J. Membrane Biol. 179, 103–111 (2001)
DOI: 10.1007/s002320010041

The Journal of
Membrane
Biology

Verapamil Block of Large-Conductance Ca-Activated K Channels in Rat Aortic Myocytes

A.A. Harper[1] **L. Catacuzzeno,**[2] **C. Trequattrini,**[2] **A. Petris,**[2] **F. Franciolini**[2]
[1]Department of Anatomy and Physiology, University of Dundee, Dundee DD1 4HN, UK
Dipartimento Biologia Cellulare e Molecolare, University of Perugia, 06123 Perugia, Italy

Received: 11 April 2000/Revised: 17 October 2000

Abstract. The effects of verapamil on the large conductance Ca-activated K (BK) channel from rat aortic smooth muscle cells were examined at the single channel level. Micromolar concentrations of verapamil produced a reversible flickering block of the BK channel activity. Kinetic analysis showed that verapamil decreased markedly the time constants of the open states, without any significant change in the time constants of the closed states. The appearance of an additional closed state — specifically, a nonconducting, open-blocked state — was also observed, whose time constant would reflect the mean residence time of verapamil on the channel. These observations are indicative of a state-dependent, open-channel block mechanism. Dedicated kinetic (group) analysis confirmed the state-dependent block exerted by verapamil. D600 (gallopamil), the methoxy derivative of verapamil, was also tested and found to exert a similar type of block, but with a higher affinity than verapamil. The permanently charged and membrane impermeant verapamil analogue D890 was used to address other important features of verapamil block, such as the sidedness of action and the location of the binding site on the channel protein. D890 induced a flickering block of BK channels similar to that observed with verapamil only when applied to the internal side of the membrane, indicating that D890 binds to a site accessible from the cytoplasmic side. Finally, the voltage dependence of D890 block was assessed. The experimental data fitted with a Langmuir equation incorporating the Woodhull model for charged blockers confirms that the D890-binding site is accessed from the internal mouth of the BK channel, and locates it approximately 40% of the membrane voltage drop along the permeation pathway.

Correspondence to: A.A. Harper

Key words: Ca-activated potassium channel — Patch clamp — Single channel — Antihypertensive agent — Arterial smooth muscle

Introduction

In low-excitability arterial smooth muscle cells the BK channels are thought to play a major role in the control of membrane potential, which is in turn of cardinal importance in the regulation of vascular tone (Somlyo, 1985; Nelson, 1993; Nelson & Quayle, 1995). In myogenic arteries, the BK channels have been shown to exert a negative feedback action to counteract pressure-induced depolarization and contraction (Nelson & Quayle, 1995). The functional relevance of this action can be appreciated by the observation that block of the BK channels results in membrane depolarization and smooth muscle contraction (Knot et al., 1998). BK channels have been implicated in the generation and maintenance of pathological conditions e.g., hypertension (Bolotina et al., 1991; Rush et al., 1992; England et al., 1993; Cox & Tulenko, 1995; Liu et al., 1995). Hypertensive states have been shown to be associated with increased expression of BK channels in vascular myocytes. In genetically hypertensive rats, the density of BK channels is 2–3 times higher than in normotensive rats (Rush et al., 1992). This increased density of BK channels returned to normal levels following pharmacological reversion of hypertension (Rush & Runnells, 1994).

In this context we have investigated the effect of the antihypertensive (and antiarrhythmic) agent verapamil (Godfrain et al., 1986; Rush et al., 1996) on the BK channel. Verapamil and other antihypertensive drugs, known primarily as calcium-antagonists, cause vasodilatation and in turn lower arterial blood pressure. This pharmacological effect is thought to be exerted by blocking high-voltage activated, L-type calcium channels

(McDonald et al., 1994; Fleckenstein-Grun, 1996), which results in a reduction of Ca influx. The present investigation of the action of verapamil on BK channel activity was instigated by reports that this drug inhibits the delayed rectifier K (DRK) channels in a number of tissues (Pancrazio et al., 1990; Rampe et al., 1993; DeCoursey, 1995; Rauer & Grismer, 1996; Trequattrini et al., 1998; Catacuzzeno et al., 1999). The efficacy of K channel block is in some instances higher than that reported for Ca channels, suggesting that the pharmacological effects of this drug can be exerted through a concerted action on different ion channels. Further, to our knowledge no detailed study was available on the action of verapamil on BK channels (*cf.* Pavenstadt et al., 1991; Berweck, 1994).

In this study we found that micromolar concentrations of verapamil and its methoxy derivative D600 (gallopamil) can effectively inhibit the BK channel in these cells with an efficacy comparable to that found for Ca channels (*see* review by McDonald et al., 1994). We additionally found that verapamil's interaction with the BK channel depends on the functional state of the channel, the open state being preferred. We also investigated other important features of verapamil block such as the sidedness of action (i.e., whether verapamil accesses its binding site from the cytoplasmic or extracellular side of the membrane) and the location of the site on the channel protein (usually obtained by assessing the voltage-dependence of block). This information is not easily obtained using verapamil, a tertiary amine present in solution both as neutral and as a quaternary (charged) compound, the relative fractions depending on the pH (pa = 8.6; Retzinger et al., 1986). Whereas the charged form, which predominates at physiological pH, cannot readily cross the membrane the highly hydrophobic neutral form equilibrates rapidly on both sides of the membrane (DeCoursey, 1995), making it difficult to determine the sidedness of action of the drug. The assessment of the location of the site of action on the channel protein is problematical because verapamil block exhibits little or no voltage dependency (Pancrazio et al., 1990; but *see* Rampe et al., 1993; DeCoursey, 1995; Trequattrini et al., 1998), and the active form is ignored. To circumvent these problems we used the permanently charged, membrane impermeant verapamil analogue D890. This compound, has been used to determine the sidedness of action and voltage-dependence of verapamil block for other ion channels (Affolter & Coronado, 1986; DeCoursey, 1995; Catacuzzeno et al. 1999). These studies demonstrated that verapamil's binding site is accessed from the cytoplasmic side of the membrane, and is located well inside (*ca.* 40% of the full voltage drop) the BK channel pore. A preliminary report of some aspects of this work has been published in abstract form (Trequattrini et al., 1996).

Materials and Methods

Myocyte Isolation Procedure

Freshly isolated aortic myocytes were obtained by enzymatic dispersion. Male Wistar rats (160–380 g; Charles River-Italia, Como, Italy) were used. The animals were killed by concussion and cervical dislocation and the abdominal aorta quickly removed. The vessel was bathed in ice-cold MOPS Buffered Saline (MBS) containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 MOPS-NaOH and 10 glucose, pH 7.6. The aorta was cleared of adhering fat and connective tissue, split longitudinally and rinsed with ice-cold low Ca (0.16 mM) MBS and Isolating Solution comprising (in mM): 110 NaCl, 5 KCl, 0.16 $CaCl_2$, 2 $MgCl_2$, 10 MOPS, 0.5 NaH_2PO_4, 0.5 KH_2PO_4, 0.49 EDTA, 10 taurine, and 10 glucose, pH 7.0. The tissue was cut into squares (2–3 mm) and stored in the Isolating Medium, prepared by dissolving bovine serum albumin (essentially fatty acid free) 0.2% (w/v) in Isolating Solution, the enzymes collagenase (Worthington Type 2) 1.5 mg/ml and papain (Sigma Chemical, St. Louis, MO) 0.15 mg/ml were then added and the tissue stored at 4°C for 12–15 hr. The tissue squares were incubated in Isolating Medium at 37°C in a shaking water bath (40 per min) for a time period determined to give optimal myocyte yield for individual enzyme activity (range 15–50 min). They were then transferred to ice-cold low Ca PSS, triturated, strained through a 80 μm nylon mesh and the resulting suspension plated onto polylysine coated cover slips. The aortic myocytes used in the present work were relaxed and spindle-shaped, contracted when superfused with the α_1 agonist phenylephrine, and remained viable for several hours when maintained at room temperature.

Electrophysiology

Single-channel currents were recorded in the excised (inside-out and outside-out) configurations of the patch-clamp method (Hamill et al., 1981). Borosilicate pipettes (1.5 mm o.d., Hilgenberg GmbH, Malsfeld, Germany), pulled with a programmable puller (PUL-100; WPI, Sarasota, FL) were used. Their resistance ranged between 10 and 15 MΩ when filled with standard 140 mM KCl pipette solution. Currents were measured and amplified with a List EPC-7 amplifier (List Medical Instruments, Darmstadt, Germany), and digitized with a 12-bit A/D converter (TL-1, DMA interface; Axon Instruments, Foster City, CA). The pClamp software package (version 5.6; Axon Instruments) was used on a Compaq Pentium PC for generating the command voltage pulses, recording and archiving the currents, and preliminary analysis of the data. For online data collection, current signals were normally filtered at 1 kHz and sampled at 40 μsec/point.

Solutions

The solution perfusing the extracellular side of excised membranes contained (in mM): 140 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 MOPS, pH 7.2. To probe the K selectivity of the BK channel, KCl was replaced with an equimolar amount of NaCl. The solution perfusing the cytoplasmic side of the membrane contained (in mM): 140 KCl, 2 $MgCl_2$, 1 EGTA-K, 10 MOPS, pH 7.2. The free Ca^{2+} concentrations were obtained by adding $CaCl_2$ at concentrations calculated as described by Magleby and coworkers (Barrett et al., 1982). Experiments were carried out at room temperature (range 18–22°C with a maximum deviation of <1°C for any procedure). The following drugs were used: verapamil and D600 (Sigma), apamin and charybdotoxin (Alomone Labs, Israel) and D890

(supplied by Dr. Martin Traut from Knoll AG, Ludwigshafen, Germany). Pharmacological agents were prepared immediately before use, and were bath applied by gravity-fed superfusion at a flow rate of 2 ml/min, with complete solution exchange within the cell chamber in *ca.* 1 min.

Analysis of Data

The dwell time distributions of open and closed current levels were constructed from single-channel recordings obtained from patches containing only one active channel (assessed by the number of current levels taken at high voltages and internal Ca concentrations). Multi-exponential fits of the dwell times distributions were obtained using pClamp analysis software, and the number of exponential components necessary in each distribution was determined with the maximum likelihood method (McManus et al. 1988). An additional exponential component was accepted when the logarithm of the maximum likelihood increased at least by a factor of 3.

The state-dependence of verapamil block of the BK channel was also assessed by using the group analysis method applied to single-channel recordings. The threshold time (T_L), corresponding to the shortest closed interval that still qualifies as an interburst event, was determined by applying the interburst interval test (Sigurdson et al., 1987). In particular, T_L was assessed by plotting the mean burst durations that resulted at varied interburst intervals. On such a plot, T_L represented the shortest interburst interval beyond which the mean burst duration remained relatively insensitive to further increases of the interburst interval. The applicability of the group analysis to each individual single channel recording was considered viable when less than 5% of the total intraburst closures (in the closed dwell time histogram) exceeded T_L. For example, in the single channel recording with 3 μM verapamil shown in Fig. 4*A*, where a $T_L = 40$ msec resulted, only 0.7% of the total intraburst closings would exceed T_L. The results are given as mean ± SEM.

Results

General Features of the BK Channels in Rat Aortic Myocytes

The BK channel unitary currents were recorded from excised (inside-out and outside-out) membrane patches in symmetrical 140 mM KCl solutions. Almost all patches (60/72, 21 preparations) exhibited high conductance levels, but often too many to be reliably analyzed. Representative unitary current recordings at varying membrane potential are presented in Fig. 1*A*. The voltage dependence of the unitary current amplitude was well described by a linear regression (Fig. 1*B*), the mean single-channel conductance being 228 ± 17 pS ($n = 3$). When external K was replaced with Na, the single-channel current did not display a clear reversal potential, approaching zero current at most negative potentials (Fig. 1*B*, open symbols), congruent with the channel exhibiting a significant selectivity for K over Na ($P_{Na}/P_K <$ 0.09, as assessed from the absence of inward current at −60 mV). The mean unitary current at 0 mV with external Na was 5.66 ± 0.57 pA ($n = 6$), and the slope conductance was 138 ± 14 pS ($n = 5$) between 0 and 20 mV, in good agreement with previous reports for BK channels in vascular smooth muscle (Benham & Bolton, 1986; Langton et al., 1991), including aortic smooth muscle cells (Giangiacomo et al., 1992).

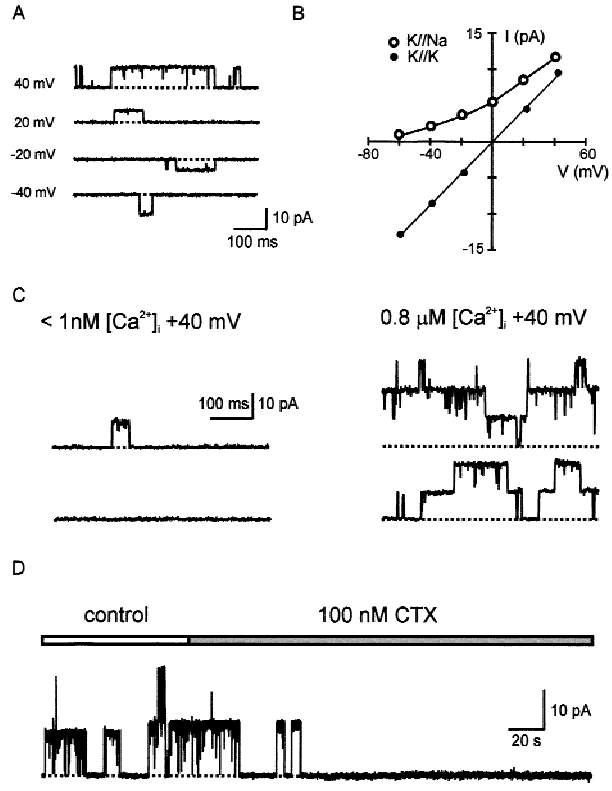

Fig. 1. General features of the BK channel in rat aortic myocytes. (*A*) Effect of varying potential on unitary BK currents recorded from an outside-out patch in presence of symmetrical 140 mM KCl and 0.8 μM $[Ca]_i$. (*B*) Current-voltage plot of mean amplitude of unitary currents recorded in symmetrical 140 mM KCl (filled symbols; $n = 3$), and after replacement of external 140 mM KCl with 140 mM NaCl (open symbols; $n = 2$). Linear interpolation of the data points in symmetrical 140 mM KCl gave a slope conductance of 228 ± 17 pS. (*C*) Effect of increasing $[Ca^{2+}]_i$ from <1 nM (1 mM EGTA and no added Ca; left) to 0.8 μM (right), at constant holding potential of +40 mV, on the BK channel activity. The recordings shown were taken from the same inside-out patch. (*D*) Single channel outside-out record in symmetrical 140 mM KCl and 0.8 μM $[Ca]_i$, holding potential +40 mV, in control conditions and upon external application of 100 nM CTX. In all panels, the closed (zero current) level is indicated by a dotted line.

The identification of the BK channel based on the K selectivity and its large conductance was confirmed by examining the Ca sensitivity of the current and its pharmacological profile. Increasing $[Ca^{2+}]_i$ from <1 nM (1 mM EGTA and no added Ca) to 0.8 μM, a marked increase in single channel activity resulted, as illustrated by the representative recordings shown in Fig. 1*C* obtained in inside-out configuration at a holding potential of +40

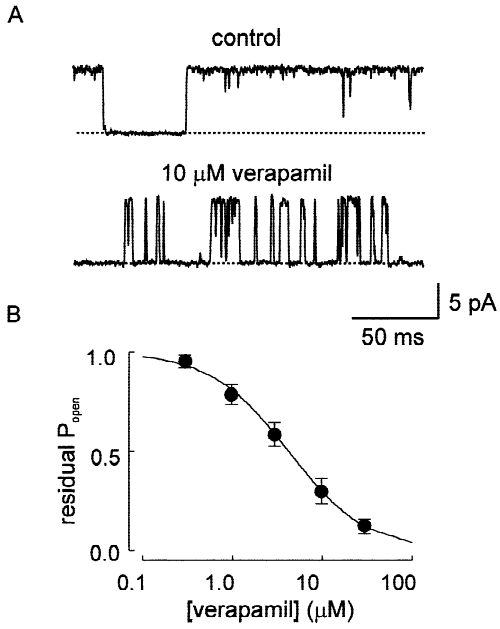

Fig. 2. Effect of verapamil on the unitary BK current. (*A*) Outside-out single-channel recordings in control conditions and after addition of 10 μM verapamil to the bath. Experimental conditions were: symmetrical 140 mM KCl, 1 μM $[Ca]_i$, and holding potential +40 mV. (*B*) Dose-response relationship showing inhibition of BK channel activity by verapamil. The residual P_{open} represents the P_{open} in presence of the blocker normalized to the open probability in control conditions (0.83 and 0.77 for the two cells presented). Each data point is the mean of two measurements from two different cells. The solid line represents the best fit of the experimental data with the Langmuir isotherm of the form $f_O = 1/(1+[B]/K_d)$, where f_O is the residual P_{open}, $[B]$ the verapamil concentration, and K_d the dissociation constant. The resulting K_d was 4.1 μM.

mV. External application of apamin (100 nM) on outside-out patches had no effect ($n = 3$, *data not shown*), while superfusion of CTX (100 nM) abolished single channel activity ($n = 4$, panel *D*), in accordance with the BK channel properties seen in other preparations (Miller et al., 1985; Latorre et al., 1989).

Verapamil Block of Unitary BK Currents

Moderate concentrations of verapamil (0.3–30 μM) produced a flickering block of the BK channel, without affecting the single channel conductance (Fig. 2*A*). The efficacy of verapamil block was not influenced by the side (internal or external) of application, consistent with the high membrane permeability of this drug (DeCoursey, 1995). The efficacy of block was assessed by constructing a dose-response curve for verapamil. Single-channel recordings sufficiently long to allow a reliable evaluation of the BK channel open probability (P_{open}) were taken at varying verapamil concentrations. P_{open} at each concentration, normalized to the P_{open} in control conditions and termed residual P_{open}, were plotted *vs.* verapamil concentration. Data from two experiments were pooled, and fitted with the Langmuir isotherm which gave a K_d for verapamil of 4.1 μM (Fig. 2*B*). The data points were well fitted with the Langmuir equation suggesting that the binding reaction between verapamil and the BK channel follows a 1:1 stoichiometry, as previously reported for the blocking action of verapamil in other K channels (DeCoursey, 1995; Trequattrini et al., 1998).

Kinetic Analysis of Verapamil Block

Verapamil has been shown to block DRK currents from various tissues in a state-dependent way, displaying far greater affinity for the open state of the channel (Rampe et al., 1993; DeCoursey, 1995; Trequattrini et al., 1998). To assess whether verapamil block of the BK channel displays similar behavior we carried out a kinetic analysis on patches containing one active channel. The principal features expected for an open-channel blocker are the following. (i) The dwell open times should be reduced due to block by verapamil, which would interrupt the open events by bringing the channel into a nonconducting, open-blocked state; (ii) an extra (open-blocked) closed state should be revealed, whose time constant would reflect the mean residence time of verapamil on the channel; (iii) the closed states should not be affected, based on the assumption that verapamil does not interact with these states.

We tested these predictions by doing a kinetic analysis of single BK channels in control conditions and in presence of 3 μM verapamil. The open time distributions shown in Fig. 3*A* and *B*, fitted with two exponential components, show that the dwell open times of both components decrease significantly following application of verapamil (from $\tau_{O1(ctrl)} = 4.12$ msec and $\tau_{O2(ctrl)} = 29.8$ msec down to $\tau_{O1(ver)} = 1.81$ msec, and $\tau_{O2(ver)} = 6.46$ msec, consistent with a state-dependent mechanism of verapamil action on BK channels. The effect of verapamil on the closed states is illustrated in Fig. 3*C* and *D*, which shows the closed time distributions obtained in control conditions and in presence of 3 μM verapamil, respectively. In control conditions two exponential components were observed, whose time constants were 0.31 msec and 318.6 msec. After addition of 3 μM verapamil an additional, third, exponential component with time constant of 5.82 msec was required to fit the distribution of the closed states. This extra component represented the nonconducting, open-blocked state induced by verapamil. It is notable that the blocker did not have any detectable effect on the dwell time of the pre-existing closed components. This indicates that verapamil does not bind to them, thus qualifying as an open-channel blocker of the BK channel. Similar results were obtained in two other patches.

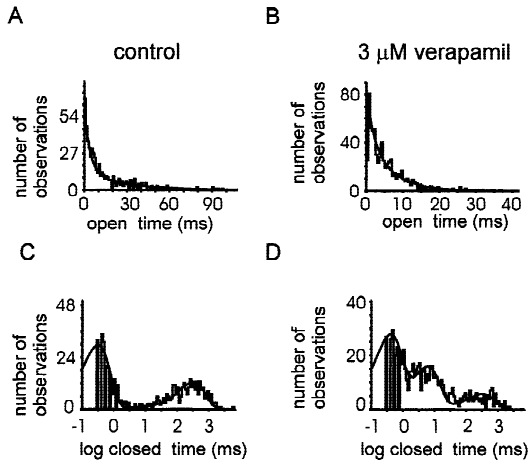

Fig. 3. Kinetic analysis of verapamil block. Open and closed dwell time distributions derived from a long (122 sec) inside-out, single-channel recording in control conditions and after addition of 3 μM verapamil. Experimental conditions were: symmetrical 140 mM KCl, 10 nM $[Ca]_i$, holding potential +100 mV. (*A* and *B*) The open time distributions were fitted with two exponential components with time constants of 4.125 msec (35.3%) and 29.85 msec (64.7%) in control conditions, and 1.805 msec (18.9%) and 6.462 msec (81.1%) in presence of verapamil. (*C* and *D*) The closed time distribution in control conditions was fitted with two exponential components (τ_1 = 0.311 msec, a_1 = 0.706; τ_2 = 318.6 msec, a_2 = 0.294) while in presence of verapamil three exponential components were necessary (τ_1 = 0.368 msec, a_1 = 0.54; τ_2 = 5.849 msec, a_2 = 0.335; τ_3 = 329.6 msec, a_3 = 0.125).

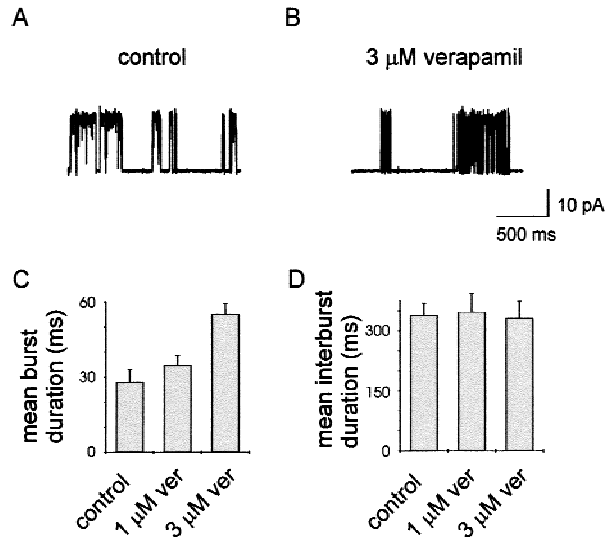

Fig. 4. Group analysis. (*A* and *B*) Single-channel recordings in inside-out configuration in control conditions and after addition of 3 μM verapamil showing that in both conditions BK channel openings are grouped in bursts, separated by long closures. Experimental conditions were: symmetrical 140 mM KCl, 10 nM $[Ca]_i$, and holding potential +100 mV. (*C* and *D*) Mean burst and interburst durations from three different patches in control conditions and in presence of either 1 or 3 μM verapamil. The threshold time defining intraburst closures in each recording analyzed was determined as described in Materials and Methods.

Group Analysis

Further evidence supporting the open channel block mechanism for verapamil inhibition of BK channels was provided by the group analysis, results shown in Fig. 4. Figure 4*A* and *B* show two representative short segments from a long inside-out recording from a patch containing only one BK channel, in control conditions and in the presence of 3 μM verapamil applied at the cytoplasmic face of the membrane (holding potential +100 mV, symmetrical 140 mM K, and 10 nM $[Ca^{2+}]_i$. The kinetic activity of the channel in both experimental conditions occurred in well-defined bursts, with short openings and closures within the bursts separated by much longer closed intervals. In the presence of verapamil, the openings were markedly shortened, as a result of verapamil block. The rational for applying the group analysis was based on the notion that if verapamil blocked the channel by interacting with the short-lived states within the burst, but not with the long closed interburst state, only the mean burst duration should increase in the presence of the blocker, with no change of the interburst duration (also *cf.* Colquhoun & Hawkes, 1995). This is because when the blocker is bound to the channel (which for an open-channel blocker can only occur within the burst), the channel would become kinetically frozen (i.e., it cannot make transitions into other states), consequently, the length of each event within the burst, and thus the burst as a whole, would increase by the fraction of time the channel has the blocker bound to it. Taking advantage of the markedly different time constants between the short (intraburst closures), and the long (interburst closures) components occurring both in the absence and presence of verapamil, we carried out the group analysis. To this end a threshold time was set in such a way that it would discriminate the intraburst from the interburst closures (*cf.* Materials and Methods). Figure 4*C* and *D* show the histograms of the burst and interburst durations recorded in control conditions and in the presence of varying concentrations of verapamil. The main observation is that verapamil markedly increases the burst duration, with no effect on the interburst duration, a result satisfying the requirements for a state-dependent blocker.

Block by D600 (gallopamil)

To probe the specificity of verapamil block on BK channels, the action of D600 (gallopamil), the methoxy derivative of verapamil, was investigated. Figure 5*A* shows representative single BK channel recordings in outside-out configuration, in control conditions and with varying D600 concentrations. D600 had similar block-

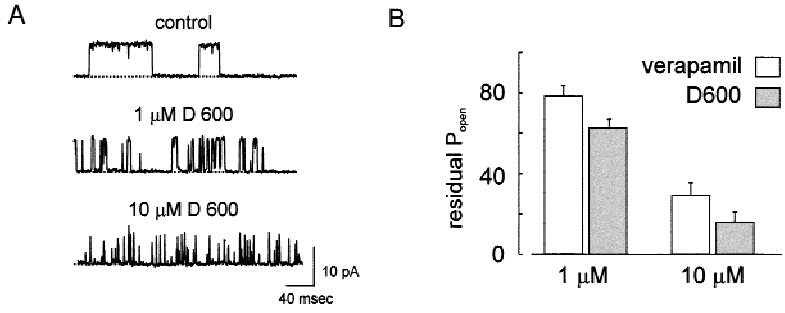

Fig. 5. Block of BK channels by D600. (*A*) Representative outside-out single-channel recordings showing the effect of increasing D600 concentrations on single BK channel activity. Experimental conditions were: symmetrical 140 mM KCl, 10 μM $[Ca]_i$, and holding potential +40 mV. At high concentrations (1 μM), D600 produced needle-shaped openings, most of which did not reach the fully open level. (*B*) Bar histograms comparing the efficacy of verapamil and D600 block. The residual P_{open} is shown for the two drugs at two concentrations. At 1 μM, D600 decreased the P_{open} by 37.3% ± 4.2 (n = 2), as compared to 21.6% ± 5.1 of verapamil. At 10 μM, the decrease in P_{open} induced by D600 is 84% ± 5.1 (n = 2), as compared to 70.7% ± 6.2 of verapamil. The data for verapamil are from Fig. 2*B*.

ing effects to verapamil, markedly decreasing the mean open time. At high concentrations (10 μM), the open events were reduced so markedly that most of them did not reach the fully open level, thus appearing as needle-like events. This occurred when the concentration of the drug was increased to the level that the apparent on-rate of block reduced the average lifetime of the open state below the resolution of our recording system. Quantitative evaluation of the efficacy of block, given in Fig. 5*B*, shows that D600 is a more effective blocker than verapamil (care was taken to have comparable P_{open} in control conditions). This is demonstrated by the observation that at same concentrations D600 depresses the P_{open} significantly more than verapamil.

Investigating the Side of Access and Location of Verapamil Binding Site by Use of D890

As verapamil can cross cell membranes very easily, and rapidly equilibrates on both sides of the membrane, information on the location of its binding site and side of access was sought by using the permanently charged, membrane impermeant verapamil analogue D890. Application of D890 to the external face of outside-out membrane patches at a concentration up to 1 mM was totally ineffective on BK channel activity (n = 3; *data not shown*). By contrast, moderate D890 concentrations (5–250 μM) applied on the internal side of the membrane (inside-out patches) were able to produce a flickery block of BK channels, similar to that observed with verapamil (Fig. 6*A*, *cf.* Fig. 2). These results show that D890 binds to a site accessible only from the internal side of the membrane, as has been reported previously for Ca and DRK channels (Affolter & Coronado, 1986; DeCoursey, 1995; Catacuzzeno et al., 1999).

To gain information about the location of the binding site on the channel protein, we assessed the voltage-dependence of D890 block by measuring the fractional P_{open} reduction at different voltages, at a fixed D890 concentration in the bath. As shown in panel *B*, D890 block of BK channels showed a substantial voltage-

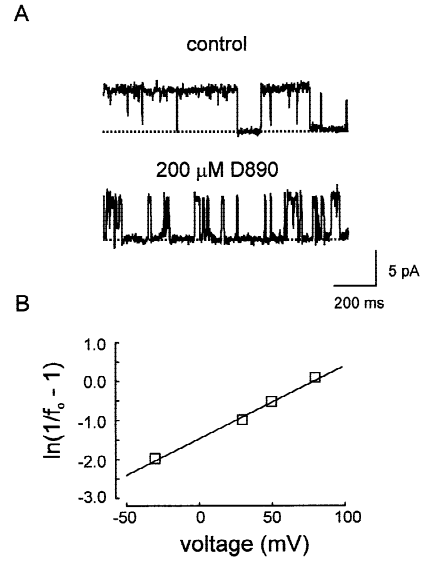

Fig. 6. D890 action on single BK channels. (*A*) Representative inside-out single-channel recordings showing the effect of D890 (200 μM) on the single BK channel activity. Experimental conditions were: symmetrical 140 mM KCl, 1 μM $[Ca]_i$, and holding potential +30 mV. (*B*) Plot of the quantity $ln(1/f_O - 1)$ *vs.* voltage, where f_O is the residual P_{open}. The solid line represents the best fit of the experimental data with Eq. (1). The best fit parameters were $K_{d(0\ mV)}$ = 869 μM and δ = 0.46.

dependence. The experimental data could be well fitted with the following modified form of the Langmuir equation which incorporates the Woodhull (1972) model for charged blockers binding to a site located inside the channel pore:

$$ln(1/f_o - 1) = ln([D890]/K_{d(0\ mV)}) - \delta zFV/RT \qquad (1)$$

where f_o is the residual P_{open} of the channel, [D890] is the blocker concentration, $K_{d(0\ mV)}$ is its dissociation constant with no applied potential, z is its valency, and δ is the fractional voltage drop sensed by the blocker to reach its binding site. The best fit δ value obtained from the plot in Fig. 6*B* was 0.46. The mean value for δ calculated from three such experiments was 0.42 ± 0.05.

These results suggest that the D890 binding site is located along the permeation pathway of BK channels, at about 40% of the voltage drop across the membrane.

Discussion

The investigation reported here focused on the effects of the antihypertensive drug verapamil on the BK channel of rat aortic myocytes. Verapamil was found to block the BK channel in this preparation with a K_d of 4.1 μM, as assessed from the decrease in P_{open} *vs.* verapamil concentration plot fitted with a Langmuir isotherm (Fig. 2). This block efficacy is comparable to that previously found for verapamil block of DRK channels (DeCoursey, 1995; Trequattrini et al., 1998), and BK channel from rabbit aortic myocytes (Pavenstadt et al., 1991). Block of the BK channel, i.e., the decrease in its P_{open}, occurred through a marked decrease of the dwell open time, without any significant change of the closed time components (Fig. 3). This effect was accompanied by the appearance of an additional exponential component to the closed time distribution (Fig. 3*D*). This extra closed component representing the nonconductive, open-blocked state, with time constant reflecting the mean residence time of verapamil on the channel's binding site. Together, these results indicated a state-dependent, open-channel block mechanism. This mechanism was subsequently confirmed by a dedicated kinetic (group) analysis carried out under conditions where BK channel activity displayed burstlike behavior. The group analysis showed that verapamil increased the mean burst duration, leaving the mean interburst time unaltered, consistent with the behavior expected for an open-channel block mechanism. We also tested the verapamil methoxy derivative D600, and found it to inhibit the BK channel with similar mechanism (i.e., producing a flickery block characterized by very brief openings; *cf.* Fig. 5*A*), but with an even higher efficacy (Fig. 5*B*).

The state-dependence of block, and in particular the preference of the drug for the open channel translates in a higher degree of inhibition the higher is the activity of the channel. This occurrence can be functionally important. From the following minimal scheme $C \leftrightarrow O \leftrightarrow O{\cdot}B$, incorporating the essential kinetic states of open-channel block (in succession: closed, open, open-blocked), it follows that the fractional reduction in P_{open} due to a given concentration of the blocker, [*B*], will be: $f_O = (1+([B]/K_B{\cdot}P_{open})^{-1}$. Here K_B is the dissociation constant of the blocker, and P_{open} is the open probability in absence of the blocker. The relation above indicates that factors that change the channel's P_{open} (i.e., $[Ca]_i$ and membrane voltage) will indirectly modulate the blocker efficacy.

The high membrane permeability of the uncharged (unprotonated) form of the phenylalkylamines verapamil and D600 precludes identification of their active site in the membrane. We used the permanently charged and membrane impermeant verapamil analogue D890 to address other important features of verapamil block such as the sidedness of action and the location of the binding site on the channel protein. At micromolar concentrations (5–250 μM), D890 induced a flickering block of BK channels only when applied to the internal side of the membrane, indicating that D890 binds to a site accessible from the cytoplasmic side. The permanently charged nature of D890 allowed us to obtain additional information about the location of the phenylalkylamines' binding site on the BK channel. For a charged molecule that binds inside the ion permeation pathway of a channel, the binding affinity will depend on the applied membrane potential (Woodhull, 1972) and on the voltage drop the blocker experiences in going from the bulk solution to the binding site along the channel pore. We found a significant voltage dependence for D890 block of the BK channel (Fig. 6), suggesting that the phenylalkylamines binding site is located deep into the permeation pathway. The experimental data fitted with a Langmuir equation incorporating the Woodhull model for charged blockers indicated that the D890 binding site is located along the permeation pathway of the BK channel at about 40% of the voltage drop across the membrane.

Another group of compounds, collectively known as quaternary ammonium (QA) ions and structurally very similar to the charged (protonated) form of verapamil, have been shown to block DRK channels by a state-dependent mechanism (Armstrong, 1969; French & Sohoukimas, 1981). Tested on BK channels, QA ions have been found to bind to a common site accessed from the internal mouth of the channel and located at about 30% of the membrane voltage drop along the permeation pathway (Villarroel et al., 1988). Given the strong structural similarity between phenylalkylamines and QA ions, together with the comparable voltage-dependence of their block it is most likely that these compounds block the BK channels by binding at the same site.

Verapamil, together with other drugs classified as Ca antagonists, has proved effective in the treatment of hypertension and other cardiovascular diseases (Abernethy & Schwartz, 1999). The verapamil-induced smooth muscle relaxation, and the consequent decrease in arterial blood pressure is generally thought to result from the inhibition of Ca influx through L-type Ca channel (Kuga et al., 1990; McDonald et al., 1994). The modulation of the BK channel conductance by verapamil was thought to be secondary to the decrease of Ca influx due to L-type Ca channel inhibition by the drug. Here we show that micromolar concentration of verapamil has also a significant inhibitory effect on the BK channels. The direct block of BK channels by verapamil may represent a significant contribution to the overall reduction

of BK P_{open} and the consequent negative feedback modulation of the membrane potential and arterial tone of pressurized arteries.

This work was supported by grants from Italian MURST 9705224541 to FF. AAH thanks the British Council/CNR Scientific Cooperation Programme, Italy for an award. We thank Dr. Martin Traut from Knoll AG (Ludwigshafen, Germany) for kindly providing us with D890.

References

Abernethy, D.R., Schwartz, J.B. 1999. Drug Therapy: Calcium-Antagonist Drugs. *New Eng. J. Med.* **341:**1447–1457

Affolter, H., Coronado, R. 1986. Sidedness of reconstituted calcium channels from muscle transverse tubules as determined by D600 and D890 blockade. *Biophys. J.* **49:**767–771

Armstrong, C.M. 1969. Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. *J. Gen. Physiol.* **545:**553–575

Barrett, J.N., Magleby, K.L., Pallotta, B.S. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* **331:**211–230

Benham, C.D., Bolton, T.B. 1986. Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol.* **381:**385–406

Berweck, S., Lepple-Wienhues, A., Stoss, M., Wiederholt, M. 1994. Large conductance calcium-activated potassium channels in cultured retinal pericytes under normal and high-glucose conditions. *Pfluegers Arch.* **427:**9–16

Bolotina, V., Gericke, N., Bregestovski, P. 1991. Kinetic differences between the Ca^{2+}-dependent K^+ channel in smooth-muscle cells isolated from normal and artherosclerotic human aorta. *Prog. Roy. Soc. Lond. B Bio.* **244:**51–55

Colquhoun, D., Hawkes, A.G. 1995. Single-Channel Recording. 503 pp. Plenum, New York

Catacuzzeno, L., Trequattrini, C., Petris, A., Franciolini, F. 1999. Mechanism of verapamil block of a neuronal delayed rectifier K channel: active form of the blocker and location of its binding domain. *Brit. J. Pharmacol.* **126:**1699–1706

Cox, R.H., Tulenko, T.N. 1995. Altered contractile and ion-channel function in rabbit portal vein with dietary atherosclerosis. *Am. J. Physiol. Heart Circ. Physiol.* **268:**H2522–H2530

DeCoursey, T.E. 1995. Mechanism of K^+ channel block by verapamil and related compounds in rat alveolar cells. *J. Gen. Physiol.* **106:**745–779

England, S.K., Woolridge, T.A., Stekiel, W.J., Rusch, N.J. 1993. Enhanced single-channel K^+ current in arterial membranes from genetically hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* **264:**H1337–H1345

Fleckenstein-Grun, G. 1996. Calcium antagonism in vascular smooth muscle cells. *Pfluegers Arch.* **432:**R53–R60

French, R.J., Shoukimas, J.J. 1981. Blockage of squid axon potassium conductance by internal tetra-N-alkylammonium ions of various sizes. *Biophys. J.* **34:**271–291

Giangiacomo KM, Garcia ML, McManus OB. 1992. Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. *Biochemistry* **31:**6719–6727

Godfrain, D., Morel, N., Wibo, M. 1986. The heterogeneity of calcium movements in cardiac and vascular smooth-muscle cells. *Scan. J. Clin. Lab. Invest.* **46:**29–39

Hamill, O.P., Marty, A., Neher, E., Sackmann, B., Sigworth, F.H. 1981. Improved patch-clamped techniques for higher resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Knot, H.J., Standen, N.B., Nelson, M.T. 1998. Ryanodine receptors regulate arterial diameter and wall $[Ca^{2+}]$ in cerebral arteries of rat via Ca^{2+}-dependent K^+ channels. *J. Physiol.* **508:**211–221

Kuga, T., Sadoshima, J., Tomoike, H., Kanaide, H., Akaike, N., Nakamura, M. 1990. Actions of Ca^{2+} antagonists on two types of Ca^{2+} channels in rat aorta smooth muscle cells in primary culture. *Circ. Res.* **67:**469–480

Langton, P.D., Nelson, M.T., Huang, Y., Standen, N.B. 1991. Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. *Am. J. Physiol. Heart Circ. Physiol.* **260:**H297–H934

Latorre, R., Oberhauser, A., Labarca, P., Alvarez, O. 1989. Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* **51:**385–399

Liu, Y., Jones, A.W., Sturek, M. 1995. Ca^{2+}-dependent K^+ current in arterial smooth-muscle cells from aldosterone-salt hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* **269:**H1246–H1257

McDonald, T.F., Pelzer, S., Trautwein, W., Pelzer, D.J. 1994. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol. Rev.* **74:**365–507

McManus, O.B., Magleby, K.L. 1988. Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat skeletal muscle. *J. Physiol.* **402:**79–120

Miller, C., Moczydlowski, D., Latorre, R., Phillips, M. 1985. Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Nature* **313:**316–318

Nelson, M.T. 1993. Ca^{2+}-activated potassium channels and ATP-sensitive potassium channels as modulators of vascular tone. *Trends Cardiovasc. Med.* **3:**54–60

Nelson, M.T., Quayle, J.M. 1995. Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol. Cell Physiol.* **268:**C799–C822

Pancrazio, J.J., Viglione, M.P., Kleiman, R.G., Kim, Y.I. 1990. Verapamil-induced blockade of voltage-activated K^+ current in small-cell lung cancer cells. *J. Pharmac. Exp. Therap.* **257:**184–191

Pavenstadt, H., Lindeman, S., Lindeman, V., Spath, M., Kunzelmann, K., Greger, R. 1991. Potassium conductance of smooth muscle cells from rabbit aorta in primary culture. *Pfluegers Arch.* **419:**57–68

Rampe, D., Wible, B., Fedida, D., Dage, R.C., Brown, A.M. 1993. Verapamil blocks a rapidly activating delayed rectifier K^+ channel cloned from human heart. *Mol. Pharmacol.* **44:**642–648

Rauer, H., Grismer, R.S. 1996. Evidence of an internal phenylalkylamine action on the voltage gated potassium channel Kv1.3. *Mol. Pharmacol.* **50:**1625–1634

Retzinger, G.S., Cohen, L., Lau, S.H., Kezdy, F.J. 1986. Ionization and surface properties of verapamil and several verapamil analogues. *J. Pharm. Sci.* **75:**976–982

Rush, N.J., Delucena, R.G., Woolridge, T.A., England, S.K., Cowley, A.W. 1992. A Ca^{2+}-dependent K^+ current is enhanced in arterial membranes of hypertensive rats. *Hypertension* **19:**301–307

Rush, N.J., Liu, Y., Pleyte, K.A. 1996. Mechanisms for regulation of arterial tone by Ca^{2+}-dependent channels in hypertension. *Clin. Exp. Pharmacol. Physiol.* **23:**1077–1082

Rush, N.J., Runnells, A.M. 1994. Remission of high blood-pressure reverses arterial potassium channel alterations. *Hypertension* **23:**941–945

Sigurdson, W.J., Morris, C.E., Brezden, B.L., Gardner, D.R. 1987. Stretch activation of a K^+ channel in molluscan heart cells. *J. Exp. Biol.* **127:**191–209

Somlyo, A.P. 1985. Excitation-contraction coupling: and the altered structure of smooth-muscle. *Circ. Res.* **57:**497–507

Trequattrini, C., Catacuzzeno, L, Petris, A., Franciolini, F. 1998. Verapamil block of the delayed rectifier K current in chick embryo dorsal root ganglion neurons. *Pfluegers Arch.* **435:**503–510

Trequattrini, C., Harper, A.A., Petris, A., Franciolini, F. 1996. Pharmacological characterization of Ca-activated K channels in arterial smooth muscle cells from rat. *Pfluegers Arch.* **431:**R343.48 (*Abstr.*)

Villarroel, A., Alvarez, O., Oberhauser, A., Latorre, R. 1988. Probing a Ca^{2+}-activated K^+ channel with quaternary ammonium ions. *Pfluegers Arch.* **413:**118–126

Woodhull, A.M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* **61:**687–708