A C-terminal peptide of the GIRK1 subunit directly blocks the G protein-activated K^+ channel (GIRK) expressed in *Xenopus* oocytes

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1. In order to find out the functional roles of cytosolic regions of a G protein-activated, inwardly rectifying potassium channel subunit we studied block of GIRK channels, expressed in *Xenopus laevis* oocytes, by synthetic peptides in isolated inside-out membrane patches.

2. A peptide (DS6) derived from the very end of the C-terminus of GIRK1 reversibly blocked GIRK activity with IC_{50} values of $7{\cdot}9 \pm 2{\cdot}0$ or $3{\cdot}5 \pm 0{\cdot}5\ \mu g\ ml^{-1}$ (corresponding to $3{\cdot}7 \pm 0{\cdot}9$ or $1{\cdot}7 \pm 0{\cdot}2\ \mu mol\ l^{-1}$) for GIRK1/GIRK5 or GIRK1/GIRK4 channels, respectively.

3. Dose dependency studies of GIRK activation by purified $\beta\gamma$ subunits of the G protein ($G_{\beta\gamma}$) showed that DS6 block of GIRK channels is not the result of competition of the peptide with functional GIRK channels for the available $G_{\beta\gamma}$.

4. Burst duration of GIRK channels was reduced, whereas long closed times between bursts were markedly increased, accounting for the channel block observed.

5. Block by the DS6 peptide was slightly voltage dependent, being stronger at more negative potentials.

6. These data support the hypothesis that the distal part of the carboxy-terminus of GIRK1 is a part of the intrinsic gate that keeps GIRK channels closed in the absence of $G_{\beta\gamma}$.

The inwardly rectifying K^+ channels of the GIRK (Kir3) family are important in the regulation of excitability in heart and brain. Opening of cardiac GIRK channels (K_{ACh}) by acetylcholine (ACh), via muscarinic m_2 receptors that activate a pertussis toxin (PTX)-sensitive G protein of the G_i/G_o family mediates the negative chronotropic effect of the vagus on heartbeat (reviewed by Kurachi, 1995). Similar K^+ channels are activated via PTX-sensitive G proteins by serotonin ($5\text{-}HT_{1A}$), δ-and μ-opioid, $GABA_B$, dopamine and some other receptors in the brain, where they are believed to act as important mediators of inhibitory neurotransmission (reviewed by North, 1989; Hille, 1992; Wickman & Clapham, 1995; Dascal, 1997).

GIRKs are members of the superfamily of inwardly rectifying K^+ channels (Kir; for review see Doupnik, Davidson & Lester, 1995) characterized by two transmembrane α-helical domains M1 and M2, a pore (P) region similar to that of voltage-dependent channels, and cytoplasmic N- and C-terminal regions. Four such proteins (subunits) form a functional channel. GIRK channels in the heart are heterotetramers of two subunits, GIRK1/KGA and GIRK4/CIR. Additional GIRK subunits, GIRK2 and GIRK3, are found in the brain where possibly they form heteromeric channels with GIRK1. GIRK1 alone cannot form functional channels, but its expression in *Xenopus* oocytes gives functional channels due to association with the GIRK5/XIR of the endogenous oocyte (see Dascal 1997 for review).

In addition to their physiological importance, GIRKs present the best studied case of ion channels directly gated by

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heterotrimeric G proteins. Membrane-delimited modulation has been described for other ion channels, notably voltage-operated Ca^{2+} channels (Wickman & Clapham, 1995), but their direct interaction with G protein subunits is less well established. Once activated by the $\beta\gamma$ subunits of the G proteins ($G_{\beta\gamma}$) GIRKs show intrinsic gating such as slow voltage-dependent relaxations and, like all other channels of the Kir superfamily, display inward rectification which is mainly due to blockade by intracellular Mg^{2+} and polyamines (see Dascal 1997 for review).

While GIRKs are activated by binding of the $\beta\gamma$ subunits (reviewed by Kurachi, 1995; Wickman & Clapham, 1995), the molecular mechanism of gating of GIRKs by $G_{\beta\gamma}$ remains obscure. The cytosolic C- and N-terminal regions of the GIRK1, and probably of the other GIRK subunits, play a role in $G_{\beta\gamma}$-dependent activation, since $G_{\beta\gamma}$ binds directly to a part of the C-terminal cytoplasmic region of GIRK1 and GIRK4 and to (a part of) the N-terminus (as reviewed by Dascal, 1997). It is unclear how the channels are transformed by binding with $G_{\beta\gamma}$ from a basal state with a low activity to a state with a high probability of opening. Because treatment with trypsin from the cytosolic side constitutively activates GIRK in a manner similar to that produced by ACh, it has been proposed (Kirsch & Brown, 1989) that a cytoplasmic segment may physically block the channel, and that binding of the G protein displaces this blocking gate. The proposed mechanism resembles the principle underlying fast inactivation of the voltage-dependent *Shaker* type potassium channels, which are blocked by a part of the N-terminal domain (Hoshi, Zagotta & Aldrich, 1990), although the biophysical and molecular details may differ significantly. The hypothetical blocking gate may reside at the C-terminus of GIRK subunits. Thus, Pessia, Bond, Kavanaugh & Adelman (1995) proposed, on the basis of work with chimeric inwardly rectifying channels, that the pore can be occluded by reversible interactions with C-terminal residues of GIRK1. We have proposed a similar hypothesis on the basis of the observation that co-expression in the oocytes of a membrane-attached C-terminal part of GIRK1, $GIRK1_{183\text{-}501}$, strongly blocks the agonist-evoked GIRK1/GIRK5 activity and, to some extent, the activity of an inward rectifier that is not gated by G proteins, ROMK1 (Dascal *et al.* 1995).

In order to elucidate the possible role of putative cytoplasmic regions of the channel in G protein-dependent and intrinsic gating of the channel, we designed synthetic peptides derived from the GIRK1 sequence. The peptides were tested in isolated inside-out patches from membranes of *Xenopus laevis* oocytes expressing GIRK1/GIRK5 (in oocytes injected with GIRK1 RNA) and GIRK1/GIRK4 channels, for their ability to modify gating behaviour. In this study we present the results obtained with one of the peptides, homologous to the very C-terminus of the GIRK1 subunit (DS6) which proved to be a potent blocker of channel activity. Part of this study has been presented in abstract form (Luchian, Dascal, Davidson, Lester & Schreibmayer, 1996).

METHODS

Solutions

The composition of physiological and buffering solutions was as follows. ND96 (mM): NaCl, 96; KCl, 2; $CaCl_2$, 1; $MgCl_2$, 1; Hepes, 5; titrated with NaOH to pH 7·8. hK (mM): KCl, 96; NaCl, 2; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 5; titrated with NaOH to pH 7·8. PG200Ca (mM): glutamate, 180; KCl, 37·5; $CaCl_2$, 1; $MgCl_2$, 1; Hepes, 10; titrated with NaOH to pH 7·5. CHAPS buffer (mM): 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS), 11·4; NaCl, 50; DTT, 3; Hepes, 20; titrated with NaOH to pH 8. Bathing solution (BS) (mM): KCl, 140; NaCl, 10; EGTA, 1; $MgCl_2$, 4; NaATP, 1; Hepes, 10; titrated with KOH to pH 7·5. Pipette solution (PS) (mM): KCl, 150; $MgCl_2$, 1; $CaCl_2$, 1; $GdCl_3$, 0·05; Hepes, 10; titrated with KOH to pH 7·5. PS was filtered through a 0·2 μm filter before use.

Oocyte preparation and RNA injection

Xenopus laevis frogs were anaesthetized in 0·15% (w/v) procaine methanesulphonate and dissected as previously described (Dascal & Lotan, 1992) by opening a small incision in the abdomen, which was sutured after removal of the desired amount of oocytes from the ovary. After recovery from anaesthesia, the animal was returned to the tank and allowed to rest for at least 4 weeks before the next surgery. The oocytes were defolliculated using collagenase, prepared and injected as previously described (Dascal & Lotan, 1992). Plasmids containing the GIRK1 (Dascal *et al.* 1993) or the GIRK4 (Ashford, Bond, Blair & Adelman, 1995) sequences were linearized with *Xho*I (GIRK1 and GIRK4), cRNA was transcribed as described in Dascal & Lotan (1992) and stored at −70 °C until use. Amounts of cRNA injected per oocyte were as follows (ng): GIRK1, 1; GIRK4, 0·05–5; IRK1, 0·5. Oocytes were kept in an incubator at 19 °C and used 3–7 days after injection for electrophysiological recordings.

Electrophysiology

Two-electrode voltage clamp experiments. Oocytes were placed into a ~500 μl recording chamber, which allowed superfusion with different solutions and exchange of the buffering solution within less than 5 s. Subsequently oocytes were impaled with agarose cushion electrodes (Schreibmayer, Lester & Dascal, 1994), the holding potential was set to −80 mV and the current was recorded using a Geneclamp500 amplifier connected via a TL-125 A/D and D/A converter (Axon Instruments) to an IBM-compatible computer. The current was low-pass filtered with a 4-pole Bessel filter at 100 Hz, digitized at 10 Hz and traces stored on hard disk for subsequent analysis. The basal inwardly rectifying potassium current was measured as the current induced by the exchange of bathing medium from ND96 to hK at −80 mV, whereas the ACh-evoked current was induced by a subsequent addition of 10 μM ACh to the hK medium.

Patch clamp experiments. For patch clamp experiments, the oocytes were placed for 3–6 min into PG200Ca medium for shrinking and the vitelline layer was removed with fine forceps. The devitellinized oocyte was placed into a recording chamber with a volume of 500 μl filled with BS. The pipettes were filled with PS solution. Inside-out patches were produced by a brief air-exposure of the pipette tip. Patch currents were recorded at −80 mV and room temperature (19 °C) using an Axopatch-1D amplifier, equipped with the IHS integrating headstage (Axon Instruments) connected via a TL-125 A/D and D/A converter to an IBM-compatible computer. Current traces were low-pass filtered at 1 kHz, using a 4-pole Bessel filter, digitized at 5 kHz, and stored on hard disk for subsequent analysis. Data analysis was performed using pCLAMP 6.0 software (Axon Instruments). Closed states longer than 4·5 times the duration of the intermediate closed time,

denoted as τ_{c2} in the text, were regarded as long-lived closures, separating individual bursts; this resulted in a minimum of erroneously counted closed times between bursts and within bursts. Peptides were applied by diluting an appropriate amount in 50 μl bathing solution and adding it directly to the recording chamber. Alternatively the recording chamber was perfused with a bathing solution containing the appropriate amount of the peptide by gravitation. Dose–response curves for DS6 inhibition of channel activity were fitted by Sigmaplot 2.0 software (Jandel Scientific, Erkrath, Germany), according to the following equation:

$$\%I_{([DS6])} = 100 - \frac{100}{1 + 10^{\log([DS6]) - \log EC_{50}/h}}, \quad (1)$$

where $\%I$ represents percentage inhibition of the channel open probability (P_o) at a certain concentration of DS6 relative to P_o in the absence of the peptide, EC_{50} represents the DS6 concentration resulting in half-maximal block and h represents the Hill coefficient. The gating parameters of the GIRK1/GIRK5 channel (Table 2) were calculated for patches containing between one and three functional channels, as judged from the maximum number of overlapping openings for a period of at least 10 min.

Statistics

Parameter sets obtained under different experimental conditions were tested for significant differences using Student's t test. The correlation between membrane potential and percentage inhibition of P_o by DS6 was tested using correlation analysis (Kreiszig, 1979).

Materials

Peptides were synthesized at the California Institute of Technology Biopolymer Synthesis Center. The sequences of the peptides were: DS6, KTRMEGNLPAKLRKMNSD; DS4, KHGNLGSE TSRYLSDLFTT (the lysine residue at the N-terminus in these two peptides is not contained in the GIRK1 sequence and has been added in order to enable coupling of the peptide to a carrier protein, bovine serum albumin, and raising of antibodies, which have been used for another study (see Dascal *et al.* 1995); DS1, KKKRQRFVDKNGRCNVQH. Recombinant $G_{\beta 1\gamma 2}$ was purified as described (Kozasa & Gilman, 1995) and stored in CHAPS buffer at −70 °C until use. Aliquots of $G_{\beta\gamma}$ solutions were thawed at 30 °C and stored on ice for up to 5 h before application to the bathing solution. Chemicals were reagent grade throughout, except chemicals for molecular biology, which were obtained from Boehringer (Mannheim, Germany). Guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) was stored in 2 μl aliquots of 0·1 M at −20 °C and used no later than 2 h after thawing. The cDNA of the IRK1 channels was kindly supplied by Dr L. Y. Jan (University of California at San Francisco, USA) and the cDNA of the m_2 receptor by Dr E. Peralta (Harvard University, Cambridge, MA, USA).

RESULTS

In the initial series of experiments we examined the effect of the DS6 peptide corresponding to GIRK1 amino acid residues 482–498, i.e. of length 17, and omitting the three

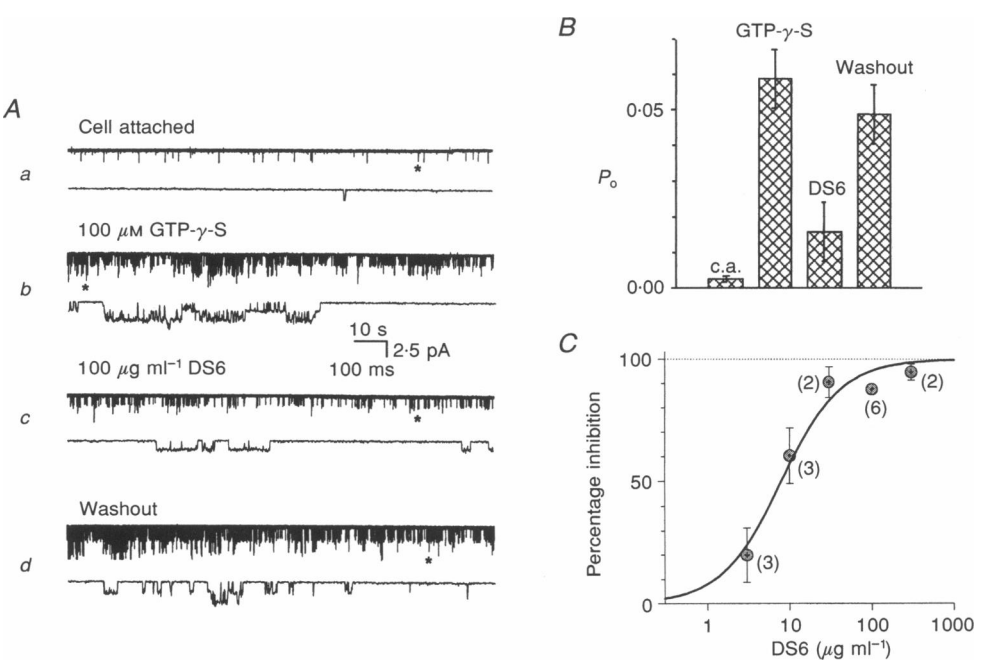

Figure 1. Block of GIRK1/GIRK5 channels by DS6 peptide applied from the cytosolic side

A, the activity of an inside-out patch containing 3 GIRK channels is shown at 2 different time scales in pairs of traces recorded at −80 mV (upper trace is shown at a compressed timescale. The segment from which the high resolution trace was taken is marked with an asterisk). *a*, basal activity of channels; *b*, after activation of channels with 100 μM GTP-γ-S; *c*, channel activity after the addition of 100 μg ml^{-1} DS6; *d*, activity after washout of the peptide. *B*, statistics of washout experiments. The hatched bars represent the open probability of 1 channel for cell-attached (c.a.), GTP-γ-S-activated, DS6-blocked and recovered channel activity. Error bars are ±S.E.M. values. *C*, dose–response curve for block of open probability of the channel by the DS6 peptide. The numbers of individual experiments for each DS6 concentration are shown in parentheses. The curve represents the best fit to the data points as explained in Methods.

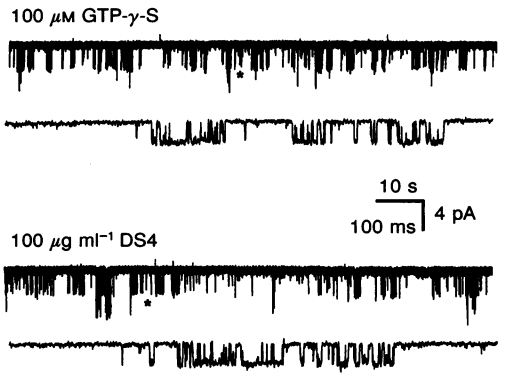

Figure 2. Application of the DS4 control peptide to a membrane patch containing GIRK1/GIRK5 channels
Channel activity at −80 mV is shown at two different time scales before (upper two traces) and after addition of 100 μg ml^{-1} DS4. The asterisk denotes the sequence that is shown at high time resolution.

amino acids at the C-terminal (with the addition of a lysine at the N-terminus of the peptide) on GIRK1/GIRK5 channels. Channel activity was quantified as NP_o, i.e. the total open probability of all the channels in the patch. When 100 μM GTP-γ-S was added to the bath solution facing the cytosolic side of the excised inside-out patch, a marked increase in channel activity could be observed, which reached steady state after about 15–20 min (see Fig. 1*Aa* and *b*). The channel activity displayed a characteristic bursting behaviour (Sakmann, Noma & Trautwein, 1983; Ivanova-Nikolova & Breitwieser, 1997; see Figs 2 and 4). After NP_o appeared to have reached a constant value, DS6 at a concentration of 100 μg ml^{-1} (corresponding to 48 μmol l^{-1}) was added to the bath. This resulted in inhibition of channel activity (Fig. 1*Ac*) by 83·4 ± 3·1 % (mean ± S.E.M.; $n = 10$). As shown in Fig. 1*Ad*, this inhibition was reversible upon washout of the peptide from the bath solution (three patches; see Fig. 1*B* for statistics of washout experiments).

Cumulative dose–response curves for peptide block (Fig. 1*C*) revealed an IC_{50} value of 7·9 ± 2·0 μg ml^{-1} (corresponding to 3·75 ± 0·95 μmol l^{-1}) with a Hill slope of 1·2 ± 0·3 ($n = 6$). Application of 10 or 100 μg ml^{-1} DS6 to excised membrane patches expressing the IRK1 channel (Kubo, Baldwin, Jan & Jan, 1993) did not lead to a noticeable change in channel activity (two patches; data not shown). Figure 2 demonstrates that application of another peptide (DS4) derived from the N-terminal part adjacent to the first putative transmembrane-spanning domain (GIRK1 amino acid residues 57–74, with the addition of an N-terminal lysine), at a concentration of 100 μg ml^{-1} (corresponding to 47 μmol l^{-1}) was ineffective (a change in NP_o of 3·2 ± 4·3 %, $n = 3$). Another N-terminal peptide, DS1 (amino acid residues 40–57) also did not block, in fact slightly enhanced, the channel opening at 100 μg ml^{-1} (corresponding to 45 μmol l^{-1}; 49·4 ± 20·1 %; $n = 3$; data not shown).

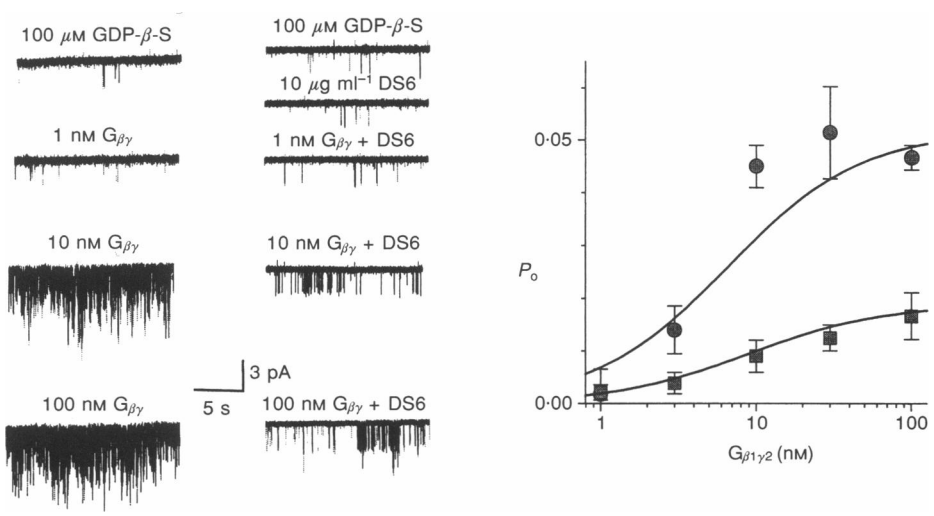

Figure 3. Dose–response traces for $G_{\beta\gamma}$ activation of GIRK1/GIRK5 channels in the absence and presence of 10 μg ml^{-1} DS6 peptide
Original current traces recorded at −80 mV from an inside-out patch are shown. The bathing solution contained 100 μM GDP-β-S and the indicated concentrations of $G_{\beta\gamma}$. Left column, control. Middle column, current traces recorded in the same experimental protocol, but 10 μg ml^{-1} DS6 added before $G_{\beta\gamma}$. Right, open probability *vs.* free concentration of $G_{\beta\gamma}$ in the presence (●) and absence (■) of DS6 (10 μg ml^{-1}). Mean values from 4 different patches are shown ±S.E.M. The continuous lines represent the best fit through the original data points.

The block of GIRK by the DS6 peptide could be due to interaction of the peptide with $G_{\beta\gamma}$, resulting in competition between the channel and the peptide for the available free $G_{\beta\gamma}$; in such a case, addition of excess $G_{\beta\gamma}$ should overcome the inhibition. Competitive inhibition by $G_{\beta\gamma}$-binding peptides has already been described (Koch, Inglese, Stone & Lefkowitz, 1993; Reuveny *et al.* 1994; Nair *et al.* 1995). Another possibility is that DS6 is part of the blocking gate of the channel which directly interacts with an acceptor site on the channel molecule; in this case, no competition between DS6 and $G_{\beta\gamma}$ is expected. To distinguish between these possibilities, we examined the dose dependence of GIRK activation by purified $G_{\beta 1\gamma 2}$ subunit heterodimer (termed $G_{\beta\gamma}$ in the following) in the presence or absence of a roughly half-maximal concentration of the DS6 peptide. Representative single channel traces from such competition experiments are shown in Fig. 3 (left and middle). It is already obvious from the original traces that the maximum open probability of the channel is considerably reduced when 10 μg ml^{-1} DS6 is present in the bathing solution. Increasing the free $G_{\beta\gamma}$ concentration up to 100 nM was unable to restore the normal channel activity (Fig. 3, right). Table 1 summarizes the dose–response experiments and shows that the only statistically significant change caused by 10 μg ml^{-1} DS6 was a ~2·5-fold decrease in maximal P_o ($P_{o(max)}$).

Table 1. Effect of DS6 peptide on activation of the GIRK1/GIRK5 channel by G protein $\beta_1\gamma_2$ subunits

	Control	DS6 (10 μg ml^{-1})
$P_{o(max)}$	0·052 ± 0·01	0·019 ± 0·00*
EC_{50}	4·75 ± 2·61	14·27 ± 4·44
h	1·21 ± 0·62	0·83 ± 0·11
n	4	4

Mean values of maximum open probability ($P_{o(max)}$), half-maximal effective concentration of $G_{\beta\gamma}$ (EC_{50}) and Hill coefficient (h) are given ±S.E.M. * Statistically significant difference at the $P < 0·05$ level. n, number of individual patches.

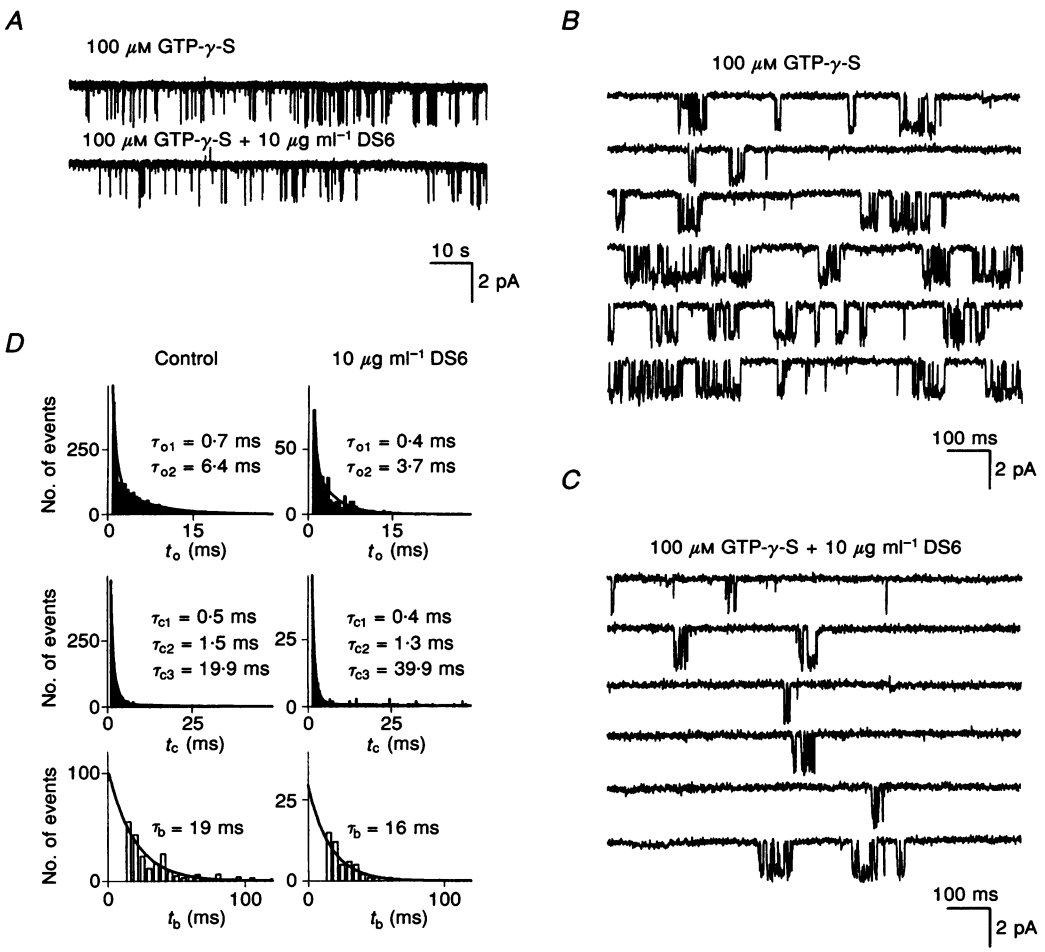

Figure 4. Single channel analysis of DS6 block

Original single channel traces recorded at a holding potential of −80 mV are shown in *A* at a compressed time scale. Single channel traces at a higher time resolution without the peptide and in the presence of 10 μg ml^{-1} DS6 are shown in *B* and *C*, respectively. *D*, representative open (top), closed (middle) and burst (bottom) duration histograms from single channel patches under control conditions (left column) and in the presence of 10 μg ml^{-1} DS6 (right column).

Table 2. Time constants of single channel recordings

	100 μM GTP-γ-S	100 μM GTP-γ-S + 10 μg ml^{-1} DS6
Open times		
τ_{o1} (ms)	0·52 ± 0·06	0·45 ± 0·07
f_{o1}	0·88 ± 0·02	0·72 ± 0·15
τ_{o2} (ms)	5·15 ± 0·54	3·55 ± 0·49
f_{o2}	0·12 ± 0·03	0·28 ± 0·16
Closed times		
τ_{c1} (ms)	0·39 ± 0·08	0·40 ± 0·04
f_{c1}	0·88 ± 0·03	0·92 ± 0·03
τ_{c2} (ms)	1·78 ± 0·30	2·81 ± 0·65
f_{c2}	0·10 ± 0·03	0·068 ± 0·03
τ_{c3} (ms)	26·4 ± 2·0	39·8 ± 6·5**
f_{c3}	0·003 ± 0·001	0·002 ± 0·001
Burst times		
τ_b (ms)	32·4 ± 3·3	15·6 ± 2·9***

τ_{o1} and τ_{o2} denote open time constants; τ_{c1} to τ_{c3}, closed time constants and τ_b, burst time duration, respectively. f is the fraction spent by the channel in each open or closed state. Mean values ±S.E.M. are given. ** $P < 0·01$; *** $P < 0·001$. Data were derived from 5 individual patches.

In order to elucidate further the molecular mechanism of DS6 interaction with GIRK, DS6 block was studied in membrane patches containing only between one and three functional GIRK1/GIRK5 channels. Channel activation was achieved by addition of 100 μM GTP-γ-S to the bathing solution; then DS6 at 10 μg ml^{-1} was added (see Fig. 4*A–C* for original records). Single channel analysis revealed that the open state of the activated channel displays at least two kinetically different states, one with a very short lifetime ($\tau_{o1} < 1$ ms) and another with a considerably longer lifetime, with a τ_{o2} of about 5 ms (Fig. 4*D* and Table 2). Closed-time histograms (Fig. 4*D*, middle) could be fitted by a three-exponential decay, with one long-lasting time constant representing closures between bursts (τ_{c3}, ~26 ms) and two shorter closed states (τ_{c1} and τ_{c2} of about 0·4 and 1·8 ms, respectively) which occur within bursts. In most cases, an additional population of very long (hundreds of milliseconds) interburst intervals could be observed, but they were too infrequent to be included in the routine fitting procedure. Table 2 summarizes the results of five experiments and shows that the main effects of 10 μg ml^{-1} of the DS6 peptide were (i) a significant increase in the duration of interburst intervals and (ii) a decrease in burst duration (both time constants were changed about 2-fold). The gating parameters within the bursts were marginally affected if at all. Single channel slope conductance (measured between −140 and −40 mV) was linear and unaffected by application of the peptide (32·9 ± 1·2 pS before and 34·6 ± 0·9 pS after DS6 addition, $n = 4$; data not shown).

In order to investigate whether the receptor site for the (charged) DS6 molecule is located within the transmembrane electrical field, block of GIRK1/GIRK5 channels by DS6

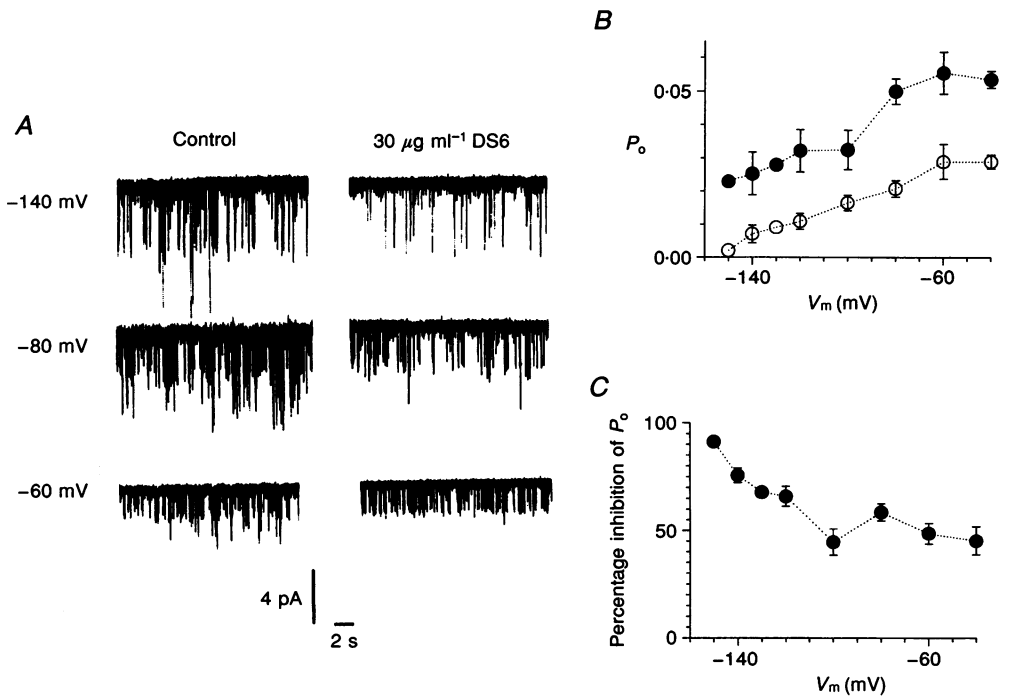

Figure 5. Voltage dependence of DS6 inhibition of GIRK1/GIRK5 channels

A, original current traces recorded at different membrane potentials under control conditions (left) and during the action of the peptide (right). *B*, P_o as a function of membrane potential (V_m) for control conditions (●) and 30 μg ml^{-1} DS6-blocked channels (○) (data from 6 different patches are shown ±S.E.M.).

was measured at different potentials (see Fig. 5A for original recordings). As can be seen from Fig. 5B, P_o exerts intrinsic voltage dependence and hence the percentage inhibition of P_o was calculated for each potential tested (Fig. 5C). As can be seen from Fig. 5C, the inhibitory effect of DS6 on P_o was stronger at more negative potentials. This dependency of P_o on transmembrane potentials was tested using correlation analysis and the correlation coefficient was found to be −0·70 with a 95% confidence interval ranging between −0·42 and −0·86. The error probability for no correlation between membrane potential (E_m) and P_o was found to be $P < 0{\cdot}0001$.

Since the G protein-activated potassium channel from atrium (K_{ACh}) is a hetero-oligomer of GIRK1 and GIRK4 subunits (Krapivinsky, Gordon, Wickman, Velimirovic, Krapivinsky & Clapham, 1995), we tested whether GIRK1/GIRK4 channels are blocked by DS6. cRNAs encoding GIRK1 and GIRK4 were injected into the oocytes at different ratios; cRNA encoding the m_2 muscarinic receptors was also co-injected in order to be able to induce channel activation upon extracellular application of ACh. Oocytes were screened using the two-electrode voltage clamp method for macroscopic whole-cell currents resulting from activation of GIRK. Figure 6A confirms that hetero-oligomeric proteins are formed between the GIRK1 and GIRK4 subunits: GIRK4 cRNA, which at this concentration was not able to induce significant currents in *X. laevis* oocytes, greatly potentiated I_{ACh} in a dose-dependent manner, when constant amounts of GIRK1 cRNA were co-injected (Fig. 6B). Subsequently experiments were performed with oocytes injected with GIRK1/GIRK4 cRNAs at a ratio of 1:10 (500 pg and 5 ng, respectively). As can be seen from our isolated patch recordings, shown in Fig. 6A, DS6 blocks hetero-oligomeric GIRK1/GIRK4 channel activity with a potency similar to GIRK1/GIRK5 channels, with a maximal inhibition of 91·9 ± 5·4% ($n = 4$) at 100 μg ml^{-1} DS6. Dose–response curves showed that the IC_{50} and Hill coefficient values are within the same order of magnitude, though not identical to GIRK1/GIRK5 channels (IC_{50}, 3·49 ± 0·53 μg ml^{-1} (corresponding to 1·67 ± 0·25 μmol l^{-1}); Hill coefficient, 0·75 ± 0·08; $n = 4$ individual patches; see Fig. 6C).

DISCUSSION

Our results demonstrate clearly that the DS6 peptide derived from the extreme C-terminal end of the GIRK1 subunit is a potent and reversible blocker of GIRK channels of GIRK1/GIRK5 and GIRK1/GIRK4 composition. Block of GIRK channels in excised patches by fusion proteins or a

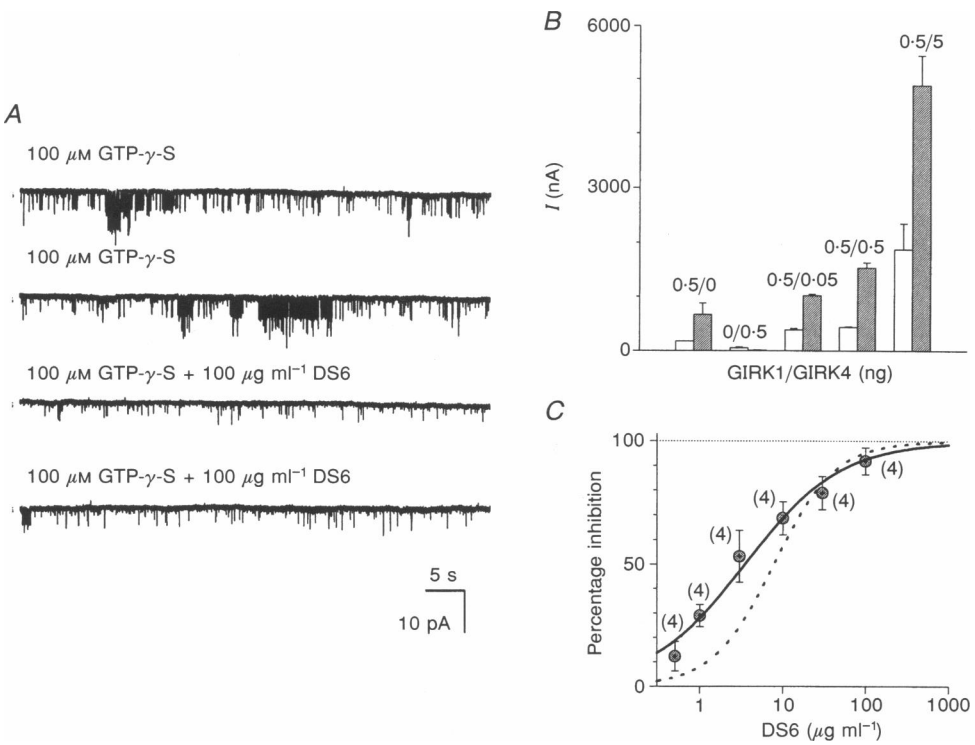

Figure 6. Effect of the DS6 peptide on GIRK1/GIRK4 channels

A, selected traces from inside-out patches containing a few hetero-oligomeric GIRK1/GIRK4 channels before (upper 2 traces) and after addition of 100 μg ml^{-1} DS6 peptide. *B*, effect of increasing amounts of cRNA encoding the GIRK4 subunit on basal (□) and ACh-induced (▨) (cf. Methods) recorded from oocytes injected with constant amounts of RNAs of GIRK1 and the muscarinic m_2 receptor. *C*, dose–response curve for DS6 block of GIRK1/GIRK4 channels. The continuous line represents best fit to the data. The dashed line corresponds to the dose–response curve for GIRK1/GIRK5 channels. Numbers in parentheses represent the number of individual patches.

peptide comprising parts of the $G_{\beta\gamma}$-binding segment of β-adrenergic receptor kinase has been observed by Reuveny *et al.* (1994) and Nair *et al.* (1995). Although direct competition experiments of these peptides with $G_{\beta\gamma}$ have not been performed, a variety of indirect evidence suggested that the mechanism of block in this case was sequestration of free $G_{\beta\gamma}$ by the peptide (Nair *et al.* 1995), in a way similar to the GDP-bound G protein α-subunit block of GIRK channels (Wickman *et al.* 1994).

As in frog atrial cells (Ivanova-Nikolova & Breitwieser, 1997), the GIRK channels expressed in *Xenopus* oocytes display a complex gating behaviour characterized by bursts of activity with variable interburst intervals. Because of the extreme complexity of gating our present analysis is somewhat preliminary and more single channel experiments will be needed in order to understand how $G_{\beta\gamma}$ alters the kinetics. However, the present level of analysis already provides several insights into the mechanism of GIRK activation and its block by DS6. A direct block by DS6 is supported by two independent observations. First, the competition experiments (Fig. 3) demonstrated that DS6 blocks the channel activity by reducing the maximal open probability rather than by competing with $G_{\beta\gamma}$. The blocking effect of DS6 could not be overcome by increasing the concentration of free $G_{\beta\gamma}$ to 100 nM, a concentration about 20- to 40-fold higher than the apparent affinity for $G_{\beta\gamma}$ in this preparation (see Table 1 and Schreibmayer *et al.* 1996). Although there was an apparent shift in the EC_{50} for $G_{\beta\gamma}$ in the presence of DS6, it was not statistically significant (Table 1) and could result from the ambiguities in measuring P_o at very low levels of channel activity, as was the case in the presence of the peptide. Second, burst duration is most probably independent of $G_{\beta\gamma}$ concentration (Ivanova-Nikolova & Breitwieser, 1997), whereas DS6 significantly shortens the bursts; therefore the changes in gating kinetics caused by DS6 are incompatible with a competition mechanism in which DS6 effectively reduces the free concentration of $G_{\beta\gamma}$. The shallow, but negative, correlation between membrane potential and blocking efficiency of DS6 excludes the hypothesis that the positively charged peptide crosses significant amounts of electrical distance across the membrane when diffusing to its receptor site on the channel molecule. Hence the peptide does not go into the pore of the channel, and blocking is more compatible with the idea of a large inhibitory gate at the channel mouth that occludes it at rest. The present results fit into the framework of the hypothesis proposed in the introduction, that activation of the GIRK channel by $G_{\beta\gamma}$ involves the removal of a blocking gate that normally blocks the channel; a hallmark of this removal is the appearance of bursts of channel openings. This hypothesis further implies that the constitutively active Kir channels, such as Kir1 (Ho *et al.* 1993) or Kir2 (Kubo *et al.* 1993) lack a blocking gate (cf. Pessia *et al.* 1995). The intraburst closed–open transitions in Kir channels probably reflect an intrinsic gating process, because they persist in the absence of the presumptive blocking gate (after trypsin treatment; Kirsch & Brown, 1989) or Mg^{2+} and polyamine cations (Ficker, Taglialatella, Wilbe, Henley & Brown, 1994; Yamada & Kurachi, 1995). The fact that DS6 does not appreciably alter the parameters of intraburst transitions suggests that DS6 does not interfere with the intrinsic gating machinery of the activated channel. (This does not preclude a degree of interaction between the blocking gate and permeant or blocking ions.) The only apparent change in the intraburst gating parameters – a reduction in the intraburst open time constant, τ_{o2} – is mild and statistically insignificant, implying that DS6 does not act as a fast channel blocker. Furthermore, any possible contribution of the apparent change in τ_{o2} to the overall reduction in P_o caused by DS6 is counterbalanced by the increase in the proportion of these openings (Table 2). Thus, our results conform to a model in which DS6 mimics the blocking gate by binding to an acceptor site and blocking the ion flow through the channel, while the interaction with $G_{\beta\gamma}$ remains unaltered. Indeed, the main effects of DS6 on single GIRK1 channels were (i) a marked and significant reduction in burst duration and (ii) a prolongation of silent periods between bursts. The greatly prolonged interburst intervals probably indicate permanent occupancy of the channel at a time scale of many tens of milliseconds, as expected for a high-affinity blocking gate, whereas the reduced burst duration reveals that the peptide may interact with the active channel, i.e. with the open or closed state(s) within a burst. We note parenthetically that, under the conditions used here, in the long (interburst) closed state the acceptor site may be inaccessible to the exogenously added DS6 for most of the time, since it is occupied by the channel's own blocking gate. Assuming that durations of prolonged interburst intervals reflect single unblocking events and reduced burst durations reflect blocking events, we estimate a calculated time constant for interburst intervals of 38·1 ms (see Appendix), which corresponds fairly well with the 39·8 ms measured in our single channel experiments. (Note, however, that the data set has been derived from patches containing 1–3 functional channels.)

Our data further support the notion (Dascal *et al.* 1995; Pessia *et al.* 1995) that the structural determinants of the putative blocking gate are situated at the end of the C-terminus of GIRK1. The segment constituting the blocking gate may not be limited to the last twenty amino acid residues; preliminary results indicate that a more proximally situated peptide, DD7 (amino acid residues 439–464 of GIRK1), also efficiently blocks the channel. So far, our data indicate that the N-terminus, at least beyond amino acid residue 40, is not involved in channel block, as witnessed by the inability of DS1 (residues 40–57) and DS4 (residues 57–74) to block the channel. At present we cannot exclude the possibility that the beginning of the N-terminus may be involved in the formation of the blocking gate. GIRK1 may not be the only subunit contributing to the formation of the blocking gate, since channels formed by GIRK2 or GIRK4 alone are $G_{\beta\gamma}$-gated (Krapivinsky, Krapivinsky, Wickman & Clapham, 1995; Kofuji, Davidson

& Lester, 1995). The C-terminus of GIRK1 is > 80 amino acids longer than that of other members of the Kir3 family, and its distal part is practically unique. However, we found that there is a limited homology between GIRK1 (rat or human), GIRK2 (mouse), GIRK4 (rat or human) and GIRK5 (*Xenopus*) within the last twenty amino acid stretch, the consensus being ExxxPxxL (residues 485–492 of rat GIRK1). It is tempting to speculate that these amino acids participate in the formation of the blocking gate.

The straightforward interpretation of our principal result, i.e. that DS6 is a blocking peptide that interacts with the channel and not with $G_{\beta\gamma}$, is that the C-terminal segment of GIRK1 is part of a blocking segment, and that $G_{\beta\gamma}$ binds elsewhere in GIRK1. Our results show that the DS6 peptide added exogenously can block the channel, but we consider it likely that the entire blocking gate includes other regions of GIRK. This is supported by our observation that peptide DD7 (residues 439–464 of GIRK1) also blocks the channel. A more elaborate model (Cohen *et al.* 1996) suggests that the channel may normally be blocked by a complex formed by the N-terminus, the heterotrimeric G protein bound to it (Huang *et al.* 1995), and the C-terminal part of the blocking gate that includes DS6 and possibly additional stretches. Activation of the G protein by the receptor may cause dissociation of G_α and $G_{\beta\gamma}$ and translocation of $G_{\beta\gamma}$ to the central portion of the C-terminus comprising the $G_{\beta\gamma}$-binding site (Slesinger *et al.* 1995; Huang *et al.* 1995; Kunkel & Peralta, 1995), followed by a conformational change resulting in channel opening. In such a model, the distal C-terminal sequence may serve as an anchor for the whole blocking complex. To provide further insights into the mechanistic details of a particular model as envisioned above, experiments involving C-terminal deletions of GIRK1 clones would be of tremendous significance. Unfortunately, attempts made to study gating properties of two C-terminal deletions (namely with the last 40 and with the last 160 amino acids deleted) were unsuccessful since none of the deleted constructs (neither expressed alone nor co-expressed with GIRK4) were able to be expressed (data not shown).

APPENDIX

Assuming that the prolonged interburst interval indeed represents unblocking of the channel by the peptide and that shortening of burst duration represents channel blocking events, the kinetics can be treated by using the Neher–Steinbach model (Neher & Steinbach, 1978) according to the scheme below:

$$C_3 \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A + DS6 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} A^*DS6, \qquad (A1)$$

where C_3 denotes the long-lived, inactive state of the channel in between bursts, A denotes all active states within a burst and A*DS6 denotes the blocked channel states. α, β, k_{on} and k_{off} represent the rate constants corresponding to inactive $\rightleftharpoons$ active interconversions and the drug blocking/unblocking processes, respectively. According to the model, the unblocked time constant (τ_U) is given as:

$$\tau_U = (\alpha + k_{on}[DS6])^{-1}, \qquad (A2)$$

where

$$\alpha = \tau_b^{-1} \text{ and } \tau_U = \tau_b^*. \qquad (A3)$$

τ_b and τ_b^* correspond to time constants of burst duration without and in the presence of DS6, respectively. [DS6] stands for the free concentration of the peptide, which was 4·75 μmol l^{-1} in our single channel experiments (molecular weight of DS6 was 2107·44). By solving eqn (A2) for k_{on} and inserting the appropriate values for τ_b and τ_b^*, we end up with a value of $k_{on} = 7{\cdot}00\ s^{-1}\ \mu mol^{-1}\ l$. From this, k_{off} can be calculated since:

$$k_{off} = k_{on} K_D \qquad (A3)$$

and $K_D = 3{\cdot}75\ \mu$mol l^{-1} (as measured in our dose–response experiments), yielding an actual value of $k_{off} = 26{\cdot}3\ s^{-1}$. Since the prolonged interburst intervals (τ_{c3} in our case) are regarded as representing the dwell time of the peptide on the channel blocker, τ_{c3} can be calculated according to:

$$\tau_b = \tau_{c3} = k_{off}^{-1}, \qquad (A4)$$

yielding a calculated value of $\tau_{c3} = 38{\cdot}1$ ms.

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Acknowledgements

We thank Gerda Breitwieser for sharing a preprint during the preparation of this manuscript. The authors greatly acknowledge financial support by the Human Frontiers Science Project (HFSP RG-379/94), the Austrian Research Foundation (Special Research Centre 'Biomembranes and Atherosclerosis', SFB007/08; W.S.) and the Israel–USA Binational Science Foundation (N.D. and H.A.L.).

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Received 8 May 1997; accepted 21 July 1997.