Role of inward K^+ channel located at carrot plasma membrane in signal cross-talking of cAMP with Ca^{2+} cascade

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Abstract Treatment of cultured carrot cells with dibutyryl cAMP or forskolin resulted in the appreciable decrease in extracellular K^+ concentration. This decrease was found to be transient and the concentration of the ion in the culture medium restored to the original level within few minutes. The cAMP-induced decrease in K^+ level in the medium was almost completely inhibited when carrot cells were incubated in the presence of K^+ channel blockers, CsCl and tetraethylammonium chloride. Appreciable amounts of $^{45}Ca^{2+}$ were discharged from $^{45}Ca^{2+}$-loaded inside-out vesicles of carrot plasma membrane by the stimulation with cAMP, however, the release of the ion was significantly inhibited in the presence of the K^+ channel blockers. The release of $^{45}Ca^{2+}$ from the vesicles was also observed when K^+ current was evoked with an ionophore, valinomycin, even in the absence of cAMP. These results suggest that the gating of some of the inward K^+ channels located at plasma membrane of cultured carrot cells is controlled by cytoplasmic concentration of cAMP and the inward K^+ current across the plasma membrane induced by the nucleotide elicits Ca^{2+} influx into the cells possibly by the activation of voltage-dependent Ca^{2+} channels.

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Key words: cAMP-sensitive channel; Inward K^+ channel; Ca^{2+} influx; Signal transduction; *Daucus carota*

1. Introduction

Signal transduction mechanisms of higher plant cells are very poorly understood as compared with those of animals and microorganisms, however, it has been demonstrated that Ca^{2+} influx into plant cells is an important event to trigger a variety of cellular responses against external stimuli [1,2]. In contrast, the physiological function of cAMP in higher plant cells has been obscure for the several decades [3,4]. Recently, we have demonstrated [5,6] that increase in cytoplasmic cAMP level of cultured carrot cells elicits Ca^{2+} influx across the plasma membrane without accompanying cAMP-dependent protein phosphorylation. We have proposed, therefore, that one of the transmembrane-signaling mechanisms in higher plants mediated by cAMP is an unique signal-cross-talking of the nucleotide with Ca^{2+} cascade [5–9]. When cAMP level in plant cells is increased in response to external stimuli, Ca^{2+} channels located at the plasma membrane are activated directly or indirectly without protein phosphorylation. Opening of the channel gates allows the increase in cytoplasmic Ca^{2+} concentration to the levels sufficient to activate several Ca^{2+} and/or Ca^{2+}/calmodulin-dependent processes. A similar mechanism of signal transduction was reported in odor-sensitive animal cells [10,11] in which cAMP-sensitive cation channels play a central role. Recently, a family of new genes encoding inward K^+ channels has been isolated from *Arabidopsis thaliana* and it has been shown that two distinct modules, a cAMP-binding domain and an ankyrin repeat motif, comprise the C-terminal half of their structures [12–15]. It appears that these findings should support our hypothesis that cAMP plays its role as a second messenger by activating cAMP-sensitive ion channels as an initial event of a series of signal transduction reactions in higher plant cells. In the present experiments, I attempted to elucidate the possible contribution of inward K^+ channels regulated by cAMP in signal cross-talking of the cyclic nucleotide with Ca^{2+} cascade in cultured carrot cells. For this purpose, I examined whether or not the increase in cAMP level in carrot cells is capable of inducing K^+ influx by the activation of a certain K^+ channel in vivo and the links between cAMP-induced inward K^+ current and Ca^{2+} influx in the cells were also studied.

2. Materials and methods

2.1. Chemicals

Forskolin, ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), valinomycin, tetraethylammonium chloride (TEA) and CsCl were purchased from Wako Pure Chemicals. ATP and cAMP were obtained from Boehringer-Mannheim while dibutyryl cAMP (Bt_2cAMP) was from Sigma. $^{45}CaCl_2$ (sp. act. 111 GBq/mmol) was obtained from New England Nuclear. All other chemicals were reagent grade.

2.2. K^+ influx into cultured carrot cells evoked by cAMP

Cultured carrot cells were grown in 70 ml of Murashige and Skoog's liquid medium [16] on an Innova 2300 rotary shaker (New Brunswick Scientific, 200 rpm) at 26°C in the presence of 4.5 μM 2,4-dichlorophenoxyacetic acid as described previously in detail [17]. Influx of K^+ into the cells was determined by measuring the loss of the ion from the medium with a K^+-selective ion electrode (Horiba, 8202-10C with a reference electrode #2535A) essentially according to the method of Sentenac et al. [15]. Cultured carrot cells in the early stationary phase (10 days old) were harvested by filtration and $\approx 2\times10^7$ cells were washed with and re-suspended in 150 ml of a modified medium of which K^+ concentration was adjusted to 3 mM. All of the K^+ salts of Murashige and Skoog's inorganic components [16] were replaced by Na^+ salts and KCl was added to the medium at the final concentration of 3 mM. The cell suspension was vigorously stirred in the modified medium for 20 min prior to the treatment with cAMP. To the suspension was added 1-ml aliquot of Bt_2cAMP (final concentration 100 μM) or forskolin (10 μM) dissolved in the modified medium and 20-ml portions of the culture were harvested at regular intervals. The cells and medium were quickly separated by vacuum filtration and K^+ concentration in the medium was determined by the ion electrode [15].

2.3. Preparation of sealed plasma membrane vesicles of cultured carrot cells

Sealed vesicles of plasma membrane of cultured carrot cells were prepared by aqueous two-phase partitioning method as described pre-

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viously in detail [6] with several modifications. In brief, microsome fraction of 10-day-old cultured carrot cells was subjected to partitioning with 10 mM Hepes-NaOH buffer (pH 7.2) containing 250 mM sucrose, 27 mM NaCl, 3 mM KCl, 5.6% (w/w) polyethylene glycol 3350 (Sigma) and 5.6% (w/w) Dextran T-500 (Pharmacia). If necessary, 27 mM NaCl+3 mM KCl in the solvent system were replaced by 30 mM NaCl, 29 mM NaCl+1 mM KCl or 28 mM NaCl+2 mM KCl in order to adjust the K^+ concentration inside the vesicles to 0, 1 or 2 mM, respectively. The resultant upper phase was removed and mixed again with freshly prepared lower phase and plasma membranes in the upper phase were recovered by centrifugation (156 000 × *g*, 30 min). Purity of the membrane was assessed by measuring several marker enzymes and the results had been reported previously [6]. The vesicles, into which various concentrations of CsCl or TEA were sealed, were prepared by adding these K^+ channel blockers directly in the two-phase solvent system prior to mixing with the microsome fractions. The plasma membrane vesicles, thus, obtained were suspended in 10 ml of 25 mM Hepes-NaOH buffer (pH 7.2) containing 250 mM sucrose, 2 mM $MgCl_2$ and 2 mM ATP and the suspensions were incubated with 2 μM $^{45}CaCl_2$ (37 kBq) at 37°C for 1 h to allow the incorporation of $^{45}Ca^{2+}$ by the action of plasma membrane-located Ca^{2+}-ATPase. Only the vesicles of inside-out orientation are able to incorporate $^{45}Ca^{2+}$ [6]. The transport reaction was terminated by the addition of EGTA at the final concentration of 2 mM and the vesicles were recovered by centrifugation (156 000 × *g*, 30 min). They were successively washed with the Hepes-sucrose buffer containing 2 mM EGTA and with the buffer without EGTA to remove the chelating reagent and served as the $^{45}Ca^{2+}$-loaded vesicles to determine Ca^{2+} channel activity. Total amount of the loaded $^{45}Ca^{2+}$ was determined after the disruption of the vesicles by mixing 200-μl aliquot of the suspensions with the same volume of 1% (w/v) sodium dodecyl sulfate [6].

2.4. Discharge of $^{45}Ca^{2+}$ from the inside-out vesicles of plasma membrane

Activity of Ca^{2+} influx across the plasma membrane of cultured carrot cells in response to K^+ current was determined by measuring the discharge of $^{45}Ca^{2+}$ from the $^{45}Ca^{2+}$-loaded vesicles of the membrane according to the methods described previously [6,8] with some modifications. The vesicles were suspended in 450 μl of 25 mM Hepes-sucrose buffer containing 0, 1, 2, 3 or 80 mM KCl (50 μg proteins/assay as determined by the method of Bradford [18]) and 5-μl aliquot of the stock solution of cAMP or valinomycin (final concentration of 1 μM for cAMP and 10 μM for valinomycin, respectively) was added to the suspensions. They were incubated at 26°C and, at regular intervals, the mixtures were filtered through nitrocellulose membrane filters (0.2 μm, Advantec) with 2 ml of the respective assay buffers as the carrier solvents. The filters were washed with another 2 ml of the buffers and the combined filtrates containing the discharged $^{45}Ca^{2+}$ were mixed with a commercial scintillation cocktail (Amersham, ACS II) to determine their radioactivities. To test the effect of K^+ channel blockers on the cAMP-induced discharge of $^{45}Ca^{2+}$ from the vesicles, K^+ concentrations inside and outside the vesicles were adjusted to 3 and 80 mM, respectively, and the incubation time was fixed at 3 min.

3. Results

3.1. K^+ influx into cultured carrot cells induced by cAMP

We have demonstrated [5,6] that cytoplasmic level of cAMP in cultured carrot was appreciably elevated by the treatment of the cells with Bt_2cAMP or forskolin. Possible K^+ influx into the cells in response to these reagents was examined by measuring the loss of the ion from the medium of which K^+ concentration was adjusted to 3 mM, a presumable concentration of the ion in cell exterior of higher plants in the native environment [19,20]. Treatment of carrot cells with 100 μM Bt_2cAMP resulted in the appreciable decrease in extracellular K^+ concentration without a notable lag and, after 20–40 s of the addition of the reagent, it reached to the level of 1.8–1.9 mM (Fig. 1). This decrease was, however, transient and the concentration of the ion restored to the original level within 1 min. In repeated experiments, the transient decrease in extracellular K^+ concentration to 1.5–1.9 mM was reproducibly observed. The decrease in K^+ concentration of carrot cell exterior induced by Bt_2cAMP was almost completely inhibited when the cells were incubated in the presence of 30 mM CsCl or TEA, well-known K^+ channel blockers. Similar experiments were carried out employing forskolin, an activator of adenylyl cyclase [6,7], to confirm the occurrence of K^+ influx into cultured carrot cells with the physiological concentrations of cAMP. We have shown [5,6] that cytoplasmic level of cAMP in carrot cells in the resting state is maintained below 1 nM, however, it elevates to 10–50 nM by the treatment with 1–10 μM forskolin. As shown in Fig. 1, extracellular K^+ concentration of forskolin-treated carrot cells decreased from 3 to ≈1.5 mM, almost the comparable level that observed for Bt_2cAMP. However, unlike in the case of Bt_2cAMP, notable lag (≈2 min) was reproducibly observed prior to the initiation of the decrease in the extracellular K^+ (Fig. 1). It was assumed that a time lag is necessary before the cAMP content reaches to a sufficient concentration [6]. Loss of the extracellular K^+ induced by forskolin-treatment was essentially negligible when the cells were incubated with the K^+ channel blockers as well as in Bt_2cAMP. These observations suggest that the increased level of cAMP in cultured carrot cells induces K^+ influx across the plasma membrane and it is very likely that this process is mediated by a certain inward K^+ channel(s).

3.2. Inhibition of cAMP-induced Ca^{2+} flux by K^+ channel blockers

In the next experiments, the possibility was examined that the cAMP-induced K^+ influx is capable of stimulating the entry of Ca^{2+} into cultured carrot cells. For this purpose, $^{45}Ca^{2+}$-loaded inside-out vesicles of carrot plasma membrane were treated with cAMP and the effect of the K^+ channel blockers on the discharge of $^{45}Ca^{2+}$ was tested. Since cytoplasmic K^+ level of cultured carrot cells was estimated to be ≈80 mM as determined by the K^+ electrode, K^+ concentrations inside and outside the vesicles were adjusted to 3 and 80 mM, respectively. ≈25–30% of $^{45}Ca^{2+}$ in the sealed vesicles were discharged upon the treatment with 1 μM cAMP, the saturated concentration of the nucleotide [6], under the present experimental conditions. As shown in Fig. 2, the cAMP-induced discharge of $^{45}Ca^{2+}$ was appreciably inhibited in the presence of CsCl in a dose-dependent manner when the blocker was sealed inside the vesicles. The release of the ion from the vesicles was inhibited to ≈44% level of the control with 30 mM CsCl. In contrast, it appeared that this blocker did not show the inhibitory activity when it was mixed in the buffer outside the vesicles, as far as tested. TEA, another K^+ channel blocker, sealed in the vesicles also exhibited marked inhibitory effects on the discharge of $^{45}Ca^{2+}$ in a dose-dependent manner and, with 30 mM TEA, the release of the ion was inhibited to ≈47% level. However, unlike in CsCl, TEA inhibited the release of the ion when it was included not only in interior but also in exterior space of the vesicles. It is well-known [21,22] that TEA is able to inhibit K^+ channel mediated ion transport at both sides of plasma membranes and, therefore, the present finding should be consistent with the unique characteristic of this K^+ channel blocker. These observations, therefore, suggest that the apparent inhibitory activities of these K^+ channel blockers toward cAMP-induced

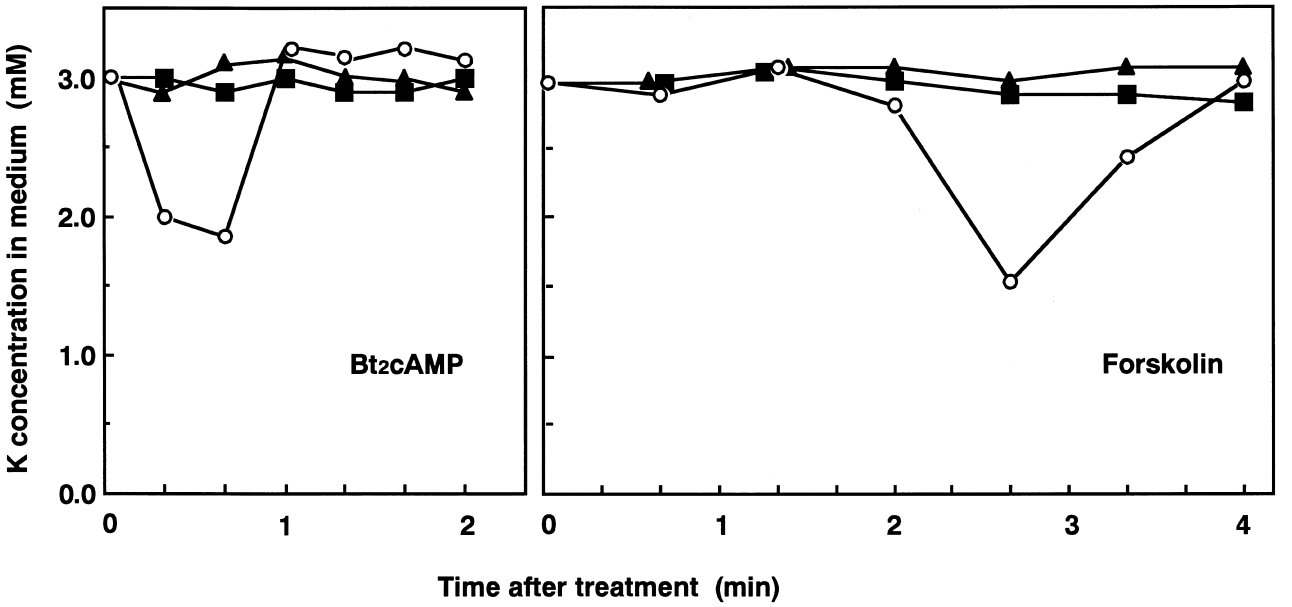

Fig. 1. Change in extracellular K^+ concentration of cultured carrot cells after the addition of Bt_2cAMP or forskolin. Cultured carrot cells were transferred into a modified medium of which K^+ concentration was adjusted to 3 mM and, after the addition of Bt_2cAMP or forskolin, change in K^+ concentration in the medium was determined with a K^+-selective electrode (○). In the parallel experiments, the cell suspension was incubated in the presence of 30 mM of K^+ channel blockers, CsCl (▲) or TEA (■). A set of the experiments was repeated 5× and the typical results are presented.

discharge of $^{45}Ca^{2+}$ are the specific effect as K^+ inhibitors but not result from a certain mechanical damage of the vesicles caused by these compounds.

We have previously reported [6] that cytoplasmic Ca^{2+} level of cultured carrot cells transiently increases by the treatment with Bt_2cAMP or forskolin as monitored by a fluorescent Ca^{2+} indicator. It appears that the decrease in extracellular K^+ concentration induced by these reagents (Fig. 1) takes place concomitant with the increase in Ca^{2+} level of the cells [6]. Together with this previous finding, the results obtained in

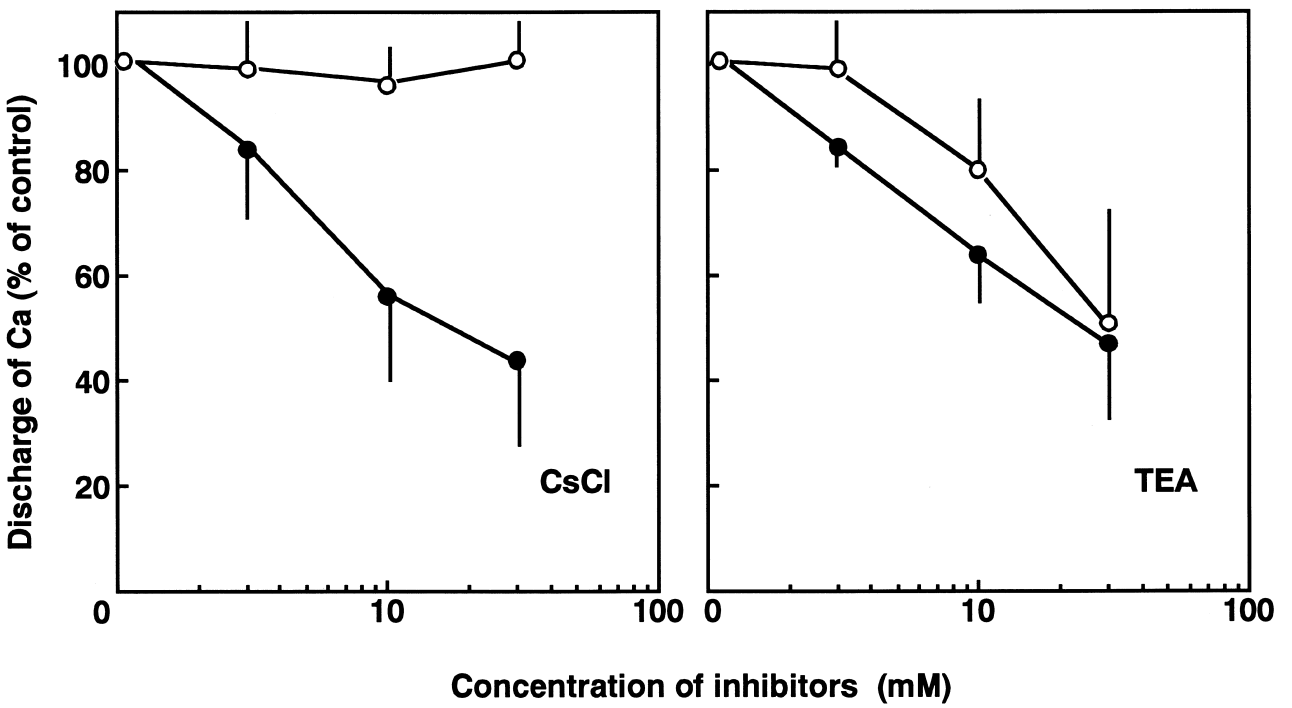

Fig. 2. Inhibition of cAMP-induced discharge of $^{45}Ca^{2+}$ from plasma membrane vesicles of carrot cells by K^+ channel blockers. Various concentrations of CsCl or TEA were included in the buffer inside (●) or outside (○) the $^{45}Ca^{2+}$-loaded vesicles of plasma membrane and 1 μM of cAMP was added to induce the discharge of the ion. Results were expressed as percentages to the control treatments as 100% in which the K^+ channel blockers were added neither inside nor outside the vesicles. Mean and S.D. values obtained from 4 independent experiments are shown.

the present study strongly suggest that cAMP-induced Ca^{2+} influx into cultured carrot cells is, at least partly, mediated by the activation of cAMP-sensitive inward K^+ channels.

3.3. Discharge of $^{45}Ca^{2+}$ from plasma membrane vesicles evoked by valinomycin or cAMP

In order to elucidate the relationship between the cAMP-induced influx of K^+ and Ca^{2+} into cultured carrot cells in detail, Ca^{2+} flux occurred in response to the K^+ current across the plasma membrane was examined under various conditions. K^+ concentration inside the membrane vesicles was fixed to 3 mM while outside the vesicles was varied in the range of 0–3 mM. Then, K^+ current across the membrane was elicited by the treatment with a K^+ ionophore valinomycin and the discharge of $^{45}Ca^{2+}$ from the vesicles was determined with time. As shown in Fig. 3, appreciable amounts of $^{45}Ca^{2+}$ were released when the K^+ concentration outside the vesicles was adjusted to 0, 1 or 2 mM. The discharge of the ion was initiated without a lag and was apparently terminated within 1 min. ≈25–29% of $^{45}Ca^{2+}$ in the vesicles were released under the present experimental condition and it appeared that time course of the discharge was essentially the same for these three K^+ concentrations. In contrast, the discharge of $^{45}Ca^{2+}$ was very low when K^+ concentrations outside and inside the vesicles were identical and controls which were not treated with valinomycin released the negligible amounts of $^{45}Ca^{2+}$. Therefore, Ca^{2+} influx into carrot cells appeared to initiate only if inward K^+ current across the plasma membrane takes place, suggesting that the entry of Ca^{2+} is triggered by sensing the alternation of K^+ distribution of both sides of plasma membrane.

In the next experiments, K^+ concentration outside the vesicles was fixed at 80 mM, the cytoplasmic concentration

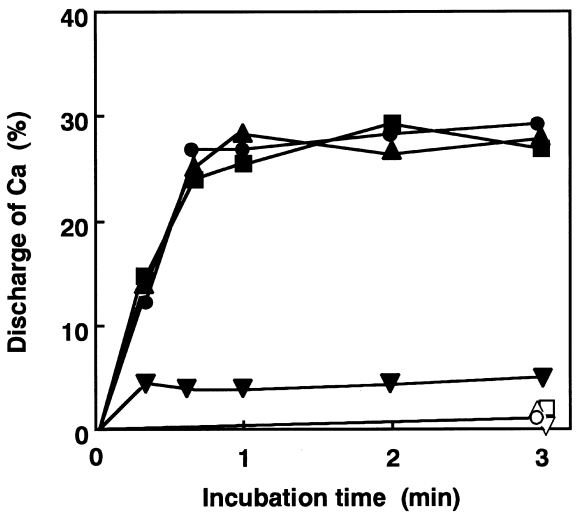

Fig. 3. Discharge of $^{45}Ca^{2+}$ from inside-out vesicles of carrot plasma membrane by the treatment with valinomycin. K^+ concentrations outside the $^{45}Ca^{2+}$-loaded vesicles were adjusted to 0 (●), 1 (▲), 2 (■) or 3 (▼) mM, while the ions inside the vesicles was 3 mM. The vesicles were treated with valinomycin (10 μ*M*) and, at regular intervals, the radioactivities of discharged $^{45}Ca^{2+}$ were determined. Control treatments which did not receive the ionophore are shown as open symbols, respectively, and the results are expressed as percentages to total $^{45}Ca^{2+}$ in the vesicles as 100%. A set of the experiments was repeated 3× and the mean values are presented. Bars for S.D. values (usually, within the range of 20% of the mean values) are not presented to avoid the confusion.

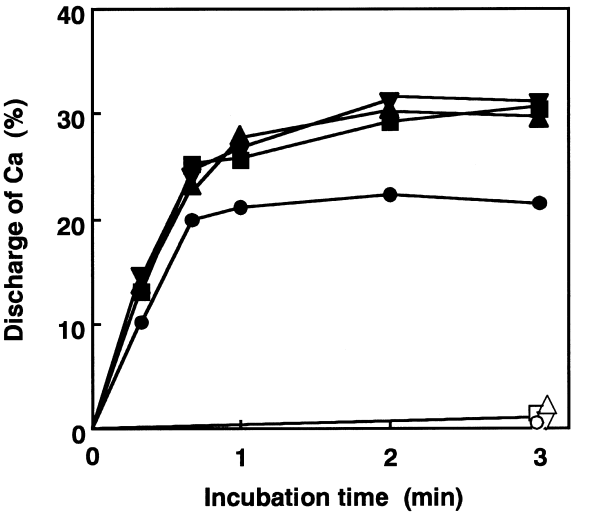

Fig. 4. Discharge of $^{45}Ca^{2+}$ from inside-out vesicles of plasma membrane by the treatment with cAMP. K^+ concentration inside the vesicles were adjusted to 0 (●), 1 (▲), 2 (■) or 3 (▼) mM, while the ion concentration outside the vesicles was 80 mM. The vesicles were treated with cAMP (1 μ*M*) and, at regular intervals, the radioactivities of discharged $^{45}Ca^{2+}$ were determined. Control treatments which did not receive cAMP are shown as open symbols, respectively, and the results are expressed as percentages to total $^{45}Ca^{2+}$ in the vesicles as 100%. A set of the experiments was repeated 3× and the mean values are presented. Bars for S.D. values are not presented to avoid the confusion.

of the ion in carrot cells and the discharge of $^{45}Ca^{2+}$ by the stimulation of cAMP was examined for various K^+ concentrations inside the vesicles (Fig. 4). The appreciable discharge of $^{45}Ca^{2+}$ was observed when K^+ concentration inside the vesicles was adjusted to 1, 2 or 3 mM. Ca^{2+} flux across the plasma membrane appeared to be terminated within 1–2 min and time course of the released ion was almost comparable for 1–3 mM of K^+ concentrations (28–32% of $^{45}Ca^{2+}$ were discharged). An appreciable discharge of $^{45}Ca^{2+}$ was also observed even when K^+ was not included inside the vesicles. However, in this case, the amount of the discharged ion was significantly lower than those observed for 1–3 mM K^+ and was 64–78% of the 'full response' in repeated experiments. These results suggest the possibility that the inward K^+ current is not solely responsible for cAMP-induced Ca^{2+} influx of cultured carrot cells. As described above, although cAMP-induced K^+ influx into cultured carrot cells was almost completely blocked in the presence of 30 mM CsCl or TEA (Fig. 1), the nucleotide-induced discharge of $^{45}Ca^{2+}$ from the membrane vesicles was only partially inhibited with the same concentration of these K^+ channel blockers (Fig. 2). In addition, I confirmed that an appreciable discharge of $^{45}Ca^{2+}$ upon the stimulation with cAMP was observed even if K^+ salts were omitted from the buffers of both sides of the membrane and were replaced by the same concentrations of Na^+ salts (data not shown). These observations clearly indicate that multiple mechanisms contribute to the cAMP-induced Ca^{2+} entry into cultured carrot cells and activation of cAMP-sensitive inward K^+ channels is not an only initial event for Ca^{2+} influx elicited by the nucleotide.

4. Discussion

In the present experiments, it has been shown that elevation

of cytoplasmic concentration of cAMP in cultured carrot cells in response to the appropriate external stimuli results in the activation of inward K^+ channels or the related structures at the plasma membrane, possibly the members of inward K^+ channel family containing the cAMP-binding domain in their structures [11–15]. It has been also demonstrated that this inward K^+ current across the membrane evokes Ca^{2+} influx into carrot cells. One of the most likely mechanisms of the relationship between these two ion currents would be as follows; cAMP-induced influx of K^+ results in the depolarization of the plasma membrane of cultured carrot and this event activates voltage-dependent Ca^{2+} channels [23] at the membrane to allow the Ca^{2+} entry into the cells. It has been assumed [12,15,24] that physiological roles of inward K^+ channels at plasma membrane of higher plant cells would be a rectifier of ions in inner and outer spaces of the membrane and a structure for uptake of the ions as a nutrient. However, the results obtained in the present study strongly suggest that some of the inward K^+ channels located at plant plasma membrane are regulated by cytoplasmic concentration of cAMP and play an important role in the signal transduction processes of higher plant cells through the unique signal cross-talking mechanism.

In the present study, it has been also indicated that the activation of cAMP-sensitive inward K^+ channel is not solely responsible for the nucleotide-induced influx of Ca^{2+} into cultured carrot cells. It is well-known that inward K^+ channels at plasma membrane are organized as tetramers and the assembly of the four identical subunits forms a single pore for the permeation of K^+ [25]. In contrast, many of other cation channels are composed of the single polypeptides in which a set of domains corresponding to the subunit of the inward K^+ channels is repeated $4\times$ to form the channel structures [25,26]. Therefore, it is reasonable to assume that cAMP-sensitive ion channels permeating the ions other than K^+ occur and function in plant plasma membrane. The activation of these unidentified channels in response to the elevation of cytoplasmic cAMP would induce depolarization or hyperpolarization of the membrane and this should be followed by gating of the voltage-dependent Ca^{2+} channels. It is also possible that, as is in animal cells, elevated levels of cAMP directly open the cyclic nucleotide-sensitive Ca^{2+} channels [10,11]. Further study is in progress in my laboratory to elucidate the overall mechanisms of cAMP-induced Ca^{2+} influx into higher plant cells mediated by the nucleotide-sensitive ion channels.

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