972; Landry, Amellal & Ruckstuhl, 1981; Douglas & Nemeth, 1982) and ATP-depletion within the cells (Montecucco et al., 1981; Ruben & Rasmussen, 1981; Corps, Hesketh & Metcalfe, 1982). Under present experimental conditions, several observations argue against the possibility of nonspecific side effects. First, the effects of calmodulin antagonists used were reversible, provided that the agents were washed out within 15 min (e.g. Fig. 3*A*). Second, the membrane resistance increased, while the hyperpolarizing responses were suppressed in the presence of calmodulin antagonists (Figs. 1 & 2). Third, the potencies (Fig. 2) of TFP, W-7 and PMZ in inhibiting repeated hyperpolarizations agreed with the known potencies and their order in inhibiting calmodulin-stimulated activation of phophodiesterase (Weiss et al., 1980; Morimoto et al., 1982). Fourth, while CPZ also inhibited the hyperpolarizing response, CPZ-sulfoxide failed to inhibit even at much higher doses (Fig. 3). Therefore, it appears that the inhibitory effect of calmodulin antagonists on repeated hyperpolarizations is not due to nonspecific effects but to their interaction with calmodulin. Calmodulin antagonists have been reported to inhibit Ca^{2+} influx under some conditions (Fleckman et al., 1981; Valverde et al., 1981; Eilam, 1983; Sasakawa et al., 1983). Thus, one may argue that the effect of the drugs might be exerted by their inhibitory effect on Ca^{2+} channels, rather than on Ca^{2+}-activated K^+ channels. However, this possibility is not compatible with the fact that TFP inhibited hyperpolarization induced by the Ca^{2+} injection as well (Fig. 4*C*, left). Therefore, these pharmacological data strongly suggest that calmodulin mediates the operation of Ca^{2+}-activated K^+ channels in L cells.

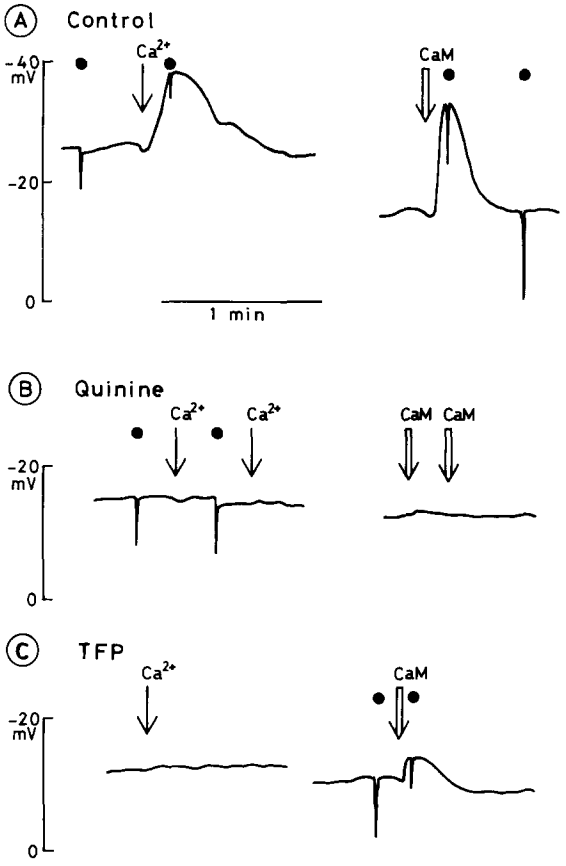

Fig. 4. Effects of pressure injections of Ca^{2+} ions (up to 10 to 100 μM) and purified calmodulin (3 to 50 μM: CaM) on the membrane potential recorded at the resting state. L cells with a relatively low frequency of potential oscillations (≤2 cycles/min) were selected for this series of experiments. 0.5 mM quinine (*B*) and 72 μM TFP (*C*) were added 5 to 20 min before Ca^{2+} or calmodulin injections. Dots are the same as in Fig. 1

The specificity for divalent cations of Ca^{2+}-activated K^+ channels in L cells also supports the above hypothesis; calmodulin-interacting cations (Sr^{2+}, Mn^{2+} and Ni^{2+}) could substitute for Ca^{2+} in the activation of the channels, but Mg^{2+} and Ba^{2+}, which cannot form TFP-sensitive complexes with calmodulin (Levin & Weiss, 1977), did not (Fig. 5). This bears similarity to previous observations that Ca^{2+}-activated K^+ channels are activated by Ca^{2+} or Sr^{2+} but not by Mg^{2+} or Ba^{2+} in erythrocyte membranes (Simons, 1976; Porzig, 1977), in *Aplysia* neurons (Gorman & Hermann, 1979), and in heart sarcolemmal membranes (Wen et al., 1984). Contrary to the present results obtained in L cells, Ni^{2+} was found to be ineffective in the molluscan neurons (Gorman & Hermann, 1979) and in the beef

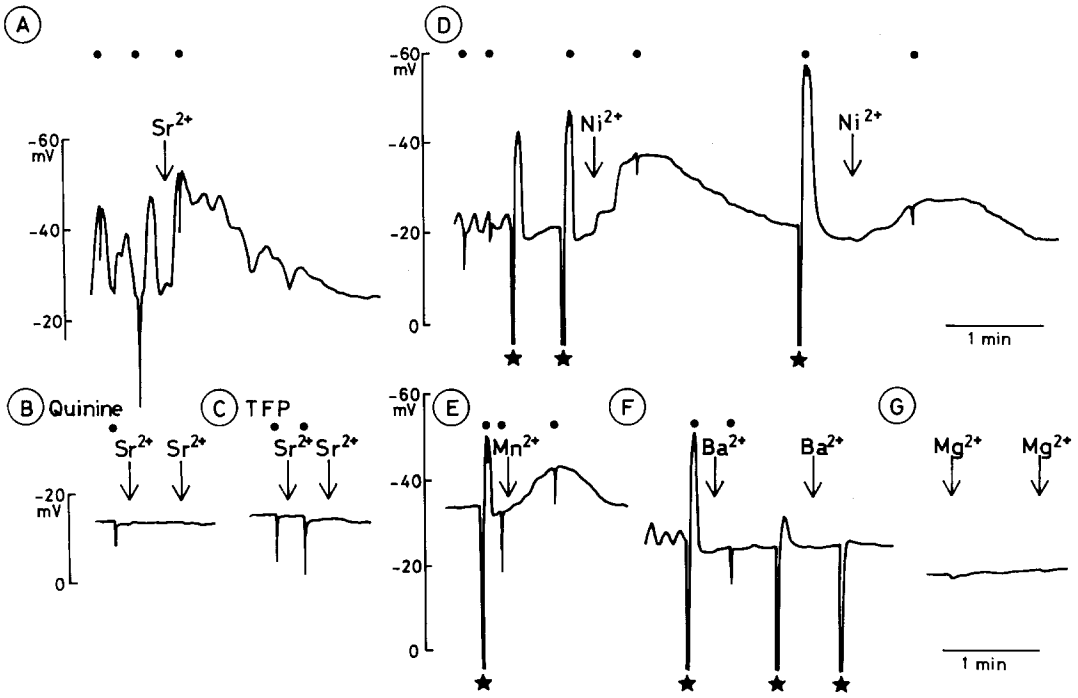

Fig. 5. Effects of pressure injections of Sr^{2+}, Ni^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+} (up to 30 to 100 μM) on the membrane potential profiles. 0.5 mM quinine (*B*) and 100 μM TFP (*C*) were added 4 and 15 min before Sr^{2+} injections, respectively. Dots and asterisks are the same as in Fig. 1

sarcolemmas (Wen et al., 1984). This discrepancy is presumably due to the species differences in the selectivity of Ca^{2+}-activated K^+ channels for cations bound to calmodulin but not necessarily to that in the cation selectivity of calmodulin.

The experiments with the intracellular injection of purified calmodulin provide most direct verification for the hypothesis. Intracellular calmodulin injection evoked quinine-sensitive hyperpolarizations and partially reversed the TFP-induced suppression of the Ca^{2+}-activated K^+ channels (Fig. 4, right). These results are in good accordance with recent observations on the effects of purified calmodulin in several cell species (Pape & Kristensen, 1984; Pershadsingh & McDonald, 1985; Hinrichsen et al., 1986). Taken together, it seems reasonable to conclude that calmodulin mediates the operation of Ca^{2+}-activated K^+ channels in the fibroblasts.

The Ca^{2+}-activated K^+ transport in red cell membrane has been reported to depend on certain cytoplasmic proteins because the dialyzed cytoplasmic protein extract facilitated Ca^{2+}-dependent Rb^+ fluxes (Sarkadi, Szebene & Gardos, 1980). However, recent single-channel recordings with excised membrane patches have demonstrated that such a cytosolic factor is not always necessary for the opening of single Ca^{2+}-activated K^+ channels (Marty, 1981; Pallotta, Magleby & Barrett, 1981; Maruyama, Gallacher & Petersen, 1983). In this light, it seems likely that a sufficient amount of calmodulin to mediate the operation of the K^+ channels is rather tightly bound to the membranes under normal conditions.

Cyclic AMP-dependent protein kinase is also known to mediate the regulation of Ca^{2+}-activated K^+ channels (de Peyer et al., 1982; Wen et al., 1984; Ewald, Williams & Levitan, 1985). The precise mechanism and significance of dual regulations of Ca^{2+}-activated K^+ channels by cyclic AMP-dependent protein kinase and calcium-calmodulin remain undetermined.

It is likely that higher concentrations of the calcium-calmodulin complex are needed for the activation of Ca pump than those for Ca^{2+}-activated K^+ channels, since calmodulin antagonists seem to inhibit Ca pump, causing sustained hyperpolarizations at lower concentrations (Fig. 2). This possibility is, in fact, in good agreement with the report that the activation of Ca pump requires higher Ca concentrations than the activation of Ca^{2+}-dependent Rb^+ permeability in human red cell membranes (Pape & Kristensen, 1984). The transient nature of the hyperpolarizing response observed in L cells might be accounted for by such a difference in the

threshold concentration of Ca-calmodulin between the K^+ channel and the Ca pump.

The authors are grateful to the late Professor Shiro Kakiuchi and Dr. Kenji Sobue, Osaka University, for a generous gift of purified calmodulin and for many helpful discussions. We thank Professor Hiroyoshi Hidaka, Mie University, for generously supplying a sample of W-7. We are indebted to Professor Motoy Kuno, Kyoto University, for reading the manuscript. This work was supported by Grants-in-Aid for Scientific Research and for Special Project Research on Mechanism of Bioelectrical Response from the Ministry of Education, Science and Culture of Japan.

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Received 15 August 1986; revised 17 December 1986