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CA^{2+}-DEPENDENT K^+ PERMEABILITY OF HEART SARCOLEMMAL VESICLES. MODULATION BY cAMP-DEPENDENT PROTEIN KINASE ACTIVITY AND BY CALMODULIN

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The Ca^{2+}-dependent K^+ permeability of heart sarcolemma vesicles was measured by following the transmembrane movement of the charge compensating tetraphenylborate anion. The increase in vesicles permeability induced by Ca^{2+} is lost when membrane proteins are dephosphorylated by an endogenous protein phosphatase and is restored by a phosphorylation process catalysed by a cAMP-dependent protein kinase. The calmodulin antagonist R 24571 lowers the Ca^{2+}-dependent K^+ permeability by decreasing the Ca^{2+} affinity of the K^+ transporting system.

It has been suggested that cAMP may regulate neuronal electric activity via phosphorylation of membrane proteins (1). Microinjection of a protein kinase inhibitor into Aplysia neurons has established the link between protein phosphorylation and the regulation of the K^+ conductance by serotonin (2). Voltage clamp experiments in which snail neurons were internally perfused (3) with the catalytic subunit of a cAMP-dependent protein kinase have demonstrated that protein phosphorylation potentiates the Ca^{2+}-dependent K^+ current (4). Ca^{2+}-activated outwardly directed K^+ currents have been also described in Purkinje fibers (5) and in ventricular muscle fibers (6), and Ca^{2+}-dependent K^+ fluxes have been demonstrated in heart sarcolemmal vesicles (7). Since sarcolemmal vesicles can be used for protein phosphorylation experiments (8) we have used them to study the effect of cAMP-dependent protein phosphorylation on the Ca^{2+}-dependent K^+ flux (channel). The data obtained have shown that the channel is indeed activated by protein phosphorylation and de-activated by protein dephosphorylation. The results presented show that calmodulin is involved in the regulation of the Ca^{2+} sensitivity of the Ca^{2+}-dependent K^+ channel.

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Abbreviations: TPB^-, tetraphenylborate, CaM, calmodulin, EDTA, ethylene diamine N',N',N',N' tetraacetic acid, EGTA ethyleneglycol-bis-(-amino-ethyl ether) N,N' tetraacetic acid, HEPES, N-2 hydroxyethylpiperazine-N'-2 ethanesulfonic acid, SDS, sodium dodecylsulfate.

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MATERIALS & METHODS

Preparation of the vesicles

Beef heart sarcolemma was prepared essentially as described by Caroni et al. (9). The following steps were modified. The first isotonic homogenization was repeated 5 times using a Polytron PT 35 homogenizer set at 5.2 for 5 sec. The second hypotonic homogenization was repeated 8 times with the same homogenizer set at 5.2 for 30 sec. Potassium salts instead of sodium salts were used in all media. The final preparation was suspended in 160 mM choline-Cl, 20 mM HEPES-Tris pH 7.4 and 0.1 mM EGTA (choline-loaded vesicles). The protein concentration was approximately 10 mg/ml. The vesicles were immediately frozen and stored at -80°C.

Measurement of tetraphenylborate uptake by the sarcolemmal vesicles

The uptake of TPB^- was monitored with a TPB^--sensitive electrode. The electrode membrane was a generous gift of Dr. D. Amman (Organic Chemistry Laboratory, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland). The vesicles were preincubated at 30°C for 5 min in a medium containing 160 mM choline-Cl (or KCl), 20 mM HEPES-Tris pH 7.4, 0.1 mM EGTA, 0.5 µM A 23187, and 30 µM TPB^-. Routinely, 3 µl of the suspension were rapidly injected into the reaction chamber which contained potassium medium supplemented with 10 µM TPB^-, 0.5 µM A 23187, and Ca^{2+} to obtain the desired final free concentration (routinely 20 µM).

Dephosphorylation and rephosphorylation of membrane proteins

In all experiments vesicles preincubated with A 23187, EGTA and TPB^- were used. Dephosphorylation of membrane proteins was carried out in the choline -HEPES medium containing 3 mM Mg^{++}, 10 µM digitoxygenin and 10 µM vanadate. The protein content was 7 mg/ml and the total volume was 35 µl. The reaction time was 5 min, at 30°C. At the end of the reaction 3 mM EDTA was added and the vesicles were cooled on ice and immediately used for the uptake experiments. In the rephosphorylation experiments, 0.6 mM ATP was added to the dephosphorylated vesicles instead of EDTA and the reaction was carried out for two additional minutes. The concentration of cAMP was 5 µM. 2 µg of protein kinase were used. The phosphorylation reaction was terminated by the addition of EDTA (3 mM) and the sample was used immediately for the uptake experiments.

Analysis of the phosphopeptides

Sarcolemma membranes were incubated under the conditions described above except that γ^{32}P-ATP (300 cpm/pmol) was used. The reaction was terminated by the addition of SDS-stop buffer and the proteins were separated electrophoretically according to Laemmly (10). The gels were fixed for 15 min in 10% trichloroacetic acid and 40% methanol, stained with Coomassie Blue, destained by diffusion and dried under vacuum. The dried gels were exposed to Kodak X-Omat AR X-ray films for two days at -70°C. The sensitivity was increased by an amplyfying screen.

Analytical methods

The protein content was estimated according to Lowry et al. (11) using bovine serum albumin as a standard.

Materials

The reagents used were of the highest purity commercially available. Valinomycin, protein kinase cAMP-dependent, and protein kinase inhibitor (type II) were from Sigma (St. Louis, Mo., USA). γ^{32}P-ATP was from Amersham International (U.K.), R 24571 was a generous gift of Dr. Van Belle (Janssen Pharmaceuticals, Beerse, Belgium), tetraphenylborate (Na salt) was from Fluka (Buchs, Switzerland) and the Ca^{2+} ionophore A 23187 was a generous gift from Ely Lilly (Indianapolis, In., USA).

RESULTS AND DISCUSSION

The sarcolemma preparation used had the characteristics described in a previous contribution from this Laboratory (9). Here, however, a new method for the measurement of the K^+ permeability of the vesicles has been used. Since the object of the study was the regulation of the $K^{\ddot{O}}$ fluxes through the membrane, we have elected to work on the fraction of the total population of vesicles having inside-out polarity. The system thus provides access to the possible regulatory component(s) of the Ca^{2+}-dependent K^+ channel from the outside medium. Previously published experiments from this Laboratory have described some properties of the Ca^{2+}-dependent K^+ flux, and have established, for example, that the channel is stimulated only by intracellular Ca^{2+}, and is unidirectional (7). One can thus measure the outflux of K^+, triggered by Ca^{2+} in the presence of A 23187, from preloaded right-side out vesicles. Inside-out vesicles preloaded with a nonpermeable cation (choline) would on the other hand be expected to accumulate K^+ from a potassium-containing medium in a Ca^{2+}-dependent way. Influx of K^+ into the vesicles creates a diffusion potential, positive inside, which can be monitored by the distribution of a negatively charged ion like TPB^-, which has already been used to monitor transmembrane potential in submitochondrial particles (12). Table I shows some properties of the experimental system. Addition of 20 μM $CaCl_2$ (free concentration) to choline-loaded vesicles clearly stimulates the uptake of TPB^-. Valinomycin (in the presence of EGTA) causes an even faster uptake of TPB^-, suggesting that the latter indeed follows the movement of K^+. Phencyclidine, a specific inhibitor of Ca^{2+}-dependent K^+ channels (13) drastically reduces the Ca^{2+}-stimulated TPB^- (K^+) uptake. The Ca^{2+}-dependent movement of charges is not

Table I. TPB^- influx into sarcolemmal vesicles

Addition to the medium	TPB^- (nmol/mg protein/sec)	
	Vesicles	
	Choline-loaded	K^+-loaded
EGTA (100 μM)	1.1	1.1
Ca^{2+} (20 μM free)	3.3	1.1
EGTA + valinomycin (1 μM)	4.2	1.2
Ca^{2+} + valinomycin (1 μM)	4.2	1.2
Ca^{2+} + phencyclidine (10 μM)	1.3	---

The numbers shown represent the average of three experiments in which each measurement was carried out in triplicate. The difference between experimental values was below 10%. The initial flux rates were calculated with the aid of a computer program for best fitting curve analysis: 40 experimental points were considered for the calculation.

observed if K^+ loaded vesicles, i.e. a system in which the K^+ concentration in the exterior and the interior of the vesicles is equal, are used. The protonophore carbonylcyanide m-chlorophenylhydrazone (1 μM) had no effect on the initial rates of TPB^- uptake (not shown). The effect of other divalent cations on the K^+ permeability has also been investigated. Ba^{2+}, Ni^{2+}, Mg^{2+}, and Co^{2+} (120 μM in the presence of 100 μM EGTA) were not able to stimulate the basal uptake. Only Zn^{2+} and Sr^{2+} could substitute for Ca^{2+}.

The regulation of channel conductivity is an important step in the function of excitable membranes. The role of cAMP in the regulation of the Ca^{2+} channel has been demonstrated in heart (14) and that of the cAMP-dependent protein kinase (catalytic subunit) in the modulation of the K^+ currents has been experimentally established in invertebrate neurons (4). The activity of the Ca^{2+}-dependent K^+ channel is considered important for the depolarization-repolarization cycle, therefore it is of great interest to establish whether the cAMP-dependent membrane protein phosphorylation in sarcolemmal vesicles (15) could be related to the modulation of the K^+ fluxes across the membrane. Heart sarcolemmal proteins in routine preparations normally exist in a partially phosphorylated state as shown by Caroni & Carafoli (8). In the case of the Ca^{2+}-dependent ATPase, the demonstration of its regulation by protein phosphorylation was only possible following dephosphorylation of the sarcolemmal membrane proteins (8). Thus we have used vesicles which had been preincubated with Mg^{2+} to facilitate the dephosphorylation of the putative substrates of protein kinases. As shown in Table II this treatment re-

Table II. Effect of dephosphorylation and rephosphorylation of membrane proteins on TPB^- influx into sarcolemmal vesicles

Reaction conditions	TPB^- uptake (nmol/mg protein/sec)
Control vesicles (+Ca^{2+})	4.1
Control vesicles (+EGTA)	1.3
Dephosphorylated vesicles (+Ca^{2+})	1.3
Dephosphorylated vesicles (+EGTA)	1.3
Vesicles dephosphorylated and then rephosphorylated by ATP (+Ca^{2+})	1.3
by ATP + cAMP (+Ca^{2+})	1.3
by ATP + cAMP + protein kinase (+Ca^{2+})	4.1
by ATP + cAMP + protein kinase (+EGTA)	1.3
by ATP + cAMP + protein kinase + phencyclidine (+Ca^{2+})	1.3

A typical experiment is shown. 4 experiments carried out under identical conditions on four different sarcolemma preparations produced very similar results. Calculation rates as in Table 1.

moves the Ca^{2+}-induced stimulation of the TPB^- (K^+) uptake, without influencing the basal TPB^- uptake. The Ca^{2+} stimulation of the K^+ permeability is restored upon incubation of the vesicles with ATP, cAMP and protein kinase. The restoring effect can be antagonized by the protein kinase inhibitor cAMP-dependent and by the channel blocker phencyclidine. These controls clearly show that the recovery of the Ca^{2+} dependent K^+ flux is due to the enzymatic activity of the kinase and is not related to a nonspecific membrane leak caused by the protein phosphorylation system. No effect of the cAMP-dependent protein kinase on the Ca^{2+}-dependent K^+ flux in the native vesicles (not preincubated with $MgCl_2$) was observed, suggesting that the proteins involved in the regulation of the K^+ channel are already phosphorylated in situ. Figure 1 shows that samples of vesicles which had been preincubated with Mg^{2+} prior to initiating the phosphorylation reaction with γ^{32}P-ATP incorporate much higher amounts of P^{32} into several proteins than vesicles not preincubated with Mg^{2+}. The phosphoprotein pattern presented here recalls that observed in other Laboratories (16) and is only offered to show that, indeed, the treatment with Mg^{2+} dephosphorylates endogenous protein substrates in sarcolemma. At the present stage it cannot be decided which of the phosphopeptides formed is associated with the regulation of the K^+ flux, or whether more than one of them is involved.

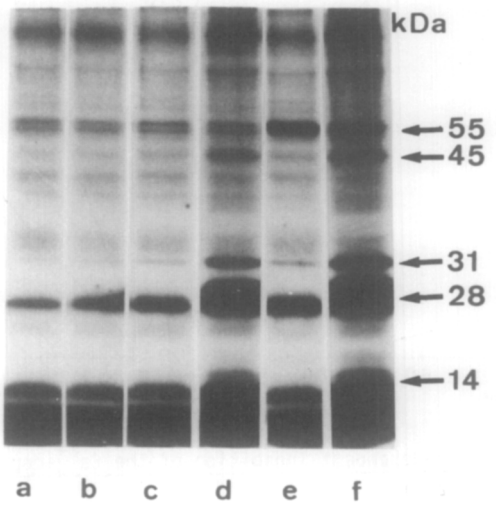
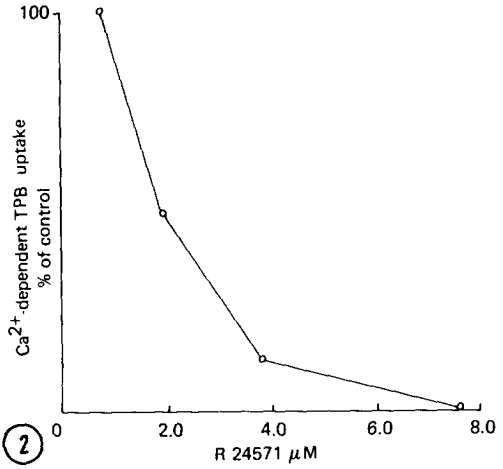


Figure 1. Effect of the preincubation of sarcolemmal vesicles with Mg^{2+} on the incorporation of ^{32}P from γ^{32}P-ATP into membrane proteins.

Full experimental details are given in the Materials and Methods section. a, c, e, no preincubation. b, d, f, membranes preincubated with $MgCl_2$. The following additions were made to the medium: γ^{32}P-ATP (a, b), γ^{32}P-ATP + cAMP (c, d), γ^{32}P-ATP + cAMP + protein kinase (e, f). The M_r of the most evident phosphopeptides was calculated from molecular weight standards run simultaneously on the same gel.

In a previous paper (7), it has been observed that trifluoperazine inhibits the Ca^{2+}-dependent K^+ flux and the suggestion has been made that CaM may be involved in the regulation of the K^+ channel. However, it is now generally accepted that trifluoperazine has side effects on the membrane integrity, especially at high concentration, thus we have tested the action of another anti-CaM compound (R 24571) which is more potent and more specific (17). The compound inhibits the Ca^{2+}-dependent K^+ flux, half maximal inhibition occuring at around 2 μM (Figure 2). Figure 3 shows that R 24571 shifts the Ca^{2+} sensitivity of the K^+ flux towards higher values. Since shifting of the Ca^{2+} affinity of CaM-dependent systems by anti-CaM compounds is well known, it could be proposed that membrane-bound CaM is involved in the regulation of the Ca^{2+}-dependent K^+ channel. One difference in the system used here with respect to other CaM-dependent systems is the apparently low affinity for Ca^{2+} of the K^+ flux (apparent K_m-10 μM, Figure 3). However, it has recently been reported (18) that the affinity of the Ca^{2+}-dependent K^+ channel could be much higher (K_m as low as 1 μM) depending on the value



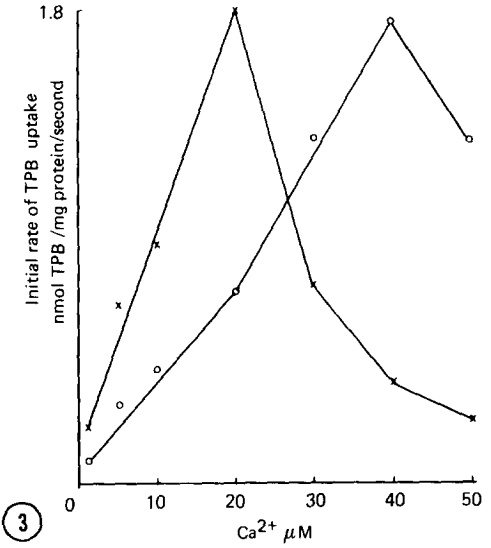


Figure 2. Concentration-dependent inhibition of the Ca^{2+}-dependent TPB^- uptake by R24571.

Full experimental details are given in the Materials and Methods section. The free Ca^{2+} concentration was 20 μM.

Figure 3. Effect of R24571 on the Ca^{2+}-dependent TPB^- uptake in the presence of different Ca^{2+} concentrations.

Full experimental details are given in the Materials and Methods section. R24571 was 3 μM. Free Ca^{2+} concentrations are as shown. X, control. O, R24571 present.

and the vector of the transmembrane potential. In the experimental system used here no means of controlling these factors were available. It may be mentioned in this context that a role of CaM in the regulation of the Ca^{2+}-dependent K^+ channel in erythrocytes has also been postulated (19). In conclusion, we propose that the Ca^{2+}-dependent K^+ channel in heart sarcolemma is maintained in the open state through phosphorylation of one or more of its components by a cAMP-dependent protein kinase. Dephosphorylation of the component(s) by an endogenous protein phosphatase reverses the channel to the closed state. Furthermore, we propose that endogenous, membrane bound CaM confers Ca^{2+} sensitivity to the channel.

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