Potassium Channels in the Cardiomyocyte Sarcolemma: Initial Opening under Influence of Hypoxia

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Abstract

Initial activation of at first ATP-sensitive and then Na^+-activated K^+ channels of sarcolemma of rat adult calcium tolerant ventricular cardiomyocytes under influence of relative hypoxia was recorded in the cell-attached configuration of the patchclamp method. It is shown that in this reaction only a part of the total population of the ATP-sensitive K^+ channels is involved and that the dynamics of their early activity is periodical.

Properties and quantitative relationships between channel activity and intracellular medium parameters ($[ATP]_i$, $[Na^+]_i$, pH_i, $pCa^{2+}{}_i$) were estimated in an inside-out configuration for the same channels. Characteristics of the single-channel currents and $[ATP]_{50\%}$ value (mean 20 μM), dependence of the ATP-sensitive K^+ channel activity on pH_i and $pCa^{2+}{}_i$, K_d value (50 mM) and Hill's coefficient (2.5) for binding Na^+ with Na^+-activated K^+ channel have not possessed anomalous peculiarities as compared with those obtained for the same types of channels before hypoxia.

It is obvious that the mechanism determining involvement of some subpopulations of channels into an initial reaction to hypoxia depends considerably on the intracellular factors associated with functional state of the heart cell.

Key words: cardiomyocyte, patch-clamp, ATP-sensitive K^+ channel, Na^+-activated K^+ channel, hypoxia.

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Alteration of potassium permeability of cardiomyocyte sarcolemma in hypoxic conditions affects resting potential, excitability, action potential duration, automatism, pacemaker activity and contraction of the heart cell. This process is significantly produced by activation of the ATP-sensitive and Na^+-activated K^+ channels [1, 9, 27].

Information on possibilities and mechanisms of these channel openings while hypoxic cell damage is in act was obtained mainly in patch-clamp experiments with anoxia, metabolic toxin, or saponin [4, 11, 17, 18, 23, 24, 32]. However, changes of K^+ currents on heart cells *in-situ* under influence of hypoxia (in the early stage particularly) are less than those observed in the cited studies. J.F. Faivre and I. Findlay [11] came to the conclusion that significant changes of action potential of cardiomyocyte occurred as a result of activation of one per each 100 ATP-sensitive K^+ channels. Taking into consideration the "spare channels" hypothesis [7, 13], the suggestion should be made that parameters determining channel activity (the concentration of ATP evoking 50% channel closure etc.) considerably varies in quantity for different channel subpopulations. In this case such heterogeneity and ranges of the parameters should determine dynamics of involving channels into cell reaction and time of this process respectively.

To prove such a suggestion a study of early reaction of the ATP-sensitive and the Na^+-activated K^+ channels on relative hypoxia in cell-attached mode was considered to estimate the quantitative dependence of channel functioning on values of intracellular medium parameters modulating these channels activity in inside-out configuration.

Materials and Methods

Adult ventricular calcium tolerant rat cardiomyocytes have been used in experiments. To obtain them heart extracted from male rat anaesthetized with Thalamonal

(0.2 ml/100 g body weight) were perfused according to Langendorff, by the DMEM medium at the volume velocity of 12 ml·min^{-1} at 37°C. Then using the medium for the preparation of cardiomyocytes (MPC) (composition reported in Tab. 1) with EGTA (0.08%) and $CaCl_2$ in the concentration 140 μM during 4-5 minutes. Then the perfusion was continued with MPC containing 0.02% Pronase E, 0.1% Albumine bovine fraction V and $CaCl_2$ in a concentration of 140 μM. After 4-5 minutes of proteolytical treatment, the ventricular tissue was minced into 2 to 3 mm pieces with scissors and transferred into a fresh portion of the same medium where 0.1% Collagenase type A (Boehringer) was added. The tissue was exposed to slight mechanical disaggregation in a specially designed disaggregation vessel [16] at 37°C during 15 minutes. A three time repetition of the procedure helped achieve 80% tissue disaggregation. The separation of the cardiomyocytes from other cells and debris in suspension was obtained in a 5% solution of the BSA fraction V in the MPC with 1 mM Ca^{2+} (MPC1) at 1 g for 15 minutes at room temperature. The separated suspension contained no less than 70% cardiomyocytes with the *in-vivo* morphology. The resting potential, evaluated according to the reverse potential in whole-cell mode of the patch-clamp method [14], was equal to 68 ± 2 mV (n = 8) for such cells.

The cells were kept in a specially constructed perfused solution box for electrophysiological experiments and placed in an incubator set of the inverted microscope MBI-17. The temperature of the extracellular medium was controlled by a miniature thermistor sensor, the pH was controlled by a pH-metre constructed on the foundation of the pH-ion-selective field transistor, the pO_2 level with the help of the ABL330 analyser (Radiometer). Under control conditions the MPC1 perfused box was saturated with a gas mixture 95% O_2 and 5% CO_2. In such a condition the PO_2 of the extracellular solution was equal to about 30 kPa.

The hypoxia was modelled by perfusing the medium with a gas mixture 95% N_2 and 5% CO_2. By controlling every 3 minutes, the PO_2 of the extracellular medium was no higher than 3 kPa. The experiments were performed at room temperature (22-24°C).

To estimate the functioning of the ion channels of the sarcolemma of cardiomyocytes, currents through single channels using the patch-clamp method [14] were recorded. Molibden glass micropipettes with resistance between 5 and 10 MΩ were employed. Current-potential conversion was conducted using a feed-back resistance of 10 GΩ. Then the signal was sent to an amplifier (SKB BP AN SSSR model). The recorded current was filtered at 300 or 1000 Hz by using a low-pass filter. The data were sampled by a twelve-bit A/D converter (PCL-718) at an interval of 1 or 0.3 ms. The single-channel currents were recorded simultaneously on magnetic tape (Teac R-81) for later analysis. The analysis of the single-channel currents was performed using an IBM PC/AT-286 computer. The original application package "CHAN" was used.

The composition of the solutions used in the experiments is shown in Table 1. The total calcium concentration was calculated from specified free Ca^{2+} concentration [10].

Results

In experiments with 40 cells kept in a medium enriched with oxygen in the cell-attached mode with K_e solution in the micropipette, inward potassium currents were recorded. They were provided by functioning K^+ channels corresponding to those previously found in rat cardio-

Table 1. The composition of the solutions used in experiments is shown: MPC - the medium for the preparation of cardiomyocytes, K_e - extracellular potassium solution, K_i - "intracellular" bath solution, K_{selec} - bath solution for the estimation of the K^+/Na^+ selectivity of channels, K_{ident} - bath solution for the identification of K^+ channels, K_{depol} - the solution for the depolarisation of cardiomyocytes, K_{iAsp^-} - the solution with the nonpenetrating through K^+ channels aniones. The concentrations of ingredients are given in mM.

Ingredient	Solution						
	MPC	K_e	K_i	K_{selec}	K_{ident}	K_{depol}	K_{iAsp^-}
NaCl	100			140			
KCl	10	140	140	140	35		
KAsp						140	140
KH_2PO_4	1.2						
$MgSO_4$	5						
Glucose	20						
Taurine	30						
MOPS	10	10	10	10	10	10	10
$CaCl_2$		I	0.3	0.3	0.3	1	0.3
EGTA			5	5	5		5
$MgCl_2$		I				1	
pH	7.2	7.2	7.2	7.2	7.2	7.2	7.2

myocytes membrane inward-rectifying K^+ channels of the Ist and the IInd types [5]. There was no activity of single channels conducting outward potassium currents within 60 minutes.

Similar experiments under hypoxic conditions (160 cells had been investigated) proved that outward currents through K^+ channels of other types had appeared (about 18% of the prepared cell-attached configurations).

Discharges of burst activity of one of the type channels appeared in 20-25 minutes of hypoxia. They repeated at minute intervals, the amplitude of the increase of their open probability (P_O) being up to 0.162 at 0 mV of the membrane potential (each P_O value was constructed from a 10-sec current recording) (Fig. 1A). The mean burst duration (τ_b) (many of them consisting of single openings) and the mean duration of the gaps between the bursts (τ_{ib}) were of about 5 ms and 300 ms respectively. Further investigation of properties of currents through these channels identified them as ATP-sensitive K^+-channels previously found in rat cardiomyocyte sarcolemma [36]. When 3 mM ATP was applied to the inner surface of the patch membrane channel, activity was reversibly blocked (Fig. 2A). The single-channel conductance with the K_e solution in the pipette was about 50 pS. In the inside-out configuration with the MPC1 in the pipette and the K_i solution in the camera, the channel conductance over the linear part of the current-voltage (I-V) relation was about 25 pS (Fig. 2C). In such conditions the outward currents demonstrated saturation at the level of 2 pA. This saturation was moved towards greater potentials and greater currents by an increase of $[K^+]_i$. The P_O value was the maximum possible when passing into the inside-out mode (up to 0.7) and then it gradually decreased (not more than 0.15 in half an hour). Under such conditions τ_b and τ_{ib} were about 150 and 10 ms respectively.

Deepening of the hypoxia (more than 40 minutes) led to the opening of the K^+ channels with greater conductance. Such channels were found much more rarely. They also possessed the burst kinetic, however their early dynamics were not periodic but gradual (Fig. 1B). By the time of the channel opening resting potential for such cells was diminished (3-7 mV) as compared with those under control conditions. In the inside-out mode with the K_i solution on the inner side of the membrane the channel was inactivated. Changing the free calcium concentration on the inner side of the membrane from $pCa^{2+} = 8$ to $pCa^{2+} = 4$ did not activate the channel. The application of K_i solution containing 50 mM Na^+ caused the opening of the channel (Fig. 2B). The channel conductance was about 75 pS at the MPC1 on the outside of the membrane (Fig. 2C). The P_O value did not depend on the membrane potential. Na^+-activated K^+ channels observed in our experiments were similar to those previously found in the guinea-pig ventricular cardiomyocyte membrane [18, 34]. The conductance of this channel (K^+-rich solution in the pipette) was

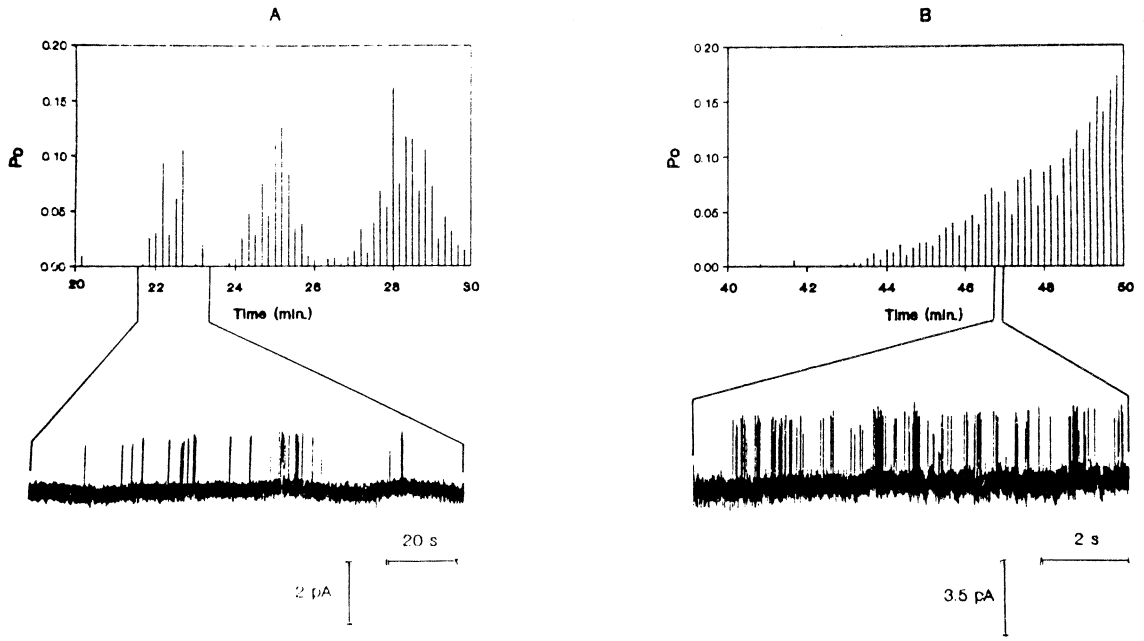

Figure 1. Dynamics of initial activity of the K^+ channels of cardiomyocytes sarcolemma in cell-attached mode in hypoxic conditions. A: Top - dynamics of P_O (each P_O value constructed from a 10-sec current recording), beginning from the 20th minute of hypoxia, bottom - the fragment of current recording from which the P_O values were constructed; the membrane potential a mV, MPC1 in the pipette and in the camera. B: Ditto, in such conditions for Na^+-activated K^+ channel, beginning from the 40th minute of hypoxia.

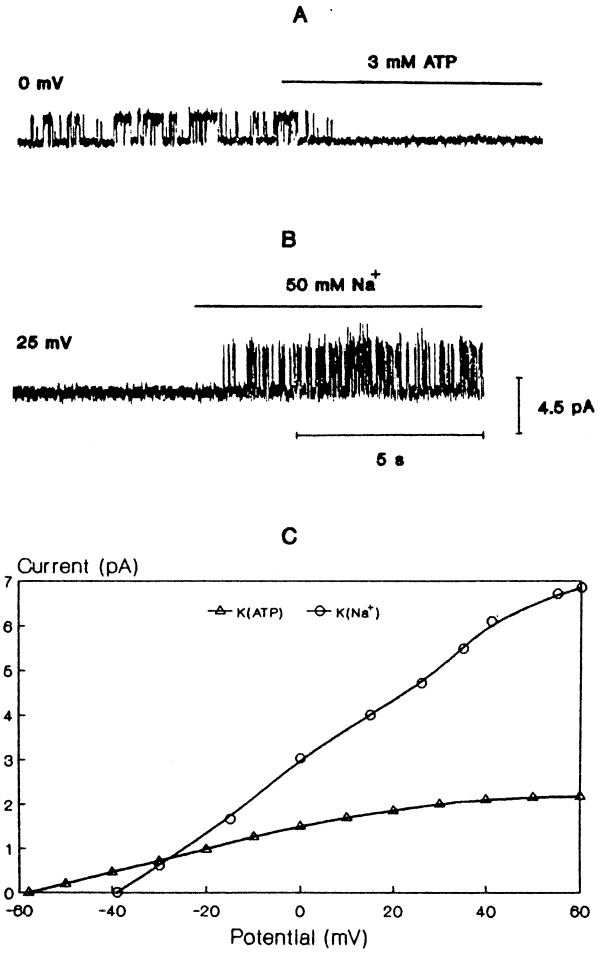

Figure 2. Recordings of outward currents through the ATP-sensitive (A) and Na^+-activated (B) K^+ channels and current-voltage relations corresponding to them (C). A: single-channel recording in the inside-out patch at 0 mV with MPC1 in the pipette and with K_i bath solution, the upward deflections on the recording corresponds to the outward current, feeding with the blocking K_i solution with $[ATP]_i$=3 mM marked line. B: single-channel recording in the inside-out mode at 25 mV with MPC1 in the pipette, the channel is activated after the change of the K_i solution upon the K_i solution with $[Na^+]$=50 mM, marked by the line, the upward deflections on the recording corresponds to the outward current. The time and current scale is given to records A and B. C: K(ATP) - current-voltage relation for the ATP-sensitive K^+-channel received in the above shown conditions, K(Na^+) - current-potential relation for Na^+-activated K^+ channel, received with the MPC1 in the pipette and with 50 mM K^+ and 90 mM Na^+ in the bath solution.

200 pS whereas in our experiments single channel conductance accounted for about 100 pS.

All cell-attached patches, in which channel openings did not occur in the hypoxic conditions, were also tested after transition into inside-out configuration. In fact each of two patches contained one or more ATP-sensitive K^+ channels. In several experiments closed Na^+-activated K^+ channels in the cell-attached mode were appearing. However, the conclusion that some of the total number of channels are involved in early response to hypoxia was statistically grounded for ATP-sensitive K^+ channels (records of Na^+-activated K^+ channels was not enough to make such an analysis).

Thus, under hypoxic conditions in the cardiomyocyte sarcolemma firstly ATP-sensitive and then Na^+-activated K^+ channels were opened in succession. Dependence of channel activity on the most important indices of the intracellular medium (such as the ATP and Na^+ concentrations ($[ATP]_i$, $[Na^+]_i$), pH_i and $pCa^{2+}{}_i$) during hypoxic damage of cell was estimated directly in the inside-out configuration.

Dependence of the ATP-sensitive K^+ channel blocking on ATP concentration at the inner side of membrane was estimated by the use of patches containing only one channel (the membrane potential was equal to 0 mV). Each patch was tested for not more than 15 minutes (beginning from the 2nd minute after patch isolation). Degree of blocking (B) was calculated in percents as follows:

$$B = 100\frac{P - P_{[ATP]}}{P},$$

where $P\text{-}P_O$ with $[ATP]_i$ = O mM and $P_{[ATP]}\text{-}P_O$ with the actual ATP concentration.

The result is shown in Fig. 3A. The ATP concentration evoking 50% channel closure ($[ATP]_{50\%}$) in the excised membrane fragment was in average 20 μM. The dependence is in conformance with the Michaelis-Menten scheme testifying that binding of one ATP molecule is enough to block the channel.

The activity of the Na^+-activated K^+ channel did not depend on $[ATP]_i$ but was defined by $[Na^+]_i$. The concentration dependence is shown in Fig. 3B. The experimental values are fitted by the theoretical curve given by:

$$P_O = \frac{P_{O\max}}{1+\left(\frac{K_d}{[Na^+]_i}\right)^n},$$

where K_d is the dissociation constant equal to 50 mM, n - a Hill's coefficient equal to 2.5. The value of Hill's coefficient over 1 testifies to the positive cooperativeness of channel activation process by the Na^+ ion.

The sensitivity of the ATP-sensitive and Na^+-activated K^+ channels to the changes of pH on the inner side of the membrane (pH_i) proved to be different. It could be visibly

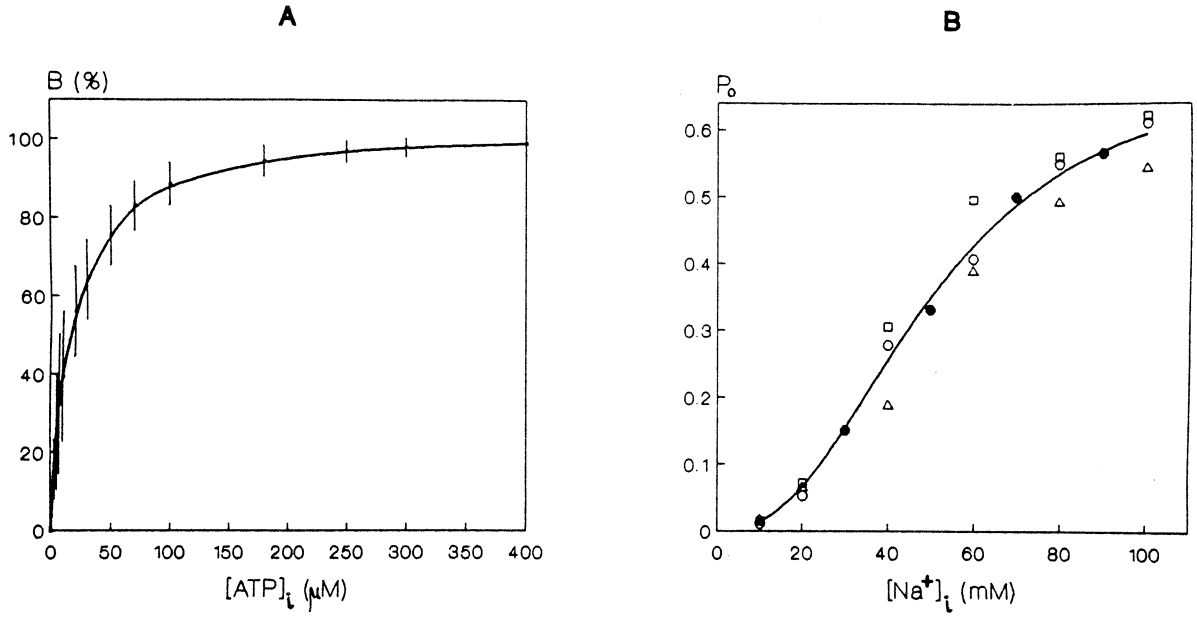

Figure 3. The dependence of the blocking of the ATP-sensitive K^+ channel activity on $[ATP]_i$ (A) and Na^+-activated K^+ channel activity on $[Na^+]_i$ (B). A: The dependence is built on the data, received on 10 patches, the vertical bar indicates the Std. Dev. B: different symbols mark measures, executed on 4 patches, the line of the theoretical curve.

seen in several experiments with patches containing channels of both types. Fig. 4A shows that if pH_i was equal to 7.0 channels of both types demonstrated high activity. After the pH_i increase up to 7.9 (Fig. 4B) the ATP-sensitive K^+ channel was inactivated significantly whereas the alteration of the kinetic of the Na^+-activated K^+ channel was scarcely detectable.

Change of P_O for the ATP-sensitive K^+-channel produced from the pH_i alterations was calculated in percentages by:

$$A = 100 \frac{P_{pH} - P_{7.2}}{P_{7.2}},$$

where P_{pH} - P_O at the actual pH_i and $P_{7.2}$ - P_O at the pH_i = 7.2. The dependence of this parameter on the acidity of the intracellular medium within the range of pH_i from 6.0 to 8.8 was nonlinear (Fig. 5). Within the physiological limits of pH_i only P_O-pH_i relation was approximately linear, the acidosis causing sufficient P_O increase.

In case of the preliminary ATP blocking of the pH_i, lowering up to 6.0 did not cause channel opening.

Apart from kinetics modulation the pH_i variation caused changes in the conductance of the ATP-sensitive K^+ channel. The pH_i elevation by 1.0 was followed by a channel conductance increase of 17 ± 4% (n = 4). The pH_i change within physiological limits was not followed by significant alterations of the current through this channel.

On 4 ATP-sensitive and 4 Na^+-activated K^+ channels their activity was compared at pCa^{2+}_i of K_i solution equal to 8, 7 and 6. The increase of the $[Ca^{2+}]_i$ within these ranges did not evoke considerable effect. Partial blocking of ATP-sensitive K^+ channel similar to the one observed earlier [17] was found at pCa^{2+} equal to 4 only.

Thus, the increase of free calcium concentration in cytosol affected insignificantly ion transport through investigated K^+ channels.

Discussion

Reported results show the possibility of opening the ATP-sensitive and Na^+-activated K^+ channels of cardiomyocyte sarcolemma in relative hypoxic conditions and ordinary physiological extracellular medium.

At first the ATP-sensitive K^+ channels were opened. Comparison of the number of these channels opened in cell-attached mode with the total number of channels found while controlling excised patches, proves that only a part of the total population of the ATP-sensitive K^+ channels is involved in an early response to hypoxia.

Our suspicion that the $[ATP]_{50\%}$ values estimated in the inside-out mode would be similar to the maximum values of the parameter reported by different authors (within the range of 9 - 580 μM) [12, 13, 20-23, 31] was not proved. Hence, inconsistency of low $[ATP]_{50\%}$ value estimated in excised patches with physiological (millimolar) intracellular ATP level in hypoxic conditions [29] still stands for the most "hypoxia sensitive" channels of this class too.

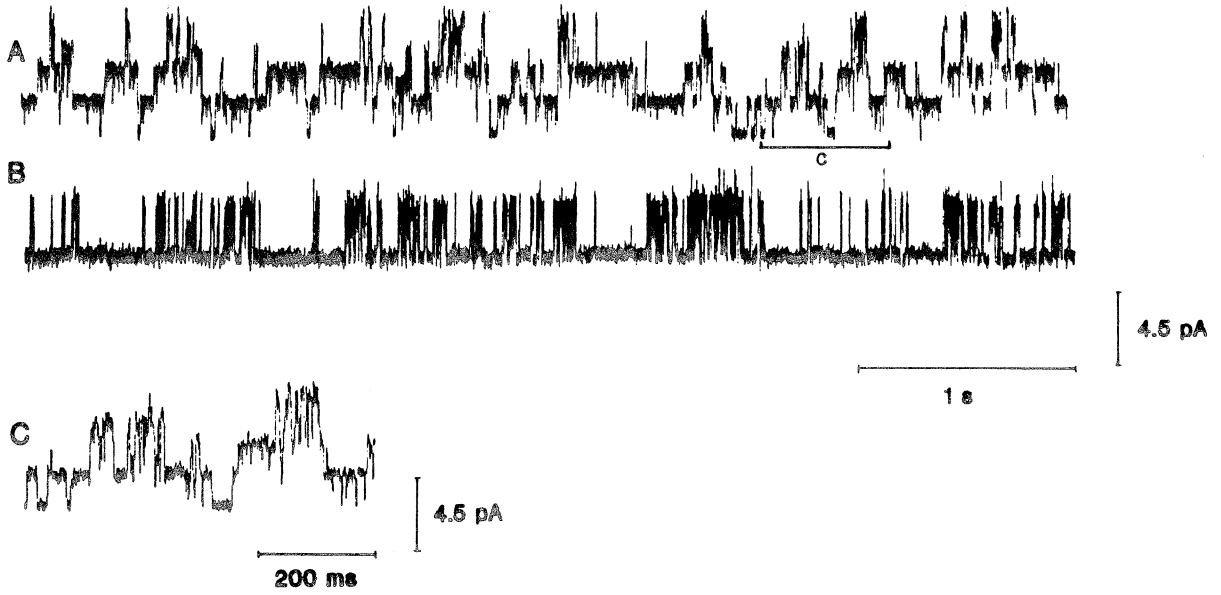

Figure 4. The difference of the dependence of ATP-sensitive and Na^+-activated K^+ channels on pH_i. A: pH_i=7.9, the recording of the currents through two ATP-sensitive K^+ channels (of less conductance) and Na^+-activated K^+ channel (of greater conductance). B: pH_i = 7.9, the current recording through still active Na^+-activated K^+ channel. C: Recording fragment extracted from the A (note different time scale bar). Recordings in the inside-out mode at 5 mV of the membrane potential with MPC1 in the pipette and with the K_i bath solution with 30 mM Na^+. The upward deflections on the recording corresponds to the outward current.

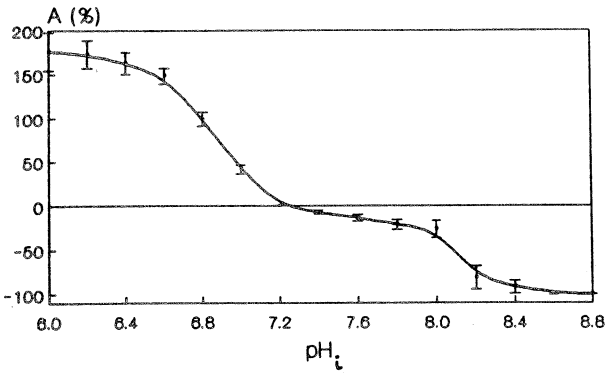

Figure 5. The dependence of the relative change of the ATP-sensitive K^+ channel activity on the pH_i shift. The dependence is constructed from the data received on 8 patches. The vertical bar indicates the Std. Dev.

This fact could be explained by the existence of compartmentalized pools of intracellular ATP with the activity of the channels [35] as well as local changes of ATP concentration in the vicinity of the plasma membrane [26]. Furthermore, periodical dynamics of initial activity of ATP-sensitive K^+ channels in the cell-attached configuration could be considered an argument for the benefit of the existence of ATP concentration oscillations in the vicinity of the sarcolemma. Differences in the ATP sensitivity of this channel in the cell-attached and the inside-out modes appear to be a possible reason for the inconsistency of the $[ATP]_{50\%}$ value with the intracellular ATP level. A similar phenomenon was observed in human ß-cells [2].

The pH_i appeared to be a factor modulating activity of the deblocked channels. The mechanism of K^+ effux modulation at the expense of reversible P_O increase functioned within a wide range of pH_i. Acidosis of the medium in the inner side of the membrane was responsible for a significant P_O increase and alkalosis caused less significant decrease of P_O. The effect of the P_O modulation was more significant than the effect of decrease of the channel conductance by the change of the pH_i. The possibility of similar pH_i modulation of the ATP-sensitive K^+ currents was explained before [8, 21] by a decrease of ATP sensitivity of the channel while lowering intracellular pH_i. We received data demonstrating direct influence of pH_i alterations upon K^+ channel activity. It is supposed that there exist multiple mechanisms of regulation of ATP-sensitive K^+ current by pH (even those affecting contrary changes of this current) in intact cells. Thus, there exist data [6] on increase of the integral outward ATP-sensitive K^+ current by lowering extracellular pH and a decrease of current by intracellular acidosis. Generally, pH_i-sensitivity of the single-channel currents could be compared with those defined before [8, 21].

Thus, the data obtained show that the ATP-sensitive K^+ channels capable to early activation in hypoxic conditions are characterized by neither extremely low affinity be-

tween ATP and site controlling channel blocking, nor by irregularly high sensitivity to intracellular acidosis. It is supposed that mechanism determining different capability of subpopulations of this class of channels in response to cell hypoxic damage should be associated with other intracellular factors, regulating the functioning of ATP-sensitive K^+ channels. These factors should probably include nucleotide [20, 21, 25, 28, 33], G-proteins [3, 19, 25] or protein kinases [15] that are able to regulate these channels activities. The suspicion [15] that a channel has several phosphorylated states with different capabilities to opening was aroused.

If duration of influence of the hypoxia were longer, Na^+-activated K^+ channels would be opening. They did not possess sensitivity to pH_i and $pCa^{2+}{}_i$ changes within physiological ranges. As the damage of the cardiomyocyte was deepening, the Na^+-dependent mechanism of K^+-effux was being superimposed on the ATP-sensitive mechanism possessing a well-developed regulation system. However, ATP-sensitive K^+ channels are able to generate a much larger current than Na^+-activated K^+-channels of higher conductance because of the significant density of the former. K_d value estimated in isolated patches was large enough (50 mM). Taking into consideration similarity of Na^+-dependence of P_O for Na^+-activated K^+ channels in the inside-out and cell-attached modes [18], the conclusion should be reached that these channels are activated only when there is a significant alteration of ion homeostasis in the heart cell. However, data have already appeared [30] that Na^+-activated K^+ channels are able to regulate action potential duration of cardiomyocytes in physiological conditions. It is questionable whether this channel capability of opening could also be variable and depends on factors connected with the functioning state.

Thus, it is obvious that a thorough study of these factors is badly needed to make further progress in investigating the mechanisms of involvement of the studied channels in the cytopathological processes in heart muscle.

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