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Distinct Modes of Blockade in Cardiac ATP-sensitive K^+ Channels Suggest Multiple Targets for Inhibitory Drug Molecules

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Abstract. Elementary K^+ currents were recorded at 19°C in inside-out patches from cultured neonatal rat cardiocytes to elucidate the block phenomenology in cardiac ATP-sensitive K^+ channels when inhibitory drug molecules, such as the sulfonylurea glibenclamide, the phenylalkylamine verapamil or sulfonamide derivatives (HE 93 and sotalol), are interacting in an attempt to stress the hypothesis of multiple channel-associated drug targets.

Similar to their adult relatives, neonatal cardiac $K_{(ATP)}$ channels are characterized by very individual open state kinetics, even in cytoplasmically well-controlled, cell-free conditions; at −7 mV, $\tau_{open(1)}$ ranged from 0.7 to 4.9 msec in more than 200 patches and $\tau_{open(2)}$ from 10 to 64 msec—an argument for a heterogeneous channel population. Nevertheless, a common response to drugs was observed. Glibenclamide and the other inhibitory molecules caused long-lasting interruptions of channel activity, after cytoplasmic application, as if drug occupancy trapped cardiac $K_{(ATP)}$ channels in a very stable, nonconducting configuration. The resultant NP_o depression was strongest with glibenclamide (apparent IC_{50} 13 nmol/liter) and much weaker with verapamil (apparent IC_{50} 9 μmol/liter), HE 93 (apparent IC_{50} 29 μmol/liter) and sotalol (apparent IC_{50} 43 μmol/liter) and may have resulted from the occupancy of a single site with drug-specific affinity or of two sites, the high affinity glibenclamide target and a distinct nonglibenclamide, low affinity target.

Changes in open state kinetics, particularly in the transition between the O_1 state and the O_2 state, are other manifestations of drug occupancy of the channel. Any inhibitory drug molecule reduced the likelihood of attaining the O_2 state, consistent with a critical reduction of the forward rate constant governing the O_1–O_2 transition. But only HE 93 (10 μmol/liter) associated (with an apparent association rate constant of 2.3×10^6 mol^{-1} sec^{-1}) to shorten significantly $\tau_{open(2)}$ to 60.6 ± 6% of the predrug value, not the expected result when the entrance in and the exit from the O_2 state would be drug-unspecifically influenced. Sotalol found yet another and definitely distinctly located binding site to interfere with K^+ permeation; both enantiomers associated with a rate close to 5×10^5 mol^{-1} sec^{-1} with the open pore thereby flicker-blocking cardiac $K_{(ATP)}$ channels. Clearly, these channels accommodate more than one drug-binding domain.

Key words: Single cardiac K_{ATP} channels — Sulfonylureas — Verapamil — Sulfonamide derivatives — Elementary properties — Channel-associated drug targets

Introduction

ATP-sensitive K^+ channels initially detected in heart muscle (Noma, 1983) and subsequently identified in many other excitable and nonexcitable tissues including insulin-secreting pancreatic β-cells (Cook & Hales, 1984), skeletal muscle (Spruce, Standen & Stanfield, 1987), smooth muscle (Standen et al., 1989) or in renal epithelium (Wang & Giebisch, 1991) may play an outstanding role in coupling energy metabolism to cellular electrophysiological events. Within the superfamily of K^+ channels, they represent a class of voltage-independent, ligand-operated ionic channels that open in response to a critical cellular ATP depletion and are sensitively modulated by both phosphorylation (Ohno, Zunkler & Trube, 1987; Takano, Qin & Noma, 1990) and a G protein (Parent & Coronado, 1989; Kirsch et al., 1990) acting at the cytoplasmic channel surface to enhance open probability. Once activated in anoxic myocardium (Benndorf, Friedrich & Hirche, 1991), $K_{(ATP)}$ channels generate a background K^+ current (Vleugels,

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Vereecke & Carmeliet, 1980) that may help maintain resting potential and, perhaps more importantly from a pathophysiological viewpoint, underlie the shortening of action potential duration in metabolically exhausted cardiac cells. Considering the functional evidence of $K_{(ATP)}$ channels for insulin secretion in pancreatic β-cells and ischemic myocardium, Cook et al. (1988) and Faivre and Findlay (1990) argued in favor of a spare channel hypothesis. Accordingly, a vanishing small fraction of about 1% (Nichols & Lederer, 1991) is functionally involved in generating transmembrane K^+ currents while the overwhelming majority remains silent, a not yet fully understood phenomenon that disagrees also with any economic principle in expression and synthesis of channel proteins.

The structure of $K_{(ATP)}$ channels remains to be determined. Recent cloning and expression experiments in rat kidney (Ho et al., 1993) identified a ROMK1 protein with a unique motif—two membrane-spanning domains and connected by the conserved, probably pore-forming H5 region—fundamentally different from the basic structural design in voltage-gated K^+ channels. Potential phosphorylation sites are accommodated in the adjacent cytosolic N- and C-terminal regions and a Walker type-A motif, a putative ATP binding site, in the latter region. Although ROMK1 channels share important elementary properties with native $K_{(ATP)}$ channels when expressed in oocytes, they are not blocked by millimolar ATP concentrations.

The pharmacological susceptibility of cardiac $K_{(ATP)}$ channels is characterized by a high sensitivity towards two distinct classes of drugs with opposite effects: K^+ channel openers represented by benzopyranes or some pyridine analogues, and the inhibitory sulfonylureas (*for review, see* Edwards & Weston, 1993) often argued to act as specific blockers that interfere with $K_{(ATP)}$ channels with a Hill coefficient of close to 1. Although the molecular block mechanism is unknown, sulfonylureas are supposed to find a channel-associated binding site whose drug occupancy reduces channel activity (the most obvious functional result of sulfonylurea interference). Other inhibitory organic molecules like verapamil (Kimura et al., 1992) or quinidine (Undrovinas et al., 1990), which are less specific blockers of K^+ conductance in heart muscle, have the same effect. From a biophysical point of view, this suggests that $K_{(ATP)}$ channels respond rather monotonically to blocking drugs. This response is surprising compared to the reaction of another member of the cardiac K^+ channel family, the outwardly rectifying 66 pS K^+ channel with a complex block phenomenology (Benz & Kohlhardt, 1994*a*), and points to the existence of multiple drug-sensitive properties of this 66 pS channel.

The present inside-out patch clamp experiments concentrated on this problem, the responsiveness of cardiac $K_{(ATP)}$ channels to blocking drugs. In identifying sulfonamide derivatives as yet other blocking molecules besides glibenclamide and verapamil the gating process as well as K^+ permeation were identified to be drug sensitive, too, supporting evidence for the hypothesis that cardiac $K_{(ATP)}$ channels bear multiple drug receptors.

Materials and Methods

Elementary K^+ currents through ATP-sensitive K^+ channels from cultured neonatal rat cardiocytes were recorded in the on-cell and the inside-out mode by using the standard patch clamp technique (Hamill et al., 1981) and an L-M/EPC 5 amplifier. Tissue disaggregation as well as cultivating and handling of the short-time (18–24 hr) cultivated cardiocytes were essentially the same as described in detail elsewhere (Kohlhardt, Fichtner & Fröbe, 1989). Of the two fractions, spherocytes and rod-shaped cells existing in cell culture, the latter type was selected for the patch clamp experiments to analyze $K_{(ATP)}$ channels in a uniform cell population at the more advanced developmental stage.

On-cell recording conditions represent a modified cell-attached mode obtained after cell permeabilization where the internal membrane surface faces an abnormal cytosolic environment but the channels remain *in situ.* Cell permeabilization was achieved by gentle horizontal vibrations of the patch pipette and became manifest as cytoplasmic hypergranulation, a rapidly developing morphological indicator of cell damage. Nevertheless, the patches remained stable for several minutes, long enough to study $K_{(ATP)}$ channels.

Elementary K^+ currents were recorded (usually at −7 mV) at an asymmetrical cationic milieu with a quasi-physiological K^+ concentration gradient (5 mmol/liter external K^+; 140 mmol/liter internal K^+) as outward currents. The records were filtered at 1 kHz with an 8-pole Bessel filter, stored on tape and digitized with a sampling rate at 5 kHz for analysis (dead time of the apparatus was 0.2 msec). The single channel analysis concentrated on i_{unit}, NP_o, open time and, in a few experiments with apparently only one functioning channel, closed time. By setting a threshold at 50% i_{unit} (Colquhoun & Sigworth, 1983) open and closed times were analyzed. By neglecting the first bin of 0.4 msec, τ_{open} and τ_{closed} resulted from the best fit of the respective histograms constructed from nonoverlapping events. In experiments without superpositions, $\tau_{closed(1)}$ and $\tau_{closed(2)}$ were determined to obtain the discriminating criterion for a burst analysis, the critical gap time that distinguishes between gaps within and gaps between bursts and resulted from the geometric mean of $\tau_{closed(1)}$ and $\tau_{closed(2)} \cdot i_{unit}$ was obtained from Gaussian event distributions; NP_o (where N means the number of channels and P_o open probability) was continuously determined for intervals mostly of 30 sec in duration to yield NP_o profiles.

Apparent IC_{50} values for glibenclamide, verapamil, HE 93 and sotalol were calculated from

$$IC_{50} = [\text{blocking agent}] \times \frac{NP_{o(\text{drug})}}{NP_{o(\text{control})} - NP_{o(\text{drug})}}.$$

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

Solutions (composition in mmol/liter)

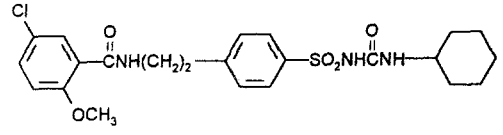
(A) Isotonic K^+ solution (used as bathing solution of the cardiocytes and facing the cytoplasmic membrane surface in the inside-out exper-

iments): KCl 140 (or 70 plus 70 sucrose); $MgCl_2$ 2; glucose 20; HEPES 10; EGTA 2; pH 7.4. Temperature 19 ± 0.5°C. (B) Pipette solution (facing the external side of the membrane): KCl 5; NaCl 135; $MgCl_2$ 2; HEPES 10; pH 7.4.

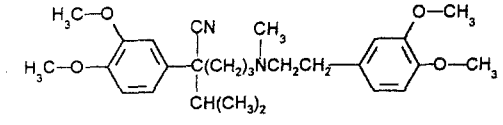
Compounds

ATP, ADP, GTP and GDP were purchased from Sigma Chemical (Munich) and freshly dissolved just before use in solution A. Glibenclamide and verapamil were purchased from Sigma, sotalol and both its enantiomers were kindly provided by Bristol-Myers (Munich) and HE 93 by Helopharm KG (Berlin). Sotalol was dissolved in dimethylsulfoxide (Sigma) to give a stock solution appropriate amounts of which were added to solution A just before use.

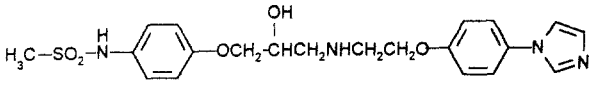
The structure formulae of the drugs used are as follows:



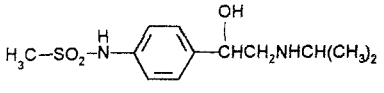
GLIBENCLAMIDE



VERAPAMIL



HE93



SOTALOL

Except glibenclamide, all other drugs were available in the hydrochloride form. A microinjection device allowed a sudden solution change and drug exposure of the cytoplasmic membrane surface governed only by the drug molecule diffusion through the aperture of the patch pipette; a major time lag was unlikely to happen since, for example, internal TEA blockade in cardiac, outwardly rectifying K^+ channels (Benz & Kohlhardt, 1994*a*) developed quasi-instantaneously when TEA was applied by using the same microinjection device.

Results

Properties of Cardiac ATP-sensitive K^+ Channels from Neonatal Cardiocytes

A first series of experiments dealt with elementary properties of neonatal cardiac $K_{(ATP)}$ channels, the less precisely identified type when compared with its adult relatives. Neonatal cardiac $K_{(ATP)}$ channels became activated just after cell permeabilization or on patch excision, but were blocked as expected when exposed cytosolically to a near-millimolar concentration of ATP. With 5–7 MΩ patch pipettes, opening events, mostly arising from multiple, (up to four) individual channels, were detected in about 70% of the patches, thus suggesting a considerable channel density. At 5 mmol/liter external K^+, the unitary conductance amounted to 23 ± 2.4 pS ($n = 5$), close to the value for γ in adult cardiac $K_{(ATP)}$ channels at comparable ionic conditions (Kakei & Noma, 1984). With 135 mmol/liter external Na^+, the coincidence between the reversal potential (−80 ± 1.2 mV; $n = 5$) and E_K (−83 mV) indicates K^+ selectivity. Inward rectification was observed in the presence of 2 mmol/liter internal Mg^{2+} or after cytosolic administration of 20 mmol/liter Na^+, but an ohmic conductance between E_{rev} and +40 mV occurred on removal of Mg^{2+} or Na^+, respectively, just as in the adult channel isoform (*for review, see* Noma & Tanako, 1991). Open time histogram analysis in more than 200 mostly inside-out patches consistently revealed a bimodal event distribution with values for $\tau_{open(1)}$ between 0.7 and 4.9 msec and for $\tau_{open(2)}$, which varied in a similarly broad range, between 10 and 64 msec. There was no correlation between the number of functional channels and $\tau_{open(2)}$ in an individual patch. At least two closed states, one in the submillisecond range and the other several-fold longer, could be attained. For the reasons mentioned above, however, a precise analysis of the closed state kinetics, including a more prolonged, probably third closed configuration, was not feasible.

Figure 1 demonstrates that $NP_{o(max)}$ strongly depends on the recording conditions, on-cell or inside-out. Maximal activity occurred just after cell permeabilization or patch excision, respectively, and was 0.58 ± 0.11 ($n = 25$) in the former recording mode but only 0.13 ± 0.03 ($n = 40$) in the latter, while the estimated value for N in an individual patch differed insignificantly (2.0 ± 0.2 *vs.* 1.7 ± 0.2). NP_o may gradually decline with time to approach a steady-state within 5 min. Rundown in channel activity was mostly accompanied by changes of open state kinetics in that $\tau_{open(2)}$ decreased and the ratio

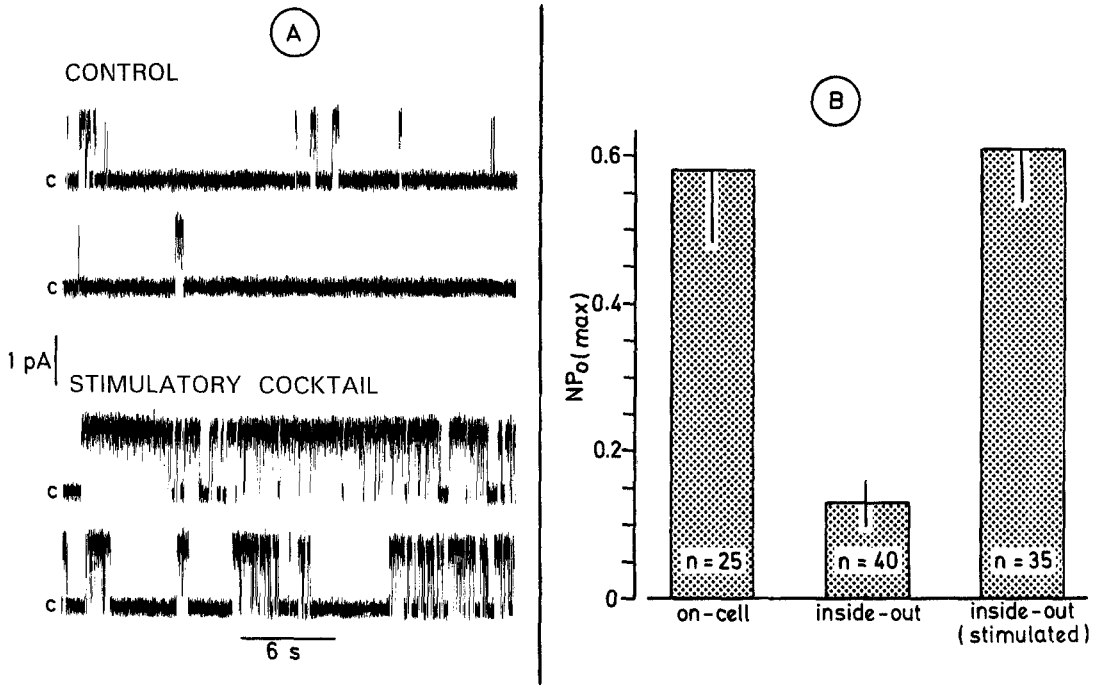


Fig. 1. (*A*) Activation of isolated cardiac $K_{(ATP)}$ channels by the cytoplasmic administration of a stimulatory cocktail consisting of Mg-ATP (50 μmol/liter), ADP (100 μmol/liter), GDP (100 μmol/liter) and GTP (100 μmol/liter). The control records were taken 4 min after patch excision. (*c*) closed configuration; upward deflections indicate outward currents. Patch 372; membrane potential –7 mV. (*B*) Comparison of $NP_{o(max)}$ of cardiac $K_{(ATP)}$ channels at different recording conditions (on-cell, inside-out without, and inside-out (stimulated) with stimulatory cocktail). Each column represents the mean of experiments as indicated; vertical bars mean SEM. Note that patch pipettes with a rather uniform resistance (5–7 MΩ) were used for the experiments.

of second-open state events to first-open state events declined. This ratio was calculated from

$$\int_{0.2\ \text{msec}}^{\infty} a\tau_{open(2)} \exp(-0.2/\tau_{open(2)}) / \int_{0.2\ \text{msec}}^{\infty} a\tau_{open(1)} \exp(-0.2/\tau_{open(1)})$$

and refers to the number of open events.

This rundown seems to be primarily related to a washout of some cytosolic metabolites. Reactivation occurred promptly when the internal membrane surface was exposed to an isotonic K^+ solution supplemented with ATP (10 μmol/liter), ADP (100 μmol/liter), GTP (100 μmol/liter) and GDP (100 μmol/liter) (Fig. 1*A*). No attempts were done to define the minimal composition of this cocktail that was necessary to stimulate $K_{(ATP)}$ channels and keep them active. Interestingly, after excision in this stimulatory cocktail, $NP_{o(max)}$ no longer differed from the value obtained in on-cell experiments (*see* Fig. 1*B*) and rundown proceeded much more slowly and less effectively. Nevertheless, there remained strong interindividual variations of $NP_{o(max)}$ and $\tau_{open(2)}$, as observed in the inside-out recording mode in the absence of the stimulatory cocktail (Fig. 2*A* and *B*). Since the estimated $P_{o(max)}$ appears to be a function of $\tau_{open(2)}$ (*see* Fig. 2), it is tempting to conclude that the dwell time in the prolonged O_2 state may govern open probability, at least under conditions where inhibitory influences are virtually absent. Similarly diverging open state kinetics were reported from adult $K_{(ATP)}$ channels in guinea pig myocytes after activation by substrate-free anoxia (Benndorf et al., 1991).

The Inhibitory Influence of Glibenclamide and the Structurally Unrelated Verapamil

To stabilize the activity of $K_{(ATP)}$ channels in cell-free conditions, they were cytosolically exposed throughout to solutions containing the stimulatory cocktail during the initial control period of 2 min, a subsequent drug administration for 1–2 min and a final drug washout lasting 2 min. A possible rundown under predrug conditions was assessed from the NP_o profile and assumed to be negligible when NP_o failed to decline during the last 60 sec of the control period preceding drug application.

As demonstrated in Fig. 3, the cytoplasmic application of glibenclamide (50 nmol/liter) reduced the activity of neonatal cardiac $K_{(ATP)}$ channels. The inhibitory ef-

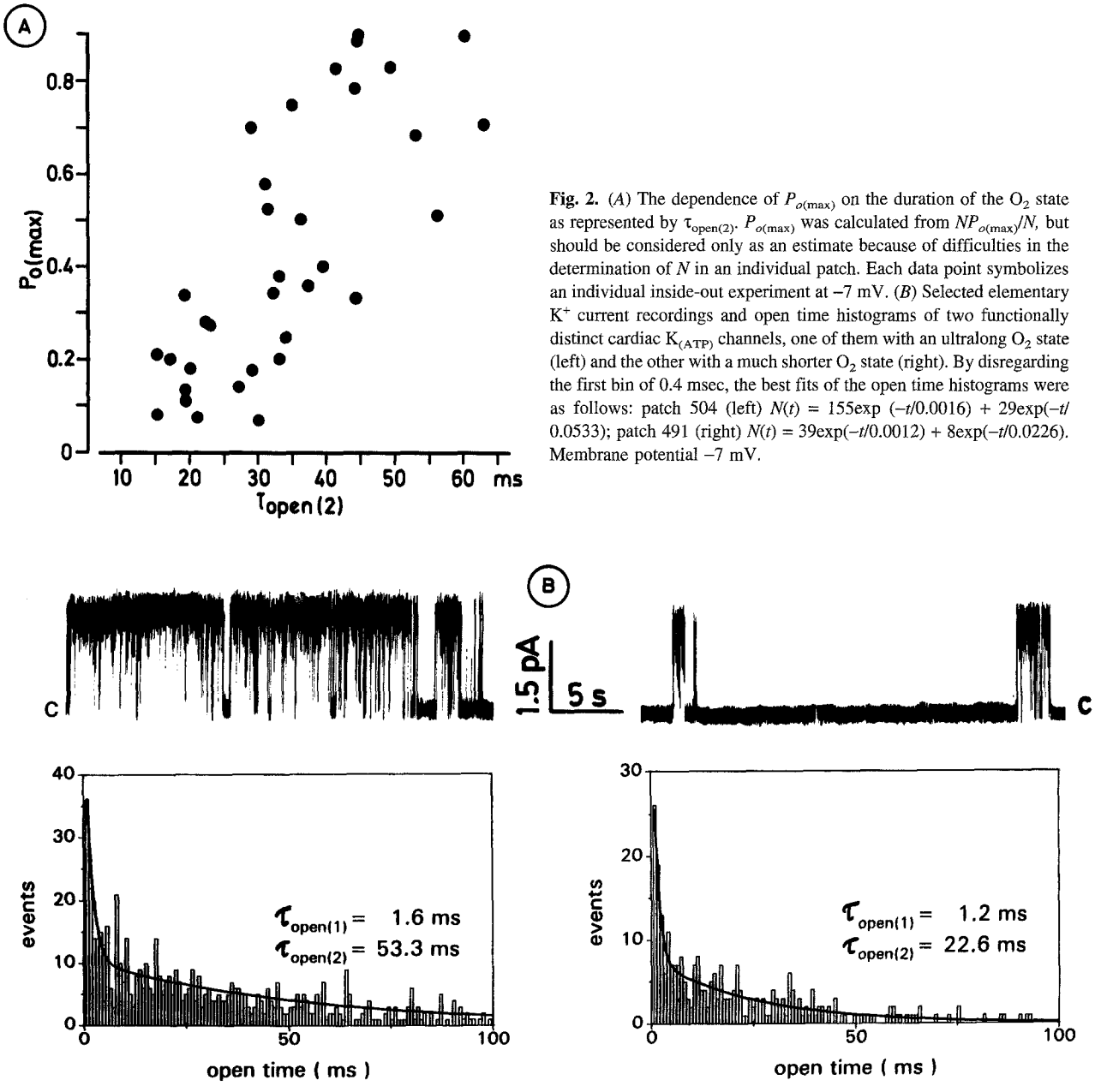


Fig. 2. (*A*) The dependence of $P_{o(max)}$ on the duration of the O_2 state as represented by $\tau_{open(2)}$. $P_{o(max)}$ was calculated from $NP_{o(max)}/N$, but should be considered only as an estimate because of difficulties in the determination of N in an individual patch. Each data point symbolizes an individual inside-out experiment at −7 mV. (*B*) Selected elementary K^+ current recordings and open time histograms of two functionally distinct cardiac $K_{(ATP)}$ channels, one of them with an ultralong O_2 state (left) and the other with a much shorter O_2 state (right). By disregarding the first bin of 0.4 msec, the best fits of the open time histograms were as follows: patch 504 (left) $N(t)$ = 155exp (−t/0.0016) + 29exp(−t/0.0533); patch 491 (right) $N(t)$ = 39exp(−t/0.0012) + 8exp(−t/0.0226). Membrane potential −7 mV.

fect on NP_o took several 10 sec to be accomplished, in the experiment illustrated in Fig. 3 about 30 sec (*see* Fig. 3*B*). This slow onset of channel blockade is remarkable with respect to the apparently free drug diffusion from the bulk phase through the aperture of the patch pipette to the internal surface of the patched membrane. This might be indicative of a delayed drug access to the channel-associated binding site or, alternatively, of a slow on-rate for the channel-drug association. The delayed block onset was consistently found in seven glibenclamide experiments and agrees with recent observations in adult cardiac $K_{(ATP)}$ channels reactivated by the K^+ channel opener SR 44866 (Findlay, 1992). Within 60 sec, NP_o declined to 19.9 + 5.7% (n = 7) of the control (i.e., mean NP_o during the last 60 sec of the control period). The calculated IC_{50} value of 13 nmol/liter is in the same order of magnitude as the value reported from whole-cell clamped ventricular myocytes (Findlay, 1992) where SR 44866 was used to activate $K_{(ATP)}$ channels, seemingly an important argument against a major overlapping of the inhibitory glibenclamide effect by an uncontrolled rundown. It should be emphasized, however, that the efficacy of glibenclamide depends on the composition of the cytosolic environment, specifically on the ATP and ADP level and also on the ATP/ADP concentration ratio (Virag, Furukawa & Hiraoka, 1993), i.e., the IC_{50} value obtained in the present inside-out experiments with very specific cytosolic environmental conditions will barely

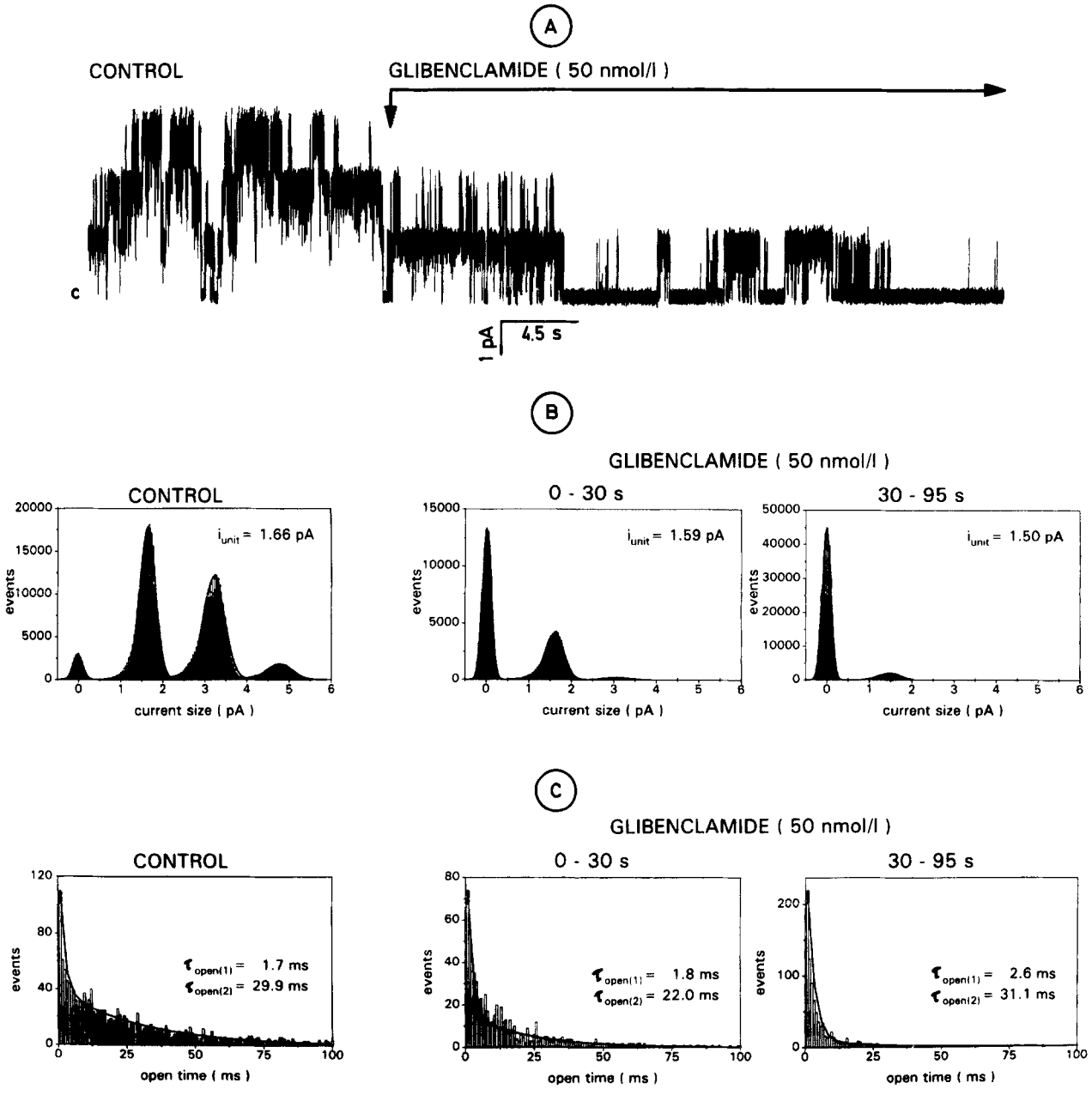


Fig. 3. The influence of glibenclamide (50 nmol/liter) on isolated cardiac $K_{(ATP)}$ channels. (*A*) Continuous records of elementary K^+ currents (*c* means closed configuration) before and after cytosolic drug application. (*B*) Amplitude histograms under control conditions, at an early (0–30 sec) and a later (30–95 sec) stage of drug administration. (*C*) Open time histograms before and after glibenclamide treatment. By disregarding the first bin of 0.4 msec, the best fits were as follows: Control $N(t)$ 121exp(−t/0.0017) + 30exp(−t/0.0299); glibenclamide (0–30 sec) $N(t)$ = 96exp(−t/0.0018) + 16exp(−t/0.0220); glibenclamide (30–95 sec) $N(t)$ = 298exp(−t/0.0026) + 8exp(−t/0.0311). Patch 760; membrane potential −7 mV.

provide a general measure of the glibenclamide sensitivity of $K_{(ATP)}$ channels.

Glibenclamide exerted its inhibitory effect without major changes of i_{unit} (*see* Fig. 3*B*): i_{unit} was 1.56 ± 0.03 pA before and 1.54 ± 0.02 pA (n = 7) after drug treatment. Since concentrations higher than 100 nmol/liter almost completely suppressed channel activity, their potential influence on K^+ permeation remains unknown.

Analogously to tolbutamide-treated $K_{(ATP)}$ channels from rat pancreatic β-cells (Gillis et al., 1989), open state kinetics also remained unaffected. In the experiment depicted in Fig. 3, for example, $\tau_{open(2)}$ was 29.9 msec under predrug conditions and 31.1 msec in a later stage of drug administration. At first glance, the increase in $\tau_{open(1)}$ from 1.7 to 2.6 msec seems paradoxical and is most probably related to analytical problems. In fact,

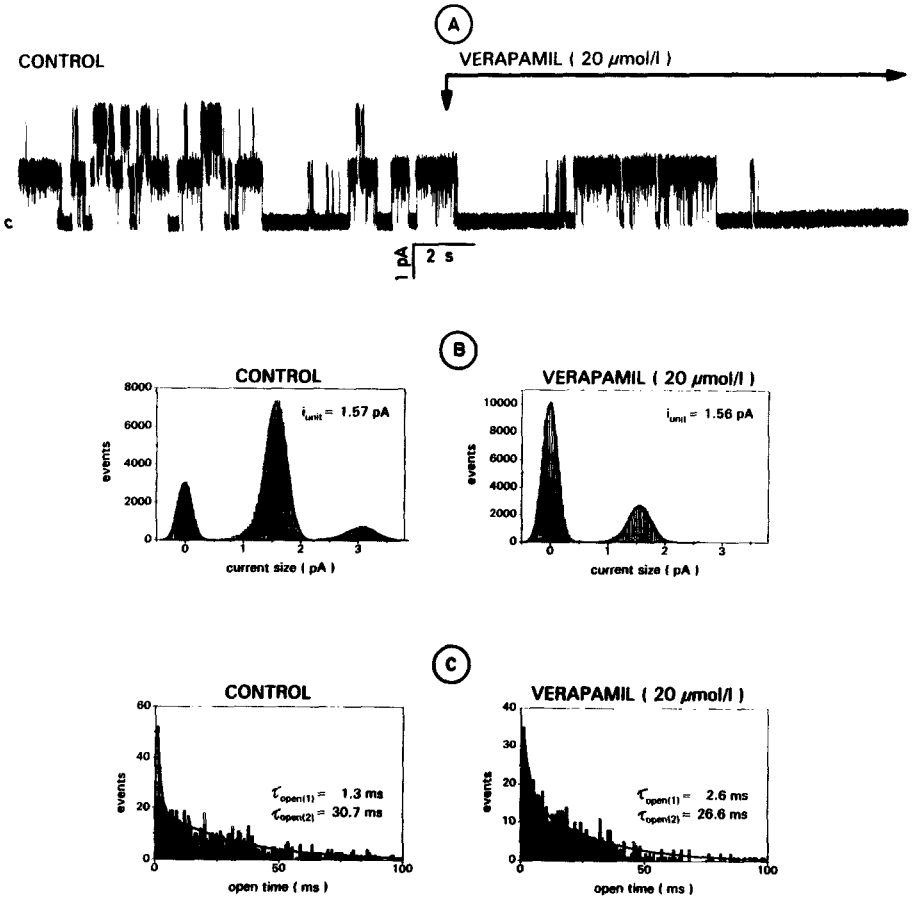


Fig. 4. The influence of verapamil (20 µmol/liter) on isolated cardiac $K_{(ATP)}$ channels. (*A*) Continuous records of elementary K^+ currents (*c* means closed configuration) before and after drug application. (*B*) Amplitude histograms under control conditions and after verapamil treatment. (*C*) Open time histograms before and after verapamil treatment. By disregarding the first bin of 0.4 msec, the best fits were as follows: Control $N(t)$ $66\exp(-t/0.0013) + 21\exp(-t/0.0307)$; verapamil $N(t)$ $27\exp(-t/0.0026) + 16\exp(-t/0.0266)$. Patch 711; membrane potential −7 mV.

$\tau_{open(1)}$ varied insignificantly to 122 ± 20% (n = 7) and $\tau_{open(2)}$ to 101 ± 13% (n = 7) of the control. The latter result surprises with respect to the strong glibenclamide effect on NP_o: in the presence of the drug, open probability is no longer a function of $\tau_{open(2)}$. The ratio of second-open state events to first-open state events significantly decreased; in the experiment shown in Fig. 3 from 6.47 to 2.22 during the first 30 sec of glibenclamide treatment and finally to 0.34 in the later stage. Thus, the short-lasting O_1 state became increasingly dominating as if glibenclamide-associated $K_{(ATP)}$ channels are hindered in attaining the O_2 state.

The inhibitory glibenclamide effect proved reversible but, as found in two washout experiments on an extended time scale, a complete NP_o recovery took more than 4 min.

Verapamil belongs to the distinct class of phenylalkylamines but was found to exert essentially the same effect on cardiac $K_{(ATP)}$ channels (Fig. 4). The cytosolic administration of 20 µmol/liter reduced NP_o to 31.0 ± 4.9% (n = 9) of the predrug value within a few seconds; the calculated IC_{50} amounted to 9 µmol/liter. Open state kinetics remained unaffected: $\tau_{open(1)}$ was 3.2 ± 0.5 msec before and 2.9 ± 0.5 msec after drug application, the respective values for $\tau_{open(2)}$ were 29.6 ± 3.7 and 28.5 ± 3.8 msec (n = 7). Kimura et al. (1992) reported similar observations with adult cardiac $K_{(ATP)}$ channels but the novel finding here is that the O_2 state not detected in their experiments is also insensitive to verapamil. Again, the ratio of second-open state events to first-open state events declined in any verapamil experiment.

Both drugs caused pauses of channel activity lasting several seconds without detectable opening events. Unfortunately, any individual patch contained more than one functional $K_{(ATP)}$ channel so that closed time kinetics could not be analyzed with reliable accuracy. Clearly, this dominance of a nonconducting channel state is the reason for the decline in NP_o.

Distinct Inhibitory Actions of Sulfonamide Derivatives

Figure 5 demonstrates the sensitivity of cardiac $K_{(ATP)}$ channels to a novel sulfonamide derivative, HE 93. HE 93 was recently found in whole-cell clamped ventricular myocytes to be effective in depressing outward currents and prolonging action potential duration in isolated cardiac Purkinje fibers (Argentieri et al., 1993). The cytoplasmic administration evoked pauses of channel activity lasting several seconds and, consequently, reduced NP_o

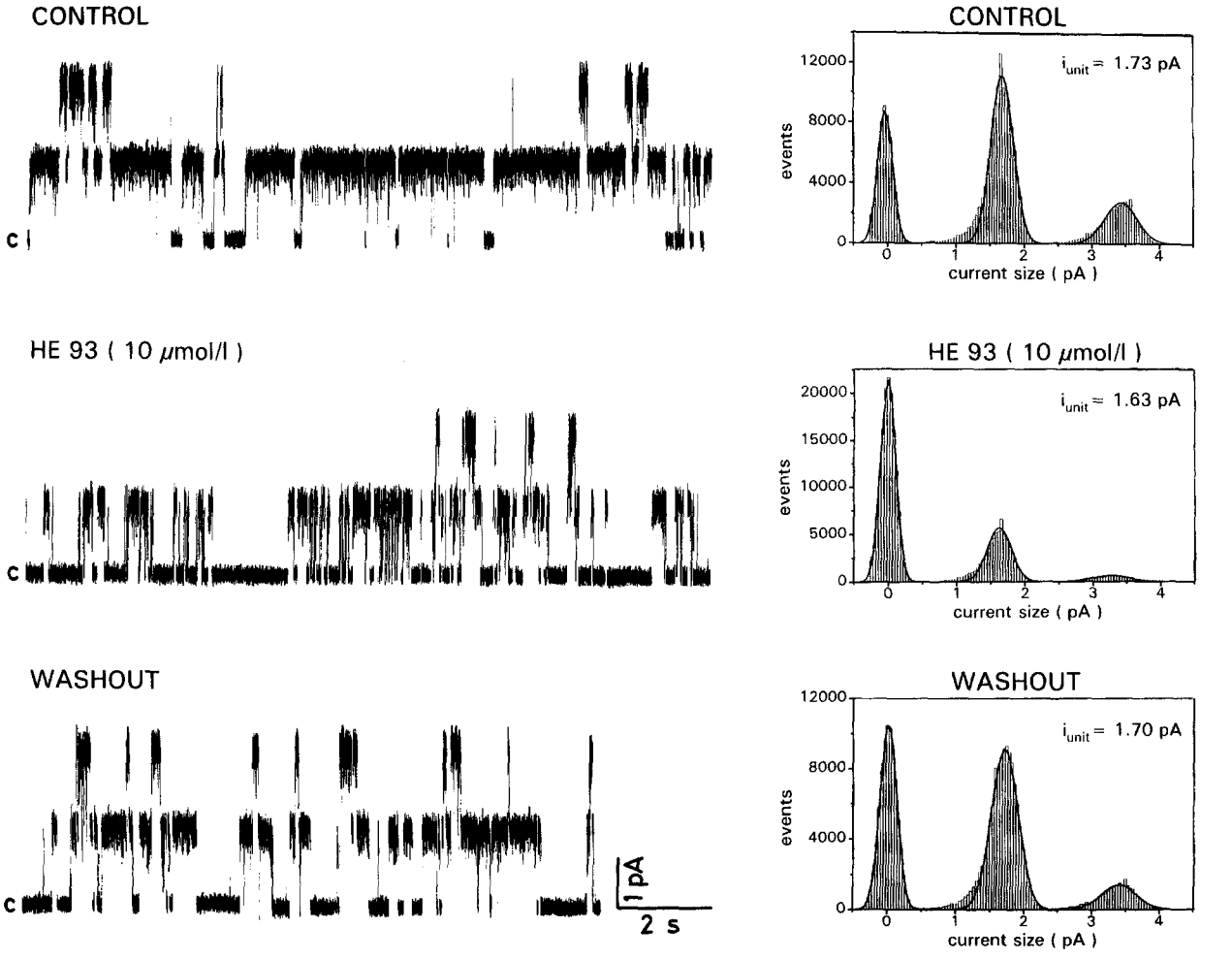


Fig. 5. The influence of HE 93 (10 μmol) on isolated cardiac $K_{(ATP)}$ channels. Elementary K^+ current recordings (*c* means closed configuration) before (upper trace) and after (middle trace) HE 93 treatment and after HE 93 washout (lower trace) with the corresponding amplitude histograms. Patch 552; membrane potential –7 mV.

(*see* Fig. 5); removal of HE 93 caused NP_o to return to the control value. With 10 μmol/liter, NP_o was 74 ± 7.2% ($n = 4$) of the control; an IC_{50} of 29 μmol/liter was calculated. The elementary current size remained unaffected even at 50 μmol/liter HE 93.

HE 93 influenced open state kinetics to shorten the O_2 state in an apparently selective fashion. In the experiment illustrated in Fig. 6, for example, $\tau_{open(2)}$ decreased reversibly from 37.5 to 26.0 msec without major changes of $\tau_{open(1)}$. Three other HE 93 experiments confirmed this result (Fig. 6): $\tau_{open(1)}$ was close to 3 msec regardless of the absence or presence of the drug but $\tau_{open(2)}$ declined significantly from 33.2 ± 3.1 to 20.5 ± 3.2 msec, i.e., to 60.6 ± 6% of the control. With 50 μmol/liter HE 93, a decrease to 40 ± 3% ($n = 2$) was obtained. In addition, the ratio of second-open state events to first-open state events decreased to about 40% of the predrug value, indicating that the O_2 state was attained with reduced likelihood.

The drug-induced open channel blockade can be modeled by a reaction scheme according to

$$\text{closed} \underset{\delta}{\overset{\gamma}{\rightleftharpoons}} \text{open}_{(1)} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{open}_{(2)} \overset{a[D]}{\underset{d}{\rightleftharpoons}} \text{blocked}$$

where closed, $open_{(1)}$, $open_{(2)}$ and blocked mean channel configurations, *a* and *d* correspond to the association and dissociation rate constant, respectively. The association rate constant can be calculated from the term ($1/\tau_{open(2)\,(drug)}$ minus $1/\tau_{open(2)\,(control)}$) to estimate the HE 93 affinity for the O_2 state but with the assumption that the drug concentration in the cytosolic environment equals the drug concentration in a compartment surrounding the channel-associated binding site. Then, HE 93 interacted with an association rate constant of $2.3 \pm 0.7 \times 10^6$ mol^{-1} sec^{-1} ($n = 4$).

Drug experiments with 10 μmol/liter HE 93 at dif-

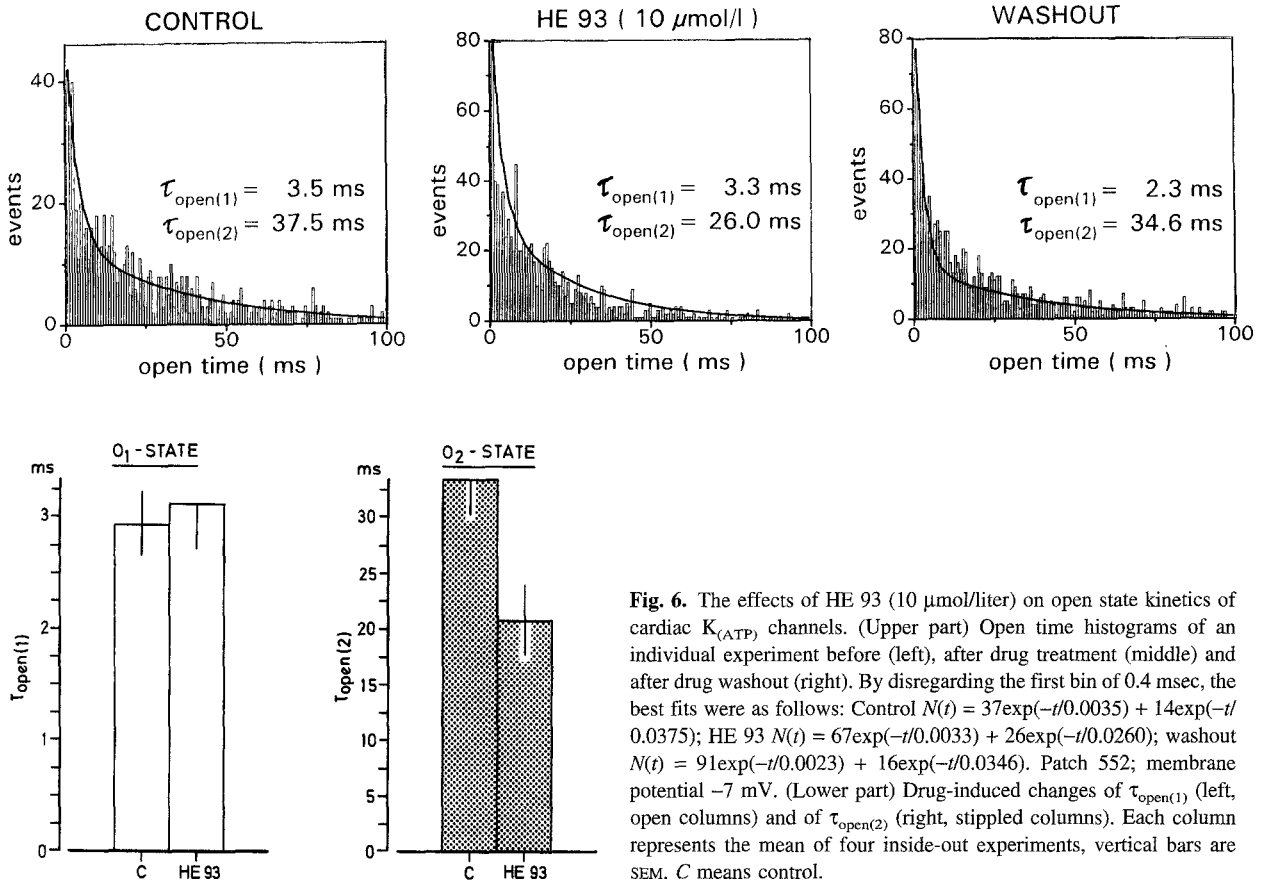


Fig. 6. The effects of HE 93 (10 μmol/liter) on open state kinetics of cardiac $K_{(ATP)}$ channels. (Upper part) Open time histograms of an individual experiment before (left), after drug treatment (middle) and after drug washout (right). By disregarding the first bin of 0.4 msec, the best fits were as follows: Control $N(t) = 37\exp(-t/0.0035) + 14\exp(-t/0.0375)$; HE 93 $N(t) = 67\exp(-t/0.0033) + 26\exp(-t/0.0260)$; washout $N(t) = 91\exp(-t/0.0023) + 16\exp(-t/0.0346)$. Patch 552; membrane potential –7 mV. (Lower part) Drug-induced changes of $\tau_{open(1)}$ (left, open columns) and of $\tau_{open(2)}$ (right, stippled columns). Each column represents the mean of four inside-out experiments, vertical bars are SEM. *C* means control.

ferent cytosolic K^+ concentrations provided no evidence to support the idea that the shortening of the O_2 state might reflect a competition of HE 93 with the permeant K^+ for a common site within the open pore. As consistently found in four inside-out experiments (Fig. 7), $\tau_{open(2)}$ did not change when the internal K^+ concentration was increased from 70 to 140 mmol/liter. The same insensitivity of open state kinetics was observed in control experiments in the absence of HE 93. The unitary current size responded to this increase in internal K^+ with a significant rise by a factor of 1.6 ± 0.03 (n = 4), thus indicating that sites involved in K^+ permeation through the pore are not yet saturated.

The block phenomenology seen with the other sulfonamide derivative tested, sotalol, is even more complex. Sotalol depresses several K^+ currents in heart muscle including the voltage-dependent, transient I_K which governs repolarization (Colatsky et al., 1982) but has not yet been proven to interact with $K_{(ATP)}$ channels. Analogously to HE 93, sotalol (100 μmol/liter) acted to depress NP_o (calculated IC_{50} = 43 μmol/liter) and selectively reduced $\tau_{open(2)}$ to 40 ± 9% (n = 3) of the predrug value. The ratio of second-open state events to first-open state events declined to about 50% of the control. Mean burst duration was shortened from 174 ± 47 to 33 ± 4 msec (n = 5). The unexpected channel response is illustrated in Fig. 8, namely, a well-resolved flicker blockade that appeared quasi-instantaneously upon drug exposure and disappeared as rapidly upon removal of sotalol. The criterion of flicker blockade, an increase of transitions between the conducting and a nonconducting state, was verified by counting events within bursts. Relating this number to burst duration yielded the ratio of events/msec that provided a normalized and time-independent measure of the transitions. With 100 μmol/liter sotalol, this ratio rose from a predrug value of 1.0 ± 0.3 to 5.7 ± 1.6 (n = 5). Experiments with 50 μmol/liter confirmed the concentration dependence of this parameter: the ratio rose from 0.95 ± 0.3 in the absence of the drug to only 2.5 ± 0.7 (n = 3), i.e., 2.6-fold, in contrast to the 5.7-fold increase with 100 μmol/liter sotalol. Both enantiomers were found to be apparently equipotent to flicker-block cardiac $K_{(ATP)}$ channels: with (+)-sotalol, the calculated association rate constant amounted to 4.3 ± 0.5 × 10^5 mol^{-1} sec^{-1} and with (–)-sotalol to 5.0 ± 1.0 × 10^5 mol^{-1} sec^{-1}.

Internal K^+ variations can modulate the sotalol-induced flicker blockade of $K_{(ATP)}$ channels (Fig. 9). This was evidenced in a last set of three inside-out experiments exposed a priori cytosolically to 100 μmol/liter (+)-sotalol from the K^+ sensitivity of the ratio of events/msec. On lowering the internal K^+ concentration

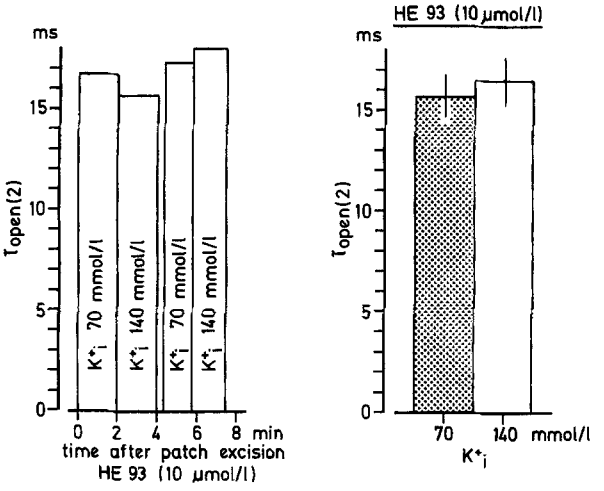


Fig. 7. Failing influence of internal K^+ variations on $\tau_{open(2)}$ in HE 93-treated cardiac $K_{(ATP)}$ channels. (Left) Experimental protocol in an individual inside-out experiment: columns represent an observation period at 70 or 140 mmol/liter K^+_i, respectively; K^+_i changes were achieved within less than 5 sec and performed at least twice between 70 and 140 mmol/liter in order to stress the reproducibility of the results. (Right) Columns symbolize mean values (vertical bars are SEM) of $\tau_{open(2)}$ at 70 and 140 mmol/liter K^+_i. $n = 4$.

from 140 to 70 mmol/liter, this ratio rose, drastically in one experiment, by a factor of 4.5, indicating enhanced flickering. The accompanying change was a significant decline in $\tau_{open(2)}$, expected results when sotalol competes with K^+ for a common site.

Discussion

The present patch clamp experiments with rat heart myocytes have shown, first of all, that neonatal cardiac $K_{(ATP)}$ channels share important elementary properties with their adult relatives. They can be modeled by a Markovian reaction scheme according to $R—O_1—O_2$, where R combines at least two closed configurations and O means open state. The O_2 state, in particular, can be left with quite individual rate constants between 15 and 90 sec^{-1} (at –7 mV), one reason for very distinct activity modes even though the cytosolic environment was under definite control. Short-time cultured cardiocytes from neonatal rats can be supposed to dispose of still the same channel population as synthesized in vivo, i.e., in the very late prenatal state since expression of $K_{(ATP)}$ channels in *Xenopus* oocytes took 4–6 days after injection of poly(A^+) mRNA from an insulin-secreting cell line (Ashcroft et al., 1988). The abnormally low TTX sensitivity of voltage-dependent Na^+ channels in embryonic skeletal muscle cells (Weiss & Horn, 1986) is a good example that prenatal cells may express ionic channels with properties differing to some extent from adult channels. Whether individual open state kinetics represent a peculiarity in neonatal cardiac $K_{(ATP)}$ channels or a feature of the whole family seems a crucial question, indeed. Cell-attached experiments in anoxic myocytes from adult guinea pigs (Benndorf et al., 1991) provide evidence in support of the second possibility.

From a formalistic viewpoint, the individuality of the O_2 state is reminiscent of another peculiarity of cardiac $K_{(ATP)}$ channels: their nonuniform ATP sensitivity. K_D values for the inhibitory ATP effect between 9 and 580 µmol/liter have been reported (Findlay & Faivre, 1991). Hill coefficients of 1 to 4 (Weiss, Venkatesh & Lamp, 1992) or 1 to even 6 (Findlay & Faivre, 1991) point to an individual stoichiometry of the ATP binding reaction that finally blocks the channel. Channels with the lowest exit rate from the O_2 state are candidates for the high activity fraction, a small percentage of the whole ensemble which is functionally relevant and governs K^+ efflux and shortening of action potential duration in ATP-depleted myocardium (Weiss et al., 1992).

To model the modulating influences on $K_{(ATP)}$ channels, Edwards and Weston (1993) proposed the existence of two sites, a phosphorylation site and a distinct regulatory site both located at the cytoplasmic channel surface. Closely associated with the phosphorylation site may be a target for nucleotide diphosphates and a binding domain for sulfonylureas. High affinity glibenclamide binding was shown with radioligands to be present also in cardiac microsomes (Fosset et al., 1988; French et al., 1991); ADP competes with glibenclamide for binding at least in HIT T15 β-cells (Niki, Nicks & Ashcroft, 1989). Nevertheless, nature and location of the glibenclamide target are unknown. Glibenclamide is assumed to act from the lipophilic channel environment (Panten et al., 1989; Findlay, 1992) but the target may be also accommodated in a cytoplasmic channel region that is accessible after external drug administration for the neutral molecule fraction via a lipophilic route or, considering the present inside-out experiments with cytosolic drug administration, directly from the bulk phase. Clearly, the observed delayed onset of glibenclamide block is not in favor of a very superficially located target, but seems little enough to provide an experimental argument in support of a glibenclamide target at an intramembrane channel portion when the lipophilic diffusion pathway is as short as some 10 Å.

Glibenclamide occupancy seems to trap $K_{(ATP)}$ channels in a rather stable, nonconducting configuration that mainly accounts for the observed NP_o depression. The drug may act with ATP cooperatively in inhibiting channel activity (Virag et al., 1993), which is not inconsistent with separate binding sites for ATP and glibenclamide (Ripoll, Lederer & Nichols, 1990). Of interest is the abnormal gating in unblocked $K_{(ATP)}$ channels. The reduced likelihood to attain the O_2 state points to another drug-sensitive reaction, the forward rate governing the O_1–O_2 transition which seems critically reduced in the

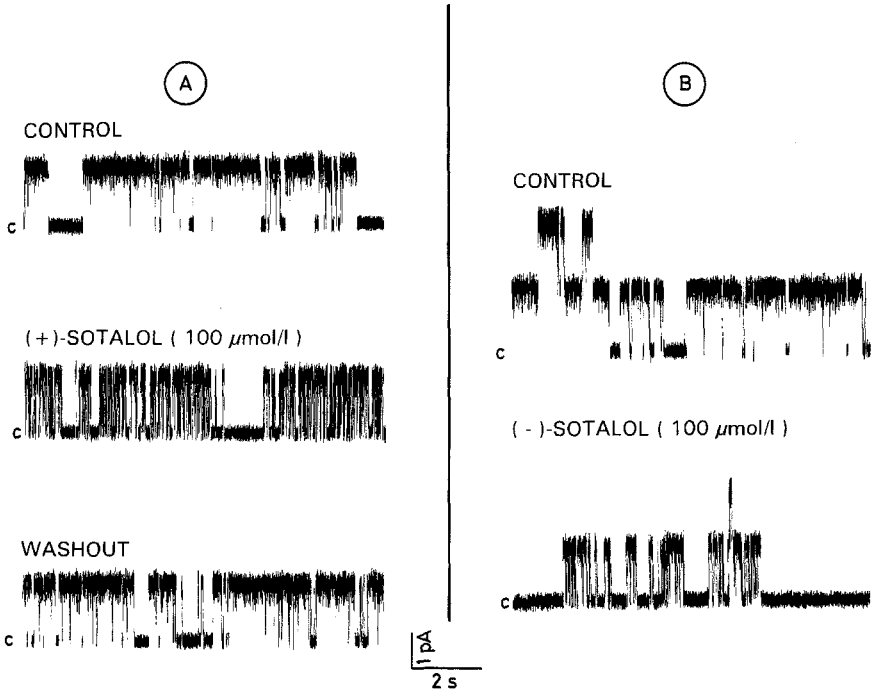


Fig. 8. The effect of (+)-sotalol (100 μmol/liter, *A*) and of (−)-sotalol (100 μmol/liter, *B*) on isolated cardiac $K_{(ATP)}$ channels. Selected elementary K^+ current recordings (*c* means closed configuration) just before and after drug treatment; the record labeled *Washout* in *A* was started 5 sec after drug removal. Patches 619 (*A*) and 624 (*B*); membrane potential −7 mV.

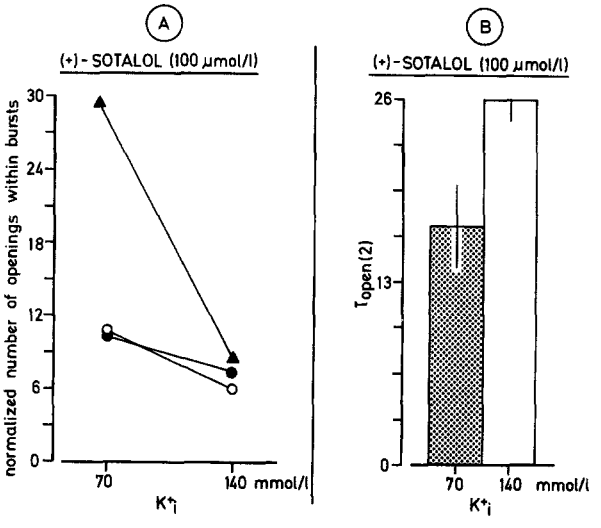


Fig. 9. (*A*) The dependence of the transition frequency between the open and a nonconducting state in (+)-sotalol-treated cardiac $K_{(ATP)}$ channels (expressed as normalized number of openings within bursts, i.e., events/msec) on internal K^+ concentration. Any symbol indicates an individual experiment. (*B*) Dependence of $\tau_{open(2)}$ in (+)-sotalol-treated cardiac $K_{(ATP)}$ channels on internal K^+ concentration. The columns symbolize mean values (vertical bars are SEM) of the same three inside-out experiments demonstrated in another form in *A*. Membrane potential −7 mV.

presence of glibenclamide. Following the mechanistic hypothesis that drug occupancy of one and the same channel site is unlikely to have distinct functional consequences, it might be tempting to postulate a second glibenclamide receptor site. In fact, some radioligand binding studies suggest multiple glibenclamide targets: apart from the high affinity site, a second site where sulfonylureas interact with a K_D far above the nanomolar range (Niki et al., 1989, 1990; Gopalakrishnan et al., 1991). However, it seems less plausible to relate the drug-sensitive O_1–O_2 transition to the latter receptor since glibenclamide in nanomolar concentrations acted similarly in reducing the likelihood of attaining the O_2 state. An allosteric reaction caused by the occupied high affinity glibenclamide receptor, which may finally affect the O_1–O_2 transition, cannot be excluded. Clearly, any molecular interpretation remains highly speculative unless relevant details of the structure-function relationship of $K_{(ATP)}$ channels are elucidated.

Less surprisingly, organic molecules, even structurally unrelated to glibenclamide, can also interact with cardiac $K_{(ATP)}$ channels. Bearing in mind methodological limitations in quantifying the inhibitory drug effect on NP_o as related to a possible rundown of channel activity and leading to an overestimation of the drug-induced NP_o depression, a rank order of the inhibitory potency with respect to the apparent IC_{50}, nevertheless, can be given, namely, glibenclamide $\gg$ verapamil > HE 93 ~ sotalol. Clearly, this rank order may be specifically determined by the present experimental conditions and the cytosolic presence of several nucleotides established to modulate the glibenclamide sensitivity of $K_{(ATP)}$ channels (*for review, see* Nichols & Lederer, 1991). Accordingly, neonatal cardiac $K_{(ATP)}$ channels may dispose of a single drug binding site capable of discriminating among several drugs whose occupancy reduces NP_o, i.e., the glibenclamide site with an affinity for nonsulfonylureas

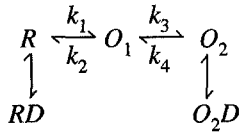
possibly too low to be important for the interaction with class III antiarrhythmic drugs. Alternatively, nonsulfonylureas might find a separate target to depress NP_o. The latter hypothesis of multiple drug receptors finds, for example, an equivalent in cardiac L-type Ca^{2+} channels where phenylalkylamines, dihydropyridines and benzodiazepines, acting together to depress open probability, interact with distinct targets at the α-subunit of the channel protein (*for review, see* Catterall & Striessnig, 1992).

Cardiac $K_{(ATP)}$ channels can respond to sulfonamides with open channel blockade. Two distinct block types can be distinguished, flicker blockade as evoked stereo-unselectively by sotalol and shortening of the O_2 state produced by HE 93. The latter block type suggests still another drug-sensitive reaction of the gating process besides the transition from the O_1 state to the O_2 state, namely, the exit rate from the O_2 state. HE 93-treated channels left the O_2 state faster, with a rate of close to 50 sec^{-1}, compared with about 30 sec^{-1} under predrug conditions. Interestingly, this resembles the influence of verapamil on the open state kinetics of cardiac, outwardly rectifying 66 pS K^+ channels (Benz & Kohlhardt, 1994*a*) or of some class 1 antiarrhythmics on chemically modified cardiac Na^+ channels (Benz & Kohlhardt, 1994*b*), stressing the notion that cardiac $K_{(ATP)}$ channels do not behave uniquely in this respect but share this particular property even with the molecularly distinct voltage-dependent Na^+ channels. Nevertheless, drug discrimination at this level can be observed: cardiac $K_{(ATP)}$ channels, in contrast to cardiac 66 pS K^+ channels (Benz & Kohlhardt, 1994*a*), were found to preserve the exit rate from the O_2 state in the presence of equimolar verapamil concentrations. HE 93 associates with kinetics close to 2×10^6 mol^{-1} sec^{-1}, an apparently intermediate affinity for a putative channel-associated binding site whose HE 93 occupancy may finally affect the exit rate from the O_2 state.

Flicker blockade of cardiac $K_{(ATP)}$ channels clearly indicates that molecules such as sotalol gain access to the open pore and, in competing with the permeant K^+, repetitively block the K^+ permeation pathway. An important part of the pore is formed by a highly conserved sequence of about 20 amino acids with a predominantly intramembranous location modeled as a hairpin, the H5 segment in outwardly rectifying K^+ channels (Ho et al., 1993; Kubo et al., 1993) which corresponds to the SS1-SS2 segment in voltage-gated K^+ channels (Kirsch et al., 1991; Hartmann et al., 1991; Yellen et al., 1991; Yool & Schwarz, 1991). Recent site-directed mutagenesis experiments (Yellen et al., 1991), probing TEA binding sites and thus sites involved in K^+ permeation, have identified single amino acids in the SS1-SS2 segment as determinants of the TEA affinity. A T441S mutation attenuated internal TEA blockade and a D431K mutation external TEA blockade. Despite structural differences, sotalol interacts as TEA with the open pore although a kinetic aspect makes both open channel blockades distinct from each other. TEA interferes with several-fold faster reaction kinetics and usually evokes a fast, nonresolvable flicker block in K^+ channels that becomes manifest as depression of i_{unit}.

Internal TEA finds a binding site at a point 25–30% distant from the cytoplasmic margin of the K^+ pore (Blatz & Magleby, 1984; Villarroel et al., 1988; Kirsch et al., 1991). There is no compelling evidence from the present inside-out experiments with cytoplasmic drug application that the internal mouth of cardiac $K_{(ATP)}$ channels also accommodates the sotalol target. This uncertainty relies on the physico-chemical properties (pK_8 ~ 8.5) of the drug. About 20% of the sotalol molecules resides at pH 7.4 in the neutral form and may therefore penetrate the lipid matrix of the membrane to reach the external channel mouth in order to block the K^+ permeation pathway only from this side. Then, the experimentally obtained association rate constant of close to 5×10^5 mol^{-1} sec^{-1} would definitely deviate from the true, i.e., higher value. Another important and similarly open question relates to the number of binding sites: a common receptor or separate receptors for TEA and sotalol since individual flicker block kinetics will not a priori stress the latter possibility.

A modeling of drug-treated cardiac $K_{(ATP)}$ channels by a Markovian reaction scheme seems mainly complicated by the uncertainty of closed state kinetics already mentioned above. The scheme



intentionally ignores transitions between several closed configurations to combine them as R. Accordingly, the NP_o reduction seen with glibenclamide, verapamil, HE 93 and sotalol may predominantly emerge from trapping the channel in the drug-associated RD state that is nonconducting. Flicker blockade by sotalol is symbolized as the O_2D state. Ignoring any possible analytical problem in determining $\tau_{open(1)}$, the apparent lack of an O_1D state may be explained by the particular reaction kinetics of sotalol, which is not fast enough for a drug interaction during the extremely short-lasting O_1 state. Of both rate constants governing transitions between O_1 and O_2, k_4 may be drug-specifically influenced since, for example, HE 93 shortens the O_2 state in contrast to glibenclamide or verapamil. Clearly, this scheme is consistent with multiple drug receptors in cardiac $K_{(ATP)}$ channels. It is

tempting to speculate that some of them may also be accommodated in the N- and C-terminal cytosolic region adjacent to either M domain of inwardly rectifying K^+ channels.

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