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Single cardiac outwardly rectifying K^+ channels modulated by protein kinase A and a G-protein

I. Benz, U. Fröbe, and M. Kohlhardt

Physiologisches Institut, Universität Freiburg, Hermann-Herder-Strasse 7, W-7800 Freiburg, Federal Republic of Germany

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Abstract. Elementary K^+ currents were recorded at 19 °C in cell-attached and in inside-out patches excised from neonatal rat heart myocytes. An outwardly rectifying K^+ channel which prevented Na^+ ions from permeating could be detected in about 10% of the patches attaining (at 5 mmol/l external K^+ and between -20 mV and $+20$ mV) a unitary conductance of 66 ± 3.9 pS. $K^+_{(outw.-rect.)}$ channels have one open and at least two closed states. Open probability and τ_{open} rose steeply on shifting the membrane potential in the positive direction, thereby tending to saturate. Open probability (at -7 mV) was as low as 3 ± 1% but increased several-fold on exposing the cytoplasmic surface to Mg-ATP (100 μmol/l) without a concomitant change of τ_{open}. No channel activation occurred in response to ATP in the absence of cytoplasmic Mg^{++}. The cytoplasmic administration of the catalytic subunit of protein kinase A (120–150 μ/ml) or GTP-γ-S (100 μmol/l) caused a similar channel activation. GDP-β-S (100 μmol/l) was also tested and found to be ineffective in this respect. This suggests that cardiac $K^+_{(outw.-rect.)}$ channels are metabolically modulated by both cAMP-dependent phosphorylation and a G-protein.

Key words: Cardiac K^+ channels – Phosphorylation – GTP – GDP – Neonatal rat heart myocytes

Introduction

Cardiac K^+ channels represent a family of ionic channels with distinctly heterogeneous elementary properties that are intimately involved in controlling heart muscle function by setting the resting potential and by influencing the shape of the cardiac action potential. Besides acetylcholine and ATP (Noma 1983), β-adrenergic catecholamines are another physiological modulator of K^+ channel activity and exert their activating effect via the formation of cAMP (for review see Szabo and Otero 1990). As shown in voltage-clamped cardiac Purkinje fibres and isolated myocytes, K^+ conductances sensitive to β-adrenergic stimulation comprise the inwardly rectifying K^+ current (Gadsby 1983), the transient outward current (Nakayama and Fozzard 1988), and the delayed rectifier K^+ current (Walsh et al. 1988). Since, surprisingly, the stimulatory influence of β-adrenergic catecholamines and protein kinase A have been reported to be temperature-dependent (Walsh et al. 1988; Walsh and Kass 1988), at least the delayed rectifier K^+ current seems to be controlled by a cascade of molecular events which are not identical in all respects with the cAMP-dependent phosphorylation in other ionic channels.

Based on permeation properties, two classes of cardiac K^+ channels can be discriminated, an inwardly rectifying K^+ channel with a conductance of 3.6 pS at 5.4 mmol/l external K^+ (Sakmann and Trube 1984) and a non-rectifying K^+ channel with a conductance of about 15 pS at a physiological external K^+ concentration reported to exist in embryonic avian (Clapham and Logothetis 1988) and adult mammalian (Yue and Marban 1988) heart muscle. The latter type represents the candidate which mediates the K^+ conductance during the repolarization phase of the cardiac action potential.

The present patch clamp experiments with neonatal rat heart myocytes newly identified a cardiac K^+ channel with outwardly rectifying properties. Evidence will be represented that its activity is metabolically regulated in that both cAMP-dependent phosphorylation and a G-protein open this K^+ channel.

Methods

Elementary K^+ currents were recorded in cell-attached and in inside-out patches excised from cultured neonatal rate heart myocytes by employing the standard patch clamp technique (Hamill et al. 1981). Cell disaggregation and the handling of the short-time (18–24 h) cultured myocytes have been already described in detail (Kohlhardt et al. 1986). To avoid spontaneous activity, the

* *Offprint requests to:* M. Kohlhardt

myocytes were kept in an isotonic K^+ solution buffered with EGTA so that nominally Ca^{++}-free conditions were achieved. Before excision, the patches were kept for at least 5 min in the cell-attached configuration. Patch pipettes were fabricated from borosilicate glass and had resistances (after filling with pipette solution) between 5 and 8 MΩ. This glass type is of advantage since it has been reported to minimize artifacts in the shape of the iv-relationship (Cota and Armstrong 1988).

Two protocols were applied to trigger elementary K^+ currents: (*i*) setting the membrane potential to values positive or negative to E_{rev}; (*ii*) stepping the membrane from E_{rev} to −10 mV or +33 mV for 370 ms at a rate of 0.2 Hz. In the latter case, the records were idealized by subtracting capacity and leakage currents. The patch clamp recordings were filtered at 1 kHz with an 8-pole Bessel filter, stored on tape and subsequently digitized with a sampling rate of 5 kHz in order to be analyzed. The dead time was 0.2 ms. The single channel analysis concentrated on the open probability (P_0) and on the open and closed time. In analyzing the channel kinetics, the 50% unitary current method (Colquhoun and Sigworth 1983) was used. Histograms were constructed from non-overlapping single channel events and fitted by the least square (x^2) method to yield τ_{open} and τ_{closed}, respectively. P_0 was determined for periods of 2 s in duration in order to give a P_0 profile over the whole life time of an individual patch. The unitary current size was determined from opening events with a minimum life time of 2 ms.

Whenever possible, the data are expressed as mean ±SEM.

Solutions (composition in mmol/l)

A. Isotonic K^+ solution (facing the cytoplasmic membrane surface of the inside-out patches): KCl 140; $MgCl_2$ 2; glucose 20; Hepes 10; EGTA 2; pH 7.4; temperature 19±0.5 °C. *B.* Pipette solution (facing the external membrane surface): KCl 5; NaCl 135; $MgCl_2$ 2; Hepes 10; pH 7.4.

Compounds

All compounds (ATP, catalytic subunit of protein kinase A, GTP-γ-S, GDP-β-S) were purchased from Sigma Chemie, Munich, and freshly dissolved in isotonic K^+ solution just before use. A microinjection device was employed in order to change the internal solution facing the cytoplasmic membrane surface in a jump-like fashion.

Results and discussion

Under quasi-physiological conditions, i.e. in the presence of an asymmetrical K^+ concentration across the membrane with 5 mmol/l at the external side, single K^+ channel events could be detected in the cell-attached as well as in the inside-out mode, which differed in elementary current size and in open time from ATP-sensitive K^+ channels, the latter being the most abundant K^+ channel observed in the present recording conditions. At −7 mV, for example, i_{unit} of the $K^+_{(ATP)}$-channel was 1.6±0.08 pA, in contrast to 2.5±0.11 pA of these particular openings. They reflect a K^+ channel with outward-rectifying properties and were seen in both spherocytes and cardiocytes with a rod-shaped morphology, the latter reflecting a more advanced developmental stage during cell culture. This particular K^+ channel was present in only 10% of a total of about 200 patches. Whether this low incidence is related to the special cell type, neonatal cardiocytes, remains still to be clarified.

Figure 1 demonstrates that outward rectification becomes prominent in a potential range of about 40 mV more positive that the reversal potential, E_{rev}. In this inside-out experiment, E_{rev} was determined to be −85 mV which coincides with the value for E_K calculated from the

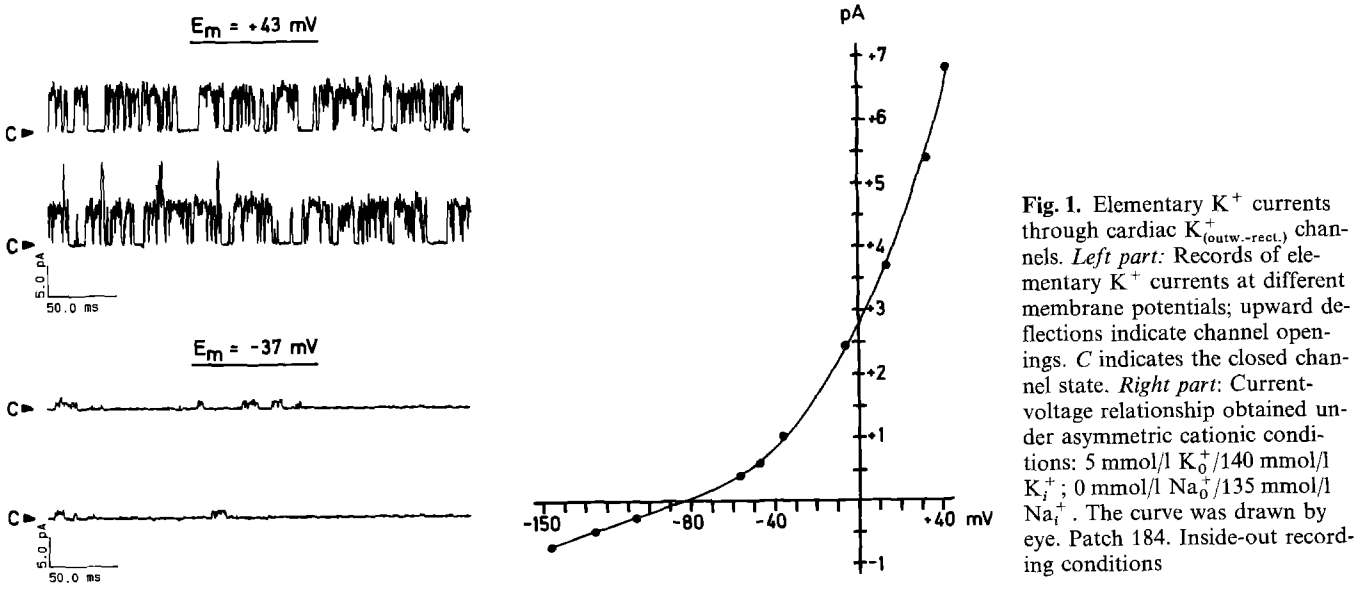

Fig. 1. Elementary K^+ currents through cardiac $K^+_{(outw.-rect.)}$ channels. *Left part:* Records of elementary K^+ currents at different membrane potentials; upward deflections indicate channel openings. *C* indicates the closed channel state. *Right part:* Current-voltage relationship obtained under asymmetric cationic conditions: 5 mmol/l K_o^+/140 mmol/l K_i^+; 0 mmol/l Na_o^+/135 mmol/l Na_i^+. The curve was drawn by eye. Patch 184. Inside-out recording conditions

Nernst equation to be −83 mV. Very similar results were obtained from the iv-relationships of three other inside-out experiments thus confirming outward rectification and K^+ selectivity. In a potential range between −20 mV and +20 mV, $K^+_{(out.-rect.)}$ channels were found to attain, at 5 mmol/l external K^+, a unitary conductance of 66 ± 3.9 pS ($n=4$) and differ in this respect, for example, from $K^+_{(ATP)}$-channels (for review see Ashcroft 1988) and from muscarinic $K^+_{(ACh)}$-channels (Soejima and Noma 1984). Still more interesting is the result that, despite the presence of 2 mmol/l Mg^{++} at the cytoplasmic membrane surface, the channel outwardly rectifies the conductance. This fundamentally contrasts with $K^+_{(ACh)}$-channels (Horie and Irisma 1987) and $K^+_{(ATP)}$-channels (Horie et al. 1987) where cytosolic Mg^{++} causes, by provoking a fast flicker blockade at membrane potentials positive to 0 mV, inward rectification of the conductance.

Membrane depolarization causes channel activation. As studied at −10 mV (see Fig. 2) and +33 mV, activation represents a fast, voltage-independent process. Ensemble averaging and the resultant macroscopic K^+ current revealed that activation proceeds within a few milliseconds (10 ms or even less). Since the macroscopic I_K showed no time-dependent relaxation, $K^+_{(outw.-rect.)}$ channels are obviously lacking an inactivation process. They would share this property with the 15 pS, non-rectifying K^+ channel (Clapham and Logothetis 1988; Yue and Marban 1988). Open probability and thus channel activity during a 370 ms lasting membrane depolarization can vary significantly from one sweep to another, in the experiment depicted in Fig. 2B between 0 and 32%. These P_0 changes seem to be reminiscent of periodical fluctuations, as if the channel activity was being slowly up- and down-regulated.

The gating of the $K^+_{(outw.-rect.)}$ channel is strongly voltage-dependent. In a systematic analysis in cell-free patches facing (cytoplasmically) a Ca^{++}-free environment, P_0 rose steeply at positive membrane potentials (see Fig. 1) and thereby tended to saturate. This behaviour predicts an outwardly rectifying macroscopic K^+ current capable of influencing the shape of the cardiac action potential, particularly in the positive potential range. However, as shown at −7 mV, the open probability is usually only small and amounted, on average, to 3 ± 1% ($n=10$). Although, in a few experiments, channel openings took several ten seconds to occur after patch excision, no systematic changes could be observed even in an extended P_0 analysis of 30 min.

Like P_0, the open state kinetics are also a function of membrane potential (Fig. 3). Open time histogram analysis consistently revealed that $K^+_{(outw.-rect.)}$ channels attain a single open state (Fig. 3A) which became increasingly prolonged when the membrane potential was shifted in the positive direction. An S-shaped $\tau_{open} - E_m$ relationship (Fig. 3B) is well-established to represent the voltage dependence of the exit rate constant which governs the transition from the conducting to a non-conducting channel configuration. Although studied under carefully controlled, identical cell-free recording conditions, τ_{open} was found to vary from one experiment to another. Two classes could be discriminated: at −7 mV, τ_{open} amounted to

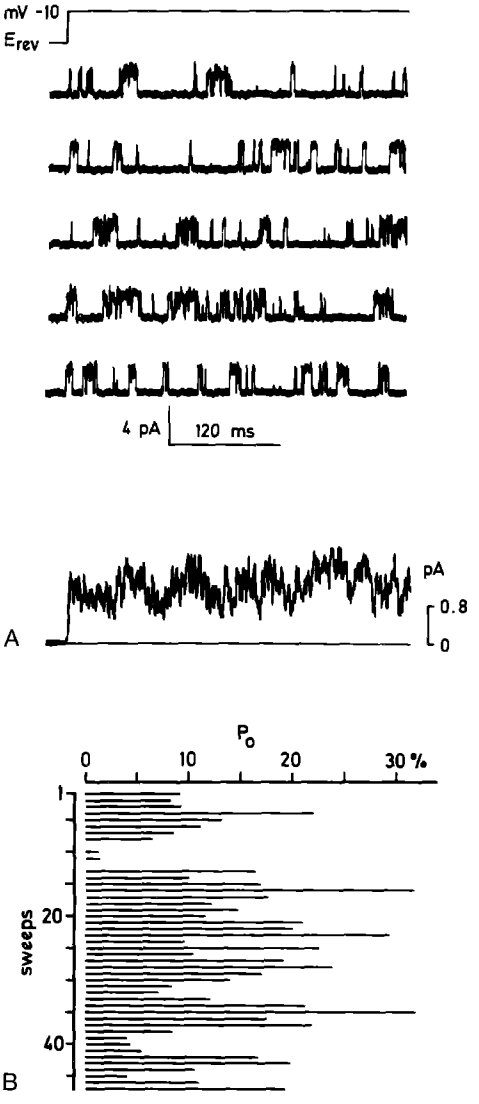

Fig. 2A, B. The response of $K^+_{(outw.-rect.)}$ channels to step depolarizations of the membrane. **A** Non-consecutive selected records of elementary K^+ currents and the ensemble average. The reconstructed macroscopic K^+ current is the ensemble average of 50 consecutive sweeps triggered at 0.2 Hz and separated from each other by an interstimulus interval of 4630 ms in duration. **B** Consecutive P_0 analysis. Each column represents an individual sweep with detectable channel openings; the abscissa numbers the sweeps collected continuously during the stimulation period. P_0 refers to the 370 ms lasting membrane depolarization. Since overlapping events could not be detected, one functioning channel was assumed. Patch 282. Holding potential −84 mV (E_{rev}), step potential −10 mV. Inside-out recording conditions

1.70 ± 0.10 ms in a series of 5 patches, but was only 0.95 ± 0.19 ms in 4 other patches. The same non-uniformity of open state kinetics has been recently reported from neuronal 38 pS K^+ channels (VanDongen et al. 1988) suggesting that heterogeneous kinetics may be a widespread property of K^+ channels.

$K^+_{(outw.-rect.)}$ channels can attain at least two closed states. A closed time analysis at +43 mV revealed a short-lasting closed state with a time constant in the submillisecond range and a long-lasting one having a time constant of about 5 ms. Thus, the channel can be mod-

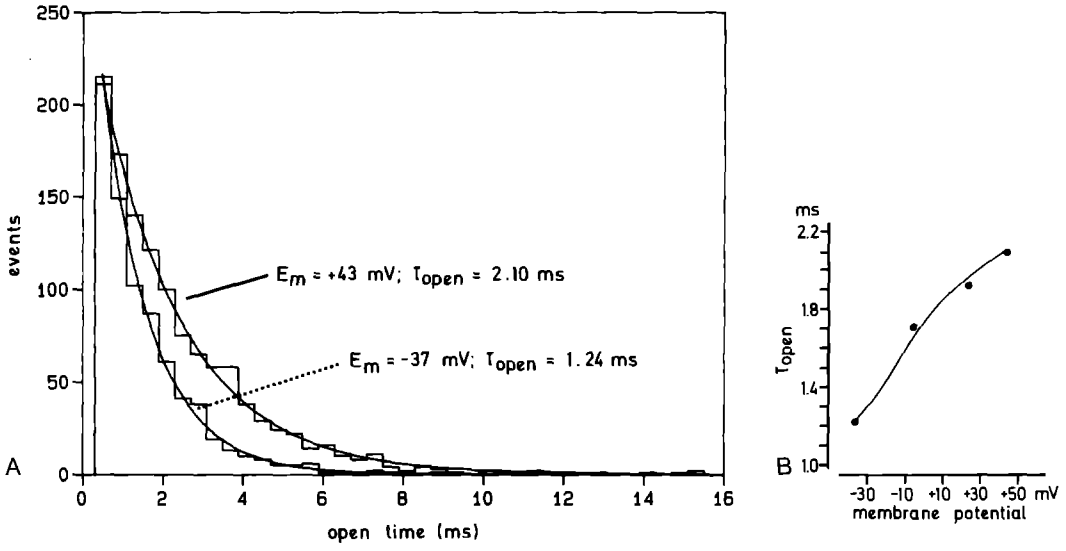

Fig. 3A, B. Voltage-dependent open state kinetics of cardiac $K^+_{(outw.-rect.)}$ channels. **A** open time histograms at different membrane potentials. By disregarding the first bin of 0.4 ms, the histograms could be best fitted by $N=316\exp(-t/0.00124)$ at -37 mV and by $N=266\exp(-t/0.00210)$ at $+43$ mV. **B** The dependence of τ_{open} on membrane potential. The curve was drawn by eye. Patch 184. Inside-out recording conditions

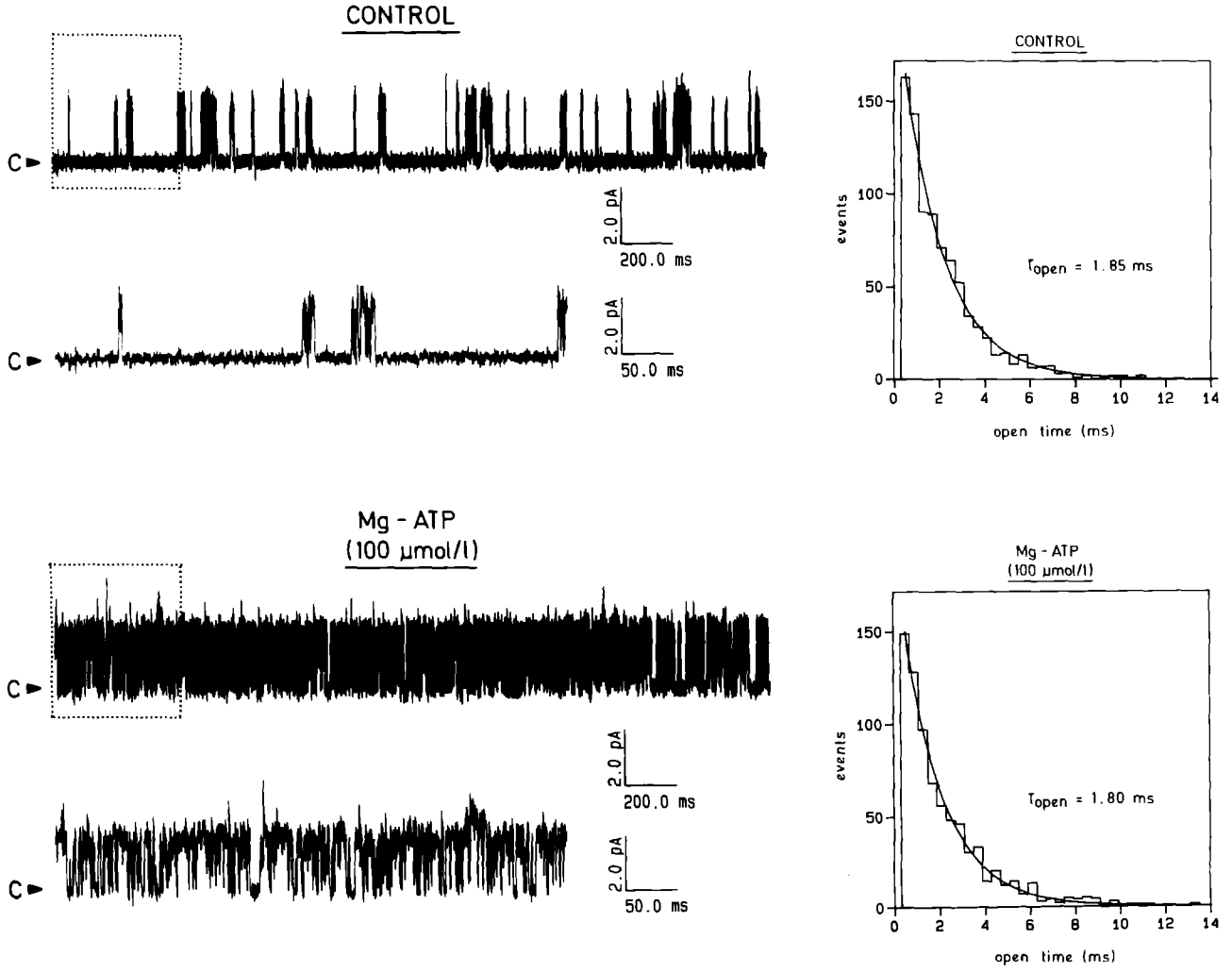

Fig. 4. The influence of ATP (100 µmol/1) on cardiac $K^+_{(outw.-rect.)}$ channels. *Left part:* Records of elementary K^+ currents before (*upper part*) and after (*lower part*) cytoplasmic ATP application in the simultaneous presence of Mg^{++} ions. That part of the records indicated by a dashed-line window is presented on an extended time scale (time scales as indicated in the records) to resolve single channel events in greater detail. Upward deflections mean channel openings; *C* indicates the closed channel state. *Right part:* Open time histograms of the same experiment before and after ATP application. The histograms could be best fitted by $N=216\exp(-t/0.00185)$ and by $N=198\exp(-t/0.00180)$, respectively. Patch 158. Membrane potential -7 mV. Inside-out recording conditions

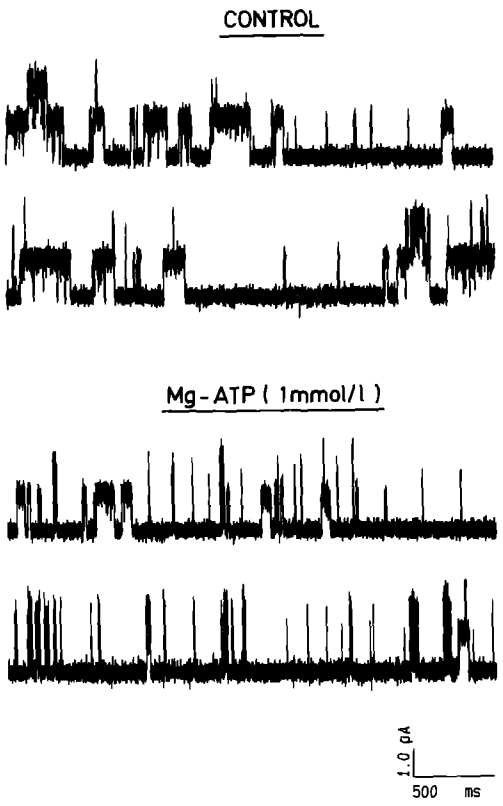

Fig. 5. Blockade of $K^+_{(ATP)}$-channels and activation of $K^+_{(outw.-rect.)}$ channels by 1 mmol/l ATP. Elementary K^+ current recordings in the absence (upper part; control) show openings of at least two individual $K^+_{(ATP)}$-channels. Note two classes of openings with an i_{unit} of 1.5 pA and 2.7 pA in the presence of 1 mmol/l ATP (lower part) and the gradual disappearance of the 1.5 pA seized events. Patch 84. Membrane potential −7 mV. Inside-out recording conditions

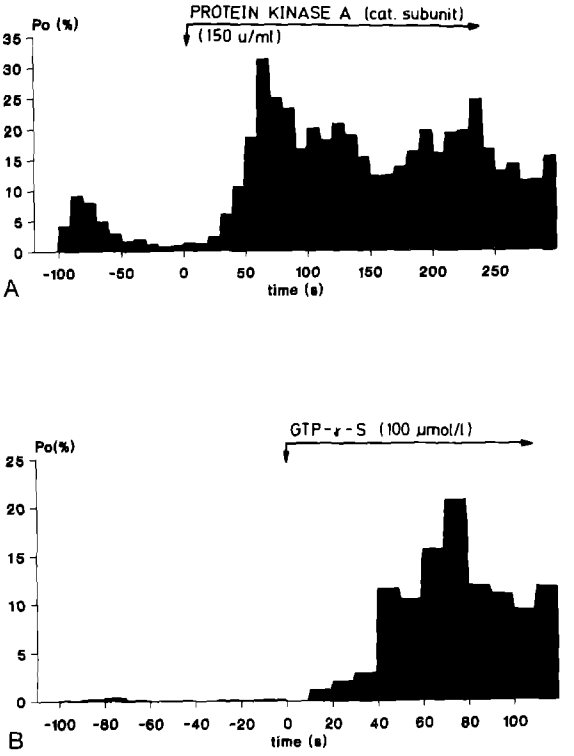

Fig. 6. A The influence of catalytic subunit of protein kinase A (150 u/ml) on open probability (P_0) of cardiac $K^+_{(outw.-rect.)}$ channels. At zero time, the cytosolic membrane surface was exposed to the catalytic subunit of protein kinase A in the simultaneous presence of Mg^{++} and 10 µmol/l ATP. Patch 53; membrane potential −7 mV. Inside-out recording conditions. **B** The influence of GTP-γ-S (100 µmol/l) on open proability (P_0) of cardiac $K^+_{(outw.-rect.)}$ channels. At zero time, the cytosolic membrane surface was exposed to GTP-γ-S in the simultaneous presence of Mg^{++}. Patch 85; membrane potential −7 mV. Inside-out recording conditions

elled by a Markovian scheme such as $C_2 - C_1 - 0$. Unfortunately, the voltage dependence of the closed state kinetics could not be analyzed in detail because the presence of more that one channel in most of the patches led to serious problems in defining the nature of long-lasting shut episodes as they occur with increasingly likelihood in a potential range more negative than +20 mV.

ATP exerts an activating effect on cardiac $K^+_{(outw.-rect.)}$ channels. When, after an initial control period of 5 min, the cytoplasmic membrane surface was exposed to 100 µmol/l of this adenine nucleotide, P_0 increased within some ten seconds in order to attain a several-fold increased level (Fig. 4). On average, maximal P_0 rose by a factor of 5.2 ± 1.8 ($n = 2$). Three experiments with the non-hydrolyzable ATP analogue, ATP-γ-S (100 µmol/l) showed a very similar response. Smaller ATP concentrations (10 µmol/l) proved to be ineffective, as shown in three other experiments. No P_0 response could be obtained when, as seen in 3 inside-out patches, 100 µmol/l ATP was administered in the absence of 2 mmol/l cytosolic Mg^{++}. It is, therefore, the Mg-ATP complex which finally induces channel activation. Figure 4 also demonstrates that even a strongly pronounced P_0 increase can develop without a concomitant change of the open state kinetics: τ_{open} changed insignificantly to $98 \pm 2\%$ ($n = 2$) of the control value.

ATP concentrations higher than 100 µmol/l were also found to be activating. As studied in a range between 1 and 5 mmol/l, ATP had a differential effect on $K^+_{(ATP)}$-channels and $K^+_{(outw.-rect.)}$ channels. Figure 5 demonstrates an inside-out patch where both K^+ channels coexisted: the former became blocked within a few seconds after administration of 1 mmol/l ATP but the latter responded with an activation and, thus, only became detectable.

To further stress the idea that Mg-ATP exerts its activating effect via a phosphorylation reaction at the channel protein, the influence of the catalytic subunit of protein kinase A was studied. Again, the patches were kept throughout at −7 mV to detect an eventual co-activation of contaminating Cl^- channels ($E_{Cl} = 0$ mV in the present experiments). The patches were excised in a solution containing 10 µmol/l Mg-ATP which served as phosphate donor but, as mentioned above, was unable to increase P_0. In fact, as shown in Fig. 6A, the cytoplasmic administration of the catalytic subunit of protein kinase A (120–150 u/ml) led to a strong increase of P_0 within some ten seconds. It was a regular finding that P_0 attained a labile steady state meaning that more or less periodical P_0 fluctuations occurred (Fig. 6A). On average, the maximal P_0 increased by a factor of 6.4 ± 0.2 ($n = 3$). τ_{open}, on the other hand, remained unaffected.

This metabolic susceptibility makes $K^+_{(outw.-rect.)}$ channels basically sensitive to β-adrenergic stimulation of heart muscle cells. The stimulatory effect of β-adrenergic catecholamines on K^+ conductance is mediated by cAMP (Tsien et al. 1972; Kass and Wiegers 1982; Nerbonne et al. 1984; Bennett et al. 1986). Phosphorylation by the cAMP-dependent protein kinase and the resultant net phosphate gain of a not yet identified channel domain can be proposed to activate the $K^+_{(outw.-rect.)}$ channel to an extent that may well be of functional relevance for the shape of the cardiac action potential and, by shortening of its plateau phase, can help in controlling transmembrane Ca^{++} influx via L-type Ca^{++} channels.

G-proteins have been recognized as being involved in the gating process of many ionic channels (for review see Brown et al. 1990). There is growing evidence that some metabolically regulated ionic channels, including cardiac L-type Ca^{++} channels (Yatani et al. 1987) and voltage-dependent Na^+ channels (Schubert et al. 1989) with a gating process which is modulated by phosphorylation, are additionally controlled by a direct interaction with a G-protein. Therefore, the effect of the non-hydrolyzable GTP analogue, GTP-γ-S (100 µmol/l) was tested in the cytoplasmic presence of 2 mmol/l Mg^{++}. Before GTP administration, the patches had been kept for 5 min in cell-free conditions in order to wash-out a possible contamination with cellular metabolites. Figure 6B shows that $K^+_{(outw.-rect.)}$ channels responded sensitively with an activation to GTP-γ-S, the maximal P_0 rose by a factor of 8.1 ± 1.4 ($n=3$). Open state kinetics, on the other hand, remained unaffected. GDP-β-S, (100 µmol/l), which preserves G-proteins in their inactivated configuration, failed in three other inside-out patches, likewise after cytoplasmic administration, to affect P_0. This supports the assumption that the channel activation seen with GTP-γ-S reflects a stimulatory influence of a G-protein on the $K^+_{(outw.-rect.)}$ channel.

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