The effect of adrenaline on the electrogenic Na^+ pump in cardiac muscle cells[1]

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Summary. Electrogenic Na^+ pump currents during K^+-activated hyperpolarizations of bullfrog atrium muscle fibres are increased by adrenaline. The log dose-response relation between these currents and activating K^+ concentrations is expressed by a sigmoidal curve, which is shifted in parallel to the left by adrenaline. It is suggested that adrenaline increases the rate of Na^+ extrusion without increasing the Na/K coupling ratio and the total number of pumping sites.

A possibility that neurotransmitters regulate the membrane potential of target cells by acting on the electrogenic Na^+ pump system of these cells has been suggested, in connection with the ionic mechanism of the slow inhibitory postsynaptic potential (slow IPSP) of sympathetic ganglion cells[2,3]. This suggestion has been supported by our recent experimental findings that the K^+-activated hyperpolarization of various kinds of excitable cells is augmented under the effect of catecholamine[4-7]; the K^+-activated hyperpolarization is generated by an activation of the electrogenic Na^+ pump when K^+ is added to extracellular K^+-free Ringer solution. These results, however, provided only indirect evidence supporting the possibility that catecholamine increases the electrogenic Na^+ pump current. In other words, direct evidence, showing that the membrane pump current during activation of an electrogenic Na^+ pump is actually increased by catecholamine, is necessary. Thus, in the present communication we measured the membrane pump current by means of the voltage clamp technique.

In the case of cardiac muscles, it has been suggested that catecholamine may augment the electrogenic Na^+ pump of these muscle cells[8-10]. Our present findings indicate that the electrogenic Na^+ pump current of these cells is clearly increased by the action of adrenaline. Furthermore, it is suggested that adrenaline increases the Na^+ pump current by increasing the rate of Na^+ extrusion without increasing the Na/K coupling ratio and the total number of pumping sites.

Material and methods. Strips (1×7 mm in size) of muscle fibre bundles excised from the atrium of bullfrog *(Rana catesbeiana)* heart were used throughout in the present experiments. The experimental apparatus, including a chamber for mounting preparations and for the voltage clamp experiment was similar to that described by Beeler and Reuter[11].

A fibre bundle preparation was tightly mounted on the Perspex chamber divided into 3 compartments by 2 rubber membranes (figure 1, B). The right and left side outer compartments were perfused with the Ringer and a test solution, respectively, and the middle compartment (2 mm of width) was perfused with sucrose solution. Platinum electrodes were placed in 2 outer compartments and used for applying clamp currents across sucrose gap. The end (less than 1 mm of length) of a fibre bundle was exposed to the solution in the left-hand chamber (test solution chamber) and membrane potential (figure 1, B) was recorded from this end. The membrane potential was measured between 2 microelectrodes filled with 3 M KCl; the 1st (40–50 MΩ) was impaled into a fibre and the 2nd (less than 5 MΩ) was placed close to fibre bundle. The intracellular microelectrode is connected to a feed-back amplifier, and the voltage clamp current for the K^+-activated hyperpolarization was applied across the sucrose gap through the pair of platinum electrodes[11]. Ionic compositions of solutions used in the present experiment are as follows. Sucrose solution; 224 mM sucrose. Ringer solution; 112 mM NaCl, 2 mM KCl, 0.18 mM $CaCl_2$, 6 mM $MgCl_2$, 2 mM $NaHCO_3$ and 2.5 mM glucose. For preparing a K^+-free Ringer solution, 2 mM KCl was omitted and KCl (0.25–5 mM) was added to K^+-free solution for K^+-containing Ringer solution. The Ca^{2+} concentration in these solutions was kept low (0.18 mM) in order to reduce intracellular Ca^{2+} accumulation[12]. Drugs added to the solutions were adrenaline bitartrate (Sigma) and ouabain (Merck).

Preparations in the test solution chamber were first perfused with K^+-free Ringer solution for 60 min, and K^+-activated hyperpolarizations were measured thereafter by replacing the perfusate to a K^+-containing (0.25–5 mM) Ringer solution. K^+-activated hyperpolarization could be repeatedly recorded with almost a constant amplitude, provided a K^+-containing Ringer solution was applied for 1 min at an interval of every 15 min.

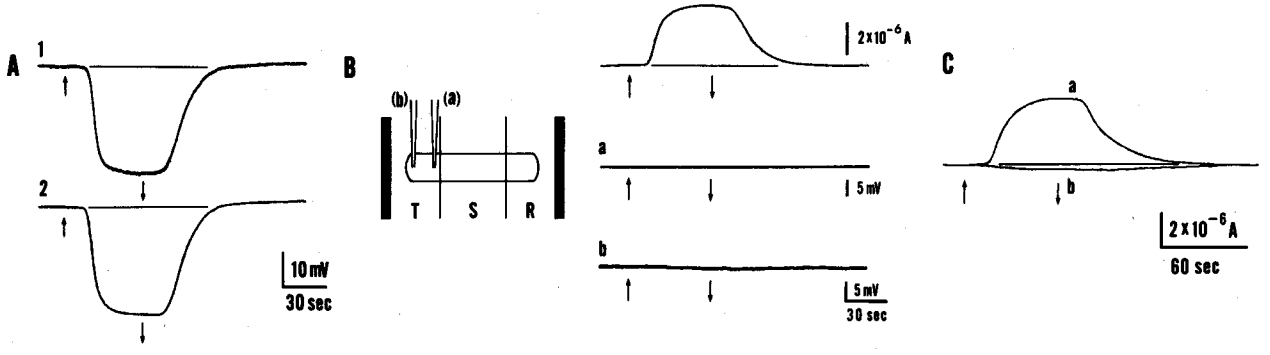

Fig. 1. *A* A comparison of the K^+(2 mM)-activated hyperpolarization recorded by a microelectrode (record 1) with that recorded simultaneously across a single sucrose gap (record 2). Arrows indicate periods of K^+ application (same B and C). *B* A test for the efficiency of the voltage clamp for the K^+-activated hyperpolarization generated at the end of a muscle bundle exposed to the test solution chamber (T); S and R are the sucrose and Ringer solutions chambers, respectively. Membrane potentials recorded by 2 microelectrodes (a) and (b) are shown in records a and b, respectively, while clamp current controled by a microelectrode (a) is shown in the right top record. *C* Clamp currents a and b are recorded from a same preparation in the absence and presence of ouabain, respectively. The electrogenic Na^+ pump current is estimated by the addition of the maximum absolute values of these 2 currents.

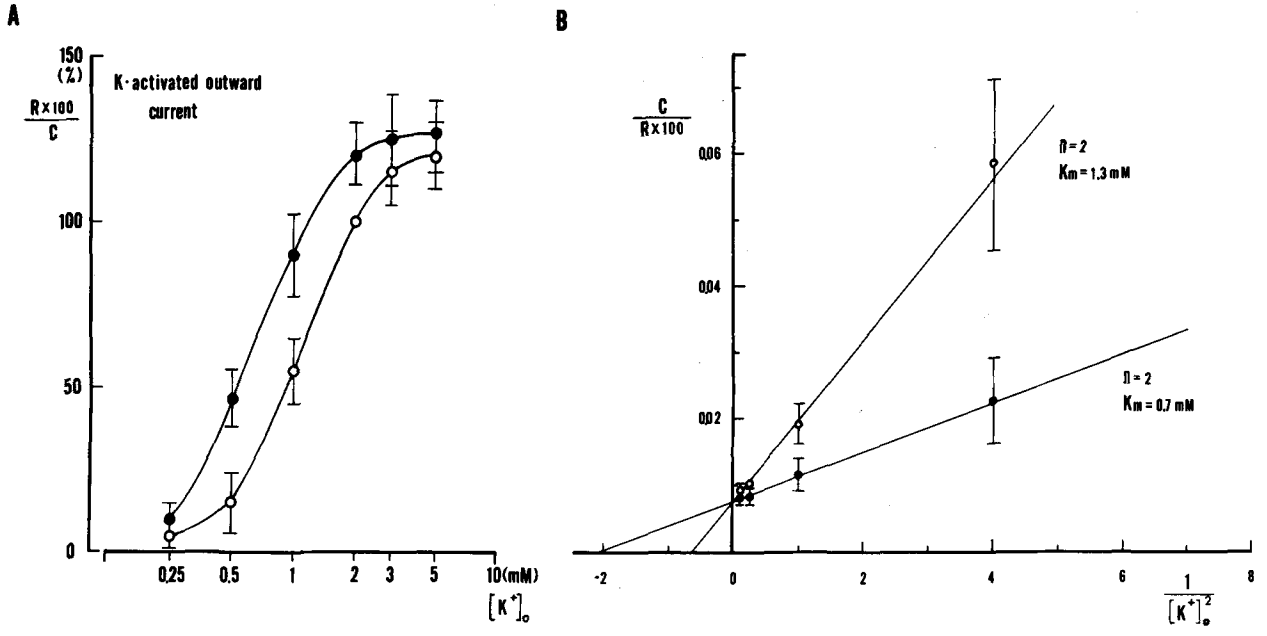

Fig. 2. *A* The log dose-response curve showing the relation between the electrogenic Na^+ pump current (K^+-activated outward current) and activating K^+ concentrations. Ordinate is relative value of K^+-activated outward current (R) and abscissa is extracellular K^+ concentration $[K^+]_o$ in log scale; the K^+(2 mM)-activated outward current (C) is taken as 100. Open and closed circles are obtained in the absence and presence of adrenaline (0.01 mM), respectively. Vertical lines at each point are standard errors obtained from different preparations. *B* Double reciprocal plots constracted from diagram A. For further details see text.

Results and discussion. Results obtained from experiments for testing reliability of the present experimental arrangement are shown in figure 1. In records A in figure 1, K^+-activated hyperpolarizations, which were recorded simultaneously by an intracellular microelectrode and also across the sucrose gap, are demonstrated. As seen in these records, the size and time course of K^+-activated hyperpolarizations recorded across the sucrose gap were almost identical with those recorded by an intracellular microelectrode. Records B of figure 1 show that the membrane potential of muscle cells in the test solution chamber was effectively clamped at the original resting level during a generation of the K^+-activated hyperpolarization. This is indicated by an absence of changes in the membrane potential recorded by a test microelectrode *b* impaled into a fibre at a distance of about 0.5 mm from a microelectrode *a* impaled close to the sucrose gap for potential control.

The net outward current (the electrogenic Na^+ pump current) during a development of the K^+-activated hyperpolarization was estimated by the following experimental procedure. Outward currents during K^+-activated hyperpolarizations of preparations were first recorded, and then these preparations were exposed to K^+-free Ringer solution containing ouabain in a concentration of 0.001 mM for 20 min. After such a treatment, a membrane depolarization instead of the K^+-activated hyperpolarization, could be measured when the perfusate was replaced to a K^+-containing solution (containing 0.001 mM ouabain). Thus, inward currents during these K^+-induced depolarizations were recorded. It was assumed in the present experiment, that the Na^+ pump current is completely blocked, while the passive ionic current is not affected by 0.001 mM ouabain. Thus, the net outward current was estimated by adding the absolute value of membrane currents obtained in the presence and absence of ouabain (see records shown in record C of figure 1).

Electrogenic Na^+ pump currents are increased when the concentration of K^+ for activation of the Na^+ pump is raised. The log dose-response relation between these currents and K^+ concentrations is expressed by a sigmoidal curve (figure 2). The double-reciprocal plot relation between the reciprocal of current values and that of the n'th power of K^+ values is expressed by a straight line when $n = 2$ (figure 2, B).

The effect of adrenaline on the electrogenic Na^+ pump current was studied by comparing the pump currents recorded in the presence of adrenaline (0.01 mM)[7] with those recorded in the absence of adrenaline in the same preparations. The log dose-response curve, derived from these experimental results, is shown in the diagram A of figure 2. As seen in this diagram, the curve is shifted in parallel to the left under the effect of adrenaline. The double-reciprocal plot of the K^+ concentration dependence of the electrogenic Na^+ pump current is shown in the diagram B of figure 2. As seen in this diagram, 2 straight lines obtained in the absence and presence of adrenaline meet at the ordinate, where the K^+ concentration is infinite. Furthermore, Hill number of K^+ action on the Na^+ pump was also 2 in the presence of adrenaline, indicating that adrenaline does not effect the cooperativity of the Na^+ pumping site. The apparent dissociation constant (K_m) for the K-membrane pumping sites complex decreased from 1.3 mM to 0.7 mM by the action of adrenaline.

According to the present experimental results the log dose-response relation between the electrogenic Na^+ pump current and the extracellular K^+ concentration appears to be expressed by a simple sigmoidal curve, namely, by a simple law of mass action. This suggests that the total number of pumping sites on the membrane may be constant irrespective to the extracellular K^+ concentration, and also that the Na/K coupling ratio for Na^+ pump may be kept constant for different extracellular K^+ concentrations. The double-reciprocal plot of the log dose-response curve, which is shown in diagram B in figure 2, suggested that

each pumping sites were activated when two molecules of K^+ attached to a pumping site.

In general, an increase of the electrogenic Na^+ pump current would be expected in the following cases: 1) an increase of the rate of Na^+ extrusion with a constant Na/K coupling ratio, 2) an increase of the Na/K coupling ratio with a constant rate of Na^+ extrusion, and 3) an increase in both the rate of Na^+ extrusion and the Na/K coupling ratio in each pumping site. According to the present experimental result, it seems reasonable to assume that adrenaline increases neither the total number of pumping sites nor the Na/K coupling ratio. Thus, it appears that adrenaline increases the rate of Na^+ extrusion by increasing the overall affinity of the pumping site to the extracellular K^+ at a fixed concentration. In other words, adrenaline increases the number of activated pumping sites per unit time at a fixed extracellular K^+ concentration.

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Inhibition of new blood vessel formation in mice by systemic administration of human rib cartilage extract[1]

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Summary. 1 M HCl-guanidine extract of human funnel chest rib cartilage administered i.v. to mice decreased specifically vasoproliferation induced by intradermal injection of allogeneic murine lymphocytes.

New blood vessel formation may be induced by tumor-angiogenesis factor (TAF) isolated from tumor cells[2], or by substance released from lymphocytes engaged in a local graft-versus-host (GVH) reaction[3]. Vasoproliferation induced by these angiogeneic factors could be inhibited by local application of cartilage fragments[4], extract[5] or isolated chondrocytes[6]. The present work was aimed to test whether the systemic (i.v.) administration of xenogeneic cartilage extract to mice could inhibit vasoproliferation induced in the course of a local GVH reaction evoked by intradermal injection of allogeneic lymphocytes.

Materials and methods. Cartilage extract was made from 42 g of costal rib cartilage removed in the course of funnel chest correcting operation performed in 11- and 13-year-old boys. Viability of chondrocytes estimated by neutral red staining was nearly 100%. Cartilage was cut into pieces and extracted with 1 M HCl-guanidine in 0.02 M phosphate buffer (pH 5.8) for 48 h at room temperature. Extract was dialyzed against distilled water at 4 °C and then centrifuged and lyophilized. About 120 mg of crude extract containing 30% of protein as estimated by the Lowry method was obtained.

In funnel chest cartilage, some focal degenerative changes could be observed[7], but we used this type of cartilage because of its excellent viability when compared with normal costal rib cartilage obtainable after autopsy.

As recipients, 8–10-week-old Swiss mice, irradiated with a single dose of 700 R (59 R/min in air) to depress their immunological response, were used. 2 h after irradiation, 1 group of recipients was injected i.v. with a single dose of 7.5 mg of cartilage extract per mouse, containing 2.5 mg of protein, dissolved in 0.2 ml of TC 199. 2 other groups were injected i.v. with 2.5 mg of either human plasma albumin (Biomed, Warsaw) or egg-white lysozyme (Reanal, Budapest) dissolved in 0.2 ml of TC 199 to check the effect of systemic administration of xenogeneic proteins on angiogenesis. Control group was injected i.v. with 0.2 ml of TC 199 only.

Vasoproliferation was induced on the following day in all irradiated and i.v. injected animals using the lymphocyte-induced-angiogenesis assay[3]. In this assay, an intradermal injection of allogeneic lymphocytes evokes a local GVH reaction resulting in pronounced angiogenesis. The intensity of the reaction, i.e. number of newly formed blood vessels, depends on the number of cells injected and reaches its peak on the 3rd day post injection. Swiss mouse recipients were injected intradermally with CFW mouse splenocytes obtained by disruption of donor spleens in a loosely fitted glass homogenizer. Each of the recipients was given 6 injections onto both flanks, consisting of 4×10^6 viable cells suspended in TC 199 in a total volume of 0.1 ml per single injection. Additional group of control mice was given 2×10^6 splenocytes to test the dose effect. 3 days later, angiogeneic response was evaluated at the inner surface of the recipient skin using the criteria of Sidky and Auerbach[3]. All extra blood vessels connected with the injection site and contrasting with the background vasculature due to their tortuosity and divarications were counted. Counting was done by a person who did not know the coding pattern of the recipients.

To prove the specific effect of cartilage extract on endothelial cell proliferation, a group of nonirradiated Swiss mice were injected i.v. with the extract in previously-used doses. Control mice were given albumin or TC 199 alone. Mitotic

Recipients injected i.v.	Mean number of blood vessels ± SD	No. of animals*
Cartilage extract	30.76 ± 7.82	12
Albumin	36.32 ± 8.55	6
Lysozyme	36.72 ± 8.00	12
TC 199 control	38.47 ± 7.80	12
TC 199 control**	29.08 ± 6.69	6

* 6 injections of splenocytes per single recipient;
** mice injected with 2×10^6 splenocytes.