Bi-stable block by 4-aminopyridine of a transient K^+ channel (Kv1·4) cloned from ferret ventricle and expressed in *Xenopus* oocytes

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1. Using the two-microelectrode, 'cut open' oocyte, and 'torn off' macropatch voltage clamp techniques, we studied the blocking effects of 4-aminopyridine (4-AP) on two cloned K^+ channels expressed in *Xenopus* oocytes, an inactivating K^+ channel isolated from ferret ventricle (FK1), and its NH_2-terminal deletion mutant (ΔNco) which lacks fast N-type inactivation.

2. Experiments with a permanently charged, impermeant 4-AP derivative, 4-aminopyridine-methyliodide, indicated that the cationic form of 4-AP blocks at an intracellular site.

3. Block accumulated from pulse to pulse and was sensitive to the applied potential during hyperpolarizing deactivating pulses, indicating trapping of 4-AP in deactivated channels. For long trains of depolarizing pulses (−90 to +50 mV, 0·1 Hz), 4-AP block increased with decreasing pulse duration. Block of FK1 was much more sensitive to pulse duration than was block of ΔNco, consistent with competition between N-type inactivation and 4-AP binding.

4. To elucidate these mechanisms further, in the absence of fast N-type inactivation the following results were obtained on ΔNco channels: (1) application of 4-AP caused the appearance of apparent inactivation; (2) 4-AP, however, did not cause cross-over of deactivating tail currents; (3) 4-AP block developed with time for potentials positive to −40 mV; and (4) trapping of 4-AP by ΔNco was insensitive to the degree of C-type inactivation.

5. We conclude that the kinetics of 4-AP block of FK1 and ΔNco channels cannot be accounted for by either a pure open channel or closed channel blocking scheme.

Many types of voltage-gated K^+ channels are inhibited by 4-aminopyridine (4-AP), although both its affinity and apparent mechanism of action vary significantly among both native cell types and cloned K^+ channels. Open-state (Hice, Swanson, Folander & Nelson, 1992; Yao & Tseng, 1994), closed-state (Yeh, Oxford, Wu & Narahashi, 1976; Kehl, 1990; Campbell, Rasmusson, Qu & Strauss, 1993), trapping (Choquet & Korn, 1992) and mixed (Thompson, 1982) mechanisms of block have all been reported. In general, the active form of 4-AP appears to be the cationic form and the site of action has been demonstrated to be the cytoplasmic side of the membrane (Oxford & Wagoner, 1989; Choquet & Korn, 1992; Hirsh & Quandt, 1993). Recently, segmental exchange mutagenesis studies between Kv2·1 and Kv3·1 channels have shown that 4-AP binding is localized to the cytoplasmic halves of membrane-spanning domains S5 and S6 (Kirsch, Shieh, Drewe, Vener & Brown, 1993). The cytoplasmic regions of S5 and S6 are located near the intracellular vestibule of the pore (Slesinger, Jan & Jan, 1993).

Both potential- and frequency-dependent blocking effects (i.e. 'use dependence') of 4-AP on various K^+ currents in many tissue types have been previously observed, including delayed rectifier-type K^+ currents of the squid axon (Yeh *et al.* 1976), frog node of Ranvier (Ulbricht & Wagner, 1976) and rabbit Schwann cells (Howe & Ritchie, 1991), and transient outward (I_{TO})-like K^+ currents in molluscan neurons ('I_A'; Thompson, 1982), rat melanotrophs ('I_K(f)'; Kehl, 1990), guinea-pig hippocampal neurons (Numann, Wadmann & Wong, 1987) and dog and ferret ventricle ('I_{TO}'; Simurda, Simurdova & Christie, 1989; Campbell *et al.* 1993). However, not all transient 'I_{TO}-like' K^+ currents display use-dependent block by 4-AP (e.g. transient K^+ current in GH_3 pituitary cells;

Oxford & Wagoner, 1989), suggesting that the presence of the phenomenon depends on the cell type studied and the specific type of K^+ channel present (see Rudy, 1988; Campbell, Rasmusson, Comer & Strauss, 1994). The complex and apparently contradictory state-dependent mechanisms of block observed for 4-AP in different voltage-gated K^+ channel types, coupled with the proximity of the binding site to a region which undergoes important conformational changes associated with activation, deactivation, inactivation and recovery from inactivation (Kirsch *et al.* 1993; Slesinger *et al.* 1993), suggest that investigation of the mechanisms of state-dependent block by 4-aminopyridine will provide significant insights into the conformational transitions associated with stabilization of each of these states. The multiplicity of mechanisms of block by 4-AP observed for different K^+ channels *in situ* suggests that there may be a molecular basis for this diversity.

This study examines the mechanism of block of FK1 channels (Comer *et al.* 1994) by 4-AP. FK1 is a transient 'I_{TO}-like' voltage-gated potassium channel isolated from a ferret ventricular cDNA library and is a member of the Kv1·4 subfamily, which includes the closely related channels RCK4 (Stühmer *et al.* 1989) and HK1 (Tamkun, Knoth, Walbridge, Kroemer, Roden & Glover, 1991). Both FK1 and the fast inactivation deletion mutant ΔNco (Comer *et al.* 1994), are used to assess the state-dependent block of 4-AP. The blocking effects of 4-AP on our Kv1·4 clone show some similarities to the block of Kv2·1 and Kv3·1 delayed rectifier-like channels (Kirsch *et al.* 1993; Kirsch & Drewe, 1993), such as an ability to become trapped within the channel, but also show differences in both time and voltage dependence. Our study presents evidence for time-dependent open-state block and closed-state trapping block, in both FK1 wild-type and ΔNco. In addition, we provide evidence for two stable 4-AP binding conformations, one of which is accessible in the open conformation and the other which is accessible in a partially deactivated conformation.

A preliminary account of this work has appeared in abstract form (Rasmusson, Zhang, Comer, Campbell & Strauss, 1994).

METHODS

Preparation of cRNA

Construction of the FK1 cDNA clone pBSMC1-12, and its inactivation mutant, ΔNco, has been previously described (Comer *et al.* 1994). Amino acids 2–146 have been removed from FK1 to make ΔNco (Comer *et al.* 1994). This deletion removes fast inactivation without significantly altering activation or permeability characteristics. Plasmids pBSMC1-12 and ΔNco were linearized using Kpn I and Xho I (Stratagene, La Jolla, CA, USA), respectively. Transcription of 2 μg of linearized DNA template was carried out at 37 °C for 2 h in 30 μl of solution containing (mM): Tris, 40; $MgCl_2$, 8; spermidine, 2; NaCl, 50; ATP, 1·3; cytosine triphosphate, 1·3; uridine triphosphate, 1·3; $m^7G(5')ppp(5')G$ capping analogue (Pharmacia Biotech, Piscataway, NJ, USA), 1·3; GTP, 0·33; 25 μM [α^{32}P]-CTP (DuPont NEN Research Products, Boston, MA, USA); 25 μM dithiothreitol; 10 units each of RNasin (Promega, Madison, WI, USA) and T3 RNA polymerase (Stratagene); pH 8·0. The resulting cRNA was phenol and chloroform extracted and checked on a denaturing gel for full-length transcription products. Following ethanol precipitation with ammonium acetate, the cRNA was redissolved in RNA-free water to a sample concentration of 10 ng (50 nl)$^{-1}$ and stored at −80 °C until used. Injection of 2–5 ng of our cRNA preparations typically gave 1–10 μA magnitude peak currents (at +50 mV) after 48 h.

Preparation of oocytes

Mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI, USA) were anaesthetized by immersion in Tricaine solution (1·5 g l^{-1} in 25 mM NaH_2PO_4, pH 6·8). Ovarian lobes were removed through a small incision in the abdominal wall. After removal of a small ovarian lobe, the frogs were sutured (twice in the abdominal wall and twice in the external skin tissue). The frogs were then allowed to recover in a small water-filled container, with their heads elevated above water level. Once the animal had recovered from anaesthesia, it was placed in a separate aquarium by itself and monitored until healed. Typically, lobes are obtained three times from a single frog. When individual frogs no longer yielded acceptable oocytes, anaesthetized frogs were killed by an overdose of Tricaine (20 g l^{-1}). The follicular layer was removed enzymatically by placing the lobes in a collagenase-containing Ca^{2+}-free OR2 solution, which contained (mM): NaCl, 82·5; KCl, 2; $MgCl_2$, 1; Hepes, 5; pH 7·4; 1–2 mg ml^{-1} collagenase (Type I, Sigma). The oocytes were gently shaken for 3 h, with an enzyme solution replacement at 1·5 h, and collagenase activity was then halted as previously described (Comer *et al.* 1994). Stage V–VI oocytes were injected with 50 nl of cRNA using a 'Nanoject' microinjection system (Drummond Scientific Co., Broomall, PA, USA) and incubated at 18 °C for 24–72 h in an antibiotic-containing Barth's solution (composition (mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2·4; $MgSO_4$, 0·82; $Ca(NO_3)_2$, 0·33; $CaCl_2$, 0·41; Hepes, 10; pH 7·4, 2% (v/v of 100 × stock) antibiotic–antimycotic (Gibco No.600-5240PG, Life Technologies Inc., Grand Island, NY, USA)).

Electrophysiological techniques

In the majority of experiments reported in this paper, oocytes were voltage clamped using a two-microelectrode 'bath clamp' amplifier (OC-725A, Warner Instruments Corp., Hamden, CT, USA) as has been described in detail elsewhere (Comer *et al.* 1994). Microelectrodes had resistances of 0·6–1·5 MΩ and were made from 1·5 mm o.d. borosilicate glass tubing (TW150F-4, WPI, Sarasota, FL, USA) using a two-stage puller (L/M-3P-A; Adams & List Associates, Ltd, Great Neck, NY, USA), filled with 3 M KCl. During recording, oocytes were continuously perfused with control ND96 solution (composition (mM): NaCl, 96; KCl, 2; $MgCl_2$, 1; $CaCl_2$, 1·8; Hepes, 10; pH 7·4, adjusted with NaOH). The saponin-permeabilized variant of the 'cut open' oocyte Vaseline-gap voltage clamp was employed for resolution of rapid events occurring during activation of the current. This technique is described in detail elsewhere (Taglialatella, Toro & Stefani, 1992; Comer *et al.* 1994). Intracellular solution contained (mM): KCl, 98; $MgCl_2$, 1·8; EGTA, 1; Hepes, 10; pH 7·4, adjusted with NaOH. The pH of the solutions was readjusted to 7·4 using HCl,

and 4-aminopyridine (Aldrich, Milwaukee, WI, USA) was added directly. Currents were recorded at room temperature (21–23 °C) and were typically filtered at 1 kHz for two-electrode clamp and torn off macropatch (see below) experiments, and at 5 kHz for cut open oocyte clamp experiments. Data were recorded on videotape using an A/D VCR adaptor (model PCM 4/8, Medical Systems Corporation, Greenvale, NY, USA) and digitized using 'pCLAMP' software (Axon Instruments, Inc., Foster City, CA, USA).

For analysis of the active form and 'sidedness' of 4-AP block, the permanently (positively) charged 4-AP derivative 4-amino-pyridine-methyliodide (4-APMI) was used (e.g. Stephens, Garrat, Robertson & Owen, 1994). The effects of extracellular 4-APMI were analysed using the standard two-microelectrode voltage clamp technique, while the effects of intracellular 4-APMI were determined by applying it to the exposed intracellular surface of torn off 'inside-out' macropatches. Macropatches were obtained on oocytes in which the vitelline membrane had been manually dissected free using a hypertonic ND96 solution (sucrose added) and then subsequently placed in a continuously perfused (1–2 ml min^{-1}) recording chamber (approximately 1 ml volume) mounted on the modified stage of an inverted microscope (Nikon Diaphot). Patch pipettes (o.d. 1·5 mm) were made from either TW150F-4 (WPI) or Corning 7052 (A-M Systems, Everett, WA, USA) glass capillaries and were filled with control (2 mM KCl) ND96 solution. After seal formation, the patches were then excised into an 'intracellular' solution composed of (mM): KCl, 98; $MgCl_2$, 1; EGTA, 1; Hepes, 5; pH 7·4. After control measurements were taken, the bath solution was changed to an identical intracellular solution containing hyperosmotically added 4-APMI. For these measurements the amplifier was an Axopatch 1-C (Axon Instruments Inc.). 4-APMI was a generous gift supplied by Dr Gerry Oxford, Department of Physiology, University of North Carolina, Chapel Hill, NC, USA.

Data are presented as means ± S.E.M. Where appropriate, estimates of changes in block are reported as changes in apparent affinity calculated from reduction in current and assuming first order binding.

RESULTS

Evidence for open channel block

Previous studies of 4-AP block on the native transient K^+ current, I_{TO}, in ferret right ventricular myocytes revealed that 4-AP blocked the channel via a closed-state blocking mechanism (Campbell *et al.* 1993). However, due to experimental limitations, Campbell *et al.* could not rule out the possibility that low affinity open-state block occurred at a rate which was slow relative to fast inactivation. To examine this possibility for the cloned FK1 channel we used an NH_2-terminal deletion mutant, ΔNco (Comer *et al.* 1994), that lacked fast inactivation. Perfusion of 10 mM 4-AP on ΔNco during continuous pulsing from a holding potential of −90 mV to +50 mV for 500 ms at 0·1 Hz resulted in a reduction and near abolition of the current (Fig. 1*A*) within 60 s. The kinetic behaviour of ΔNco was also altered by 10 mM 4-AP, producing an inactivation-like behaviour during the depolarizing pulse that was indicative of open channel block.

Figure 1*B* shows the effects of a 3 min exposure to 10 mM 4-AP while the oocyte was continuously held at −90 mV (i.e. steady-state effects) using the two-electrode voltage clamp. The peak current during the first pulse of a train of pulses to +50 mV was reduced, but not abolished. Additional block rapidly developed during the subsequent pulse. However, it was difficult to precisely quantify the degree of 4-AP block using the two-electrode voltage clamp due to the overlap of activation, drug binding and the capacitative current transient. In order to measure the degree of inhibition of current at the onset of the clamp pulse more precisely, we employed the cut open oocyte voltage clamp technique, which achieves much faster voltage clamp control than is possible using the two-microelectrode technique. Figure 1*C* shows the effects of a 3 min exposure to 10 mM 4-AP while the oocyte was continuously held at −90 mV. The top trace is the average of the first 5 ms of the final two pulses in control solution prior to 4-AP application. Trace P1 is the current recorded during the first 5 ms of the first pulse after a 3 min exposure to 4-AP. Partial blockade of the current was readily apparent at the earliest time point of activation. Fractional block during the first pulse was $47 \pm 8\%$ at $t = 1000\ \mu s$ and $41 \pm 9\%$ at $t = 2000\ \mu s$ ($n = 3$), indicating that the block was the result of a reduction in total current available for activation and not due to alterations in the time course of activation. These results indicate that 10 mM 4-AP is capable of at least partially blocking the resting channel. In summary, the blocking action of 10 mM 4-AP on ΔNco did not require, but was accelerated by, channel activation. The accelerated development of 4-AP block with increased channel activation is similar to that observed for Kv2·1 and Kv3·1 (Kirsch *et al.* 1993; Kirsch & Drewe, 1993) and the rat heart K^+ channel RHK1 (Yao & Tseng, 1994).

Since 4-AP is an organic base (pK ~9·2), at the pH values used in the present experiments (pH 7·4) approximately 98% of it exists in its charged cationic form (membrane impermeant), with the remaining approximately 2% being uncharged (membrane permeant). Therefore, the results presented in Fig. 1 do not allow determination of either the active form of 4-AP (i.e. cationic *versus* uncharged) or the sidedness of its action (i.e. extracellular, intracellular, and/or intramembranous). To help address this question, the effects of the permanently charged, membrane-impermeant 4-AP derivative 4-amino-pyridine-methyliodide (4-APMI) were determined. External application of 10 mM 4-APMI failed to produce any significant block of FK1 or ΔNco. In contrast, 10 mM 4-APMI applied to the exposed intracellular face of torn off ('inside-out') macropatches containing either FK1 or ΔNco channels resulted in a rapid and reversible block (data not shown), confirming earlier demonstrations of an intracellular site of action (e.g. Howe & Ritchie, 1991; Stephens *et al.* 1994).

Activation-dependent block is suggestive of an open channel blocking mechanism (Hille, 1992). This is supported further by the observation that application of 1 mM 4-AP caused a small, but nonetheless detectable, cross-over of the late FK1 currents at depolarized potentials (Fig. 2*A*). Such a cross-over phenomenon is consistent with either delayed activation of channels or competition with inactivation (Campbell *et al.* 1993; Yao & Tseng, 1994). The inactivation-like effects of 4-AP on the open channel conformation are also apparent at steady state following equilibration of block for low concentrations of 4-AP for the fast inactivation deletion mutant ΔNco. Furthermore, application of 0·2 mM 4-AP produced inactivation-like behaviour in ΔNco (Fig. 2*B*) in addition to reducing peak current. These effects of 4-AP on ΔNco therefore resemble the use-dependent effects of other open channel blocking compounds.

The time-dependent decrease of ΔNco during a depolarizing pulse in the presence of 4-AP (Fig. 2*B*) suggests that the degree of block at the end of the pulse is greater than that at the beginning and implies a time-dependent open channel blocking mechanism. Compounds which have affinity only for the open state of the channel frequently produce a cross-over phenomenon in deactivating tail currents (e.g. Rasmusson, Campbell, Qu

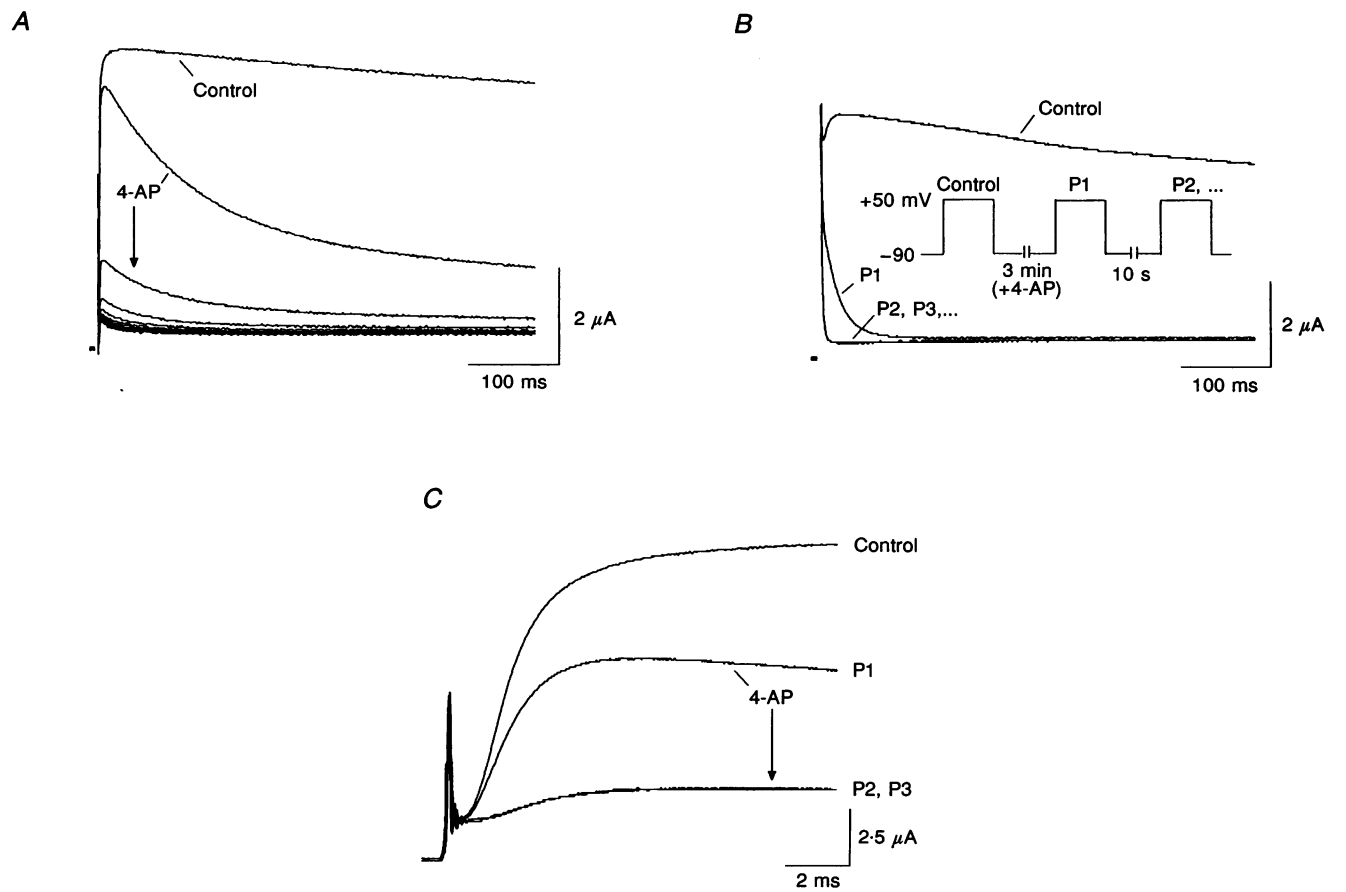


Figure 1. Effects of 4-AP application

A, effects of 10 mM 4-AP on macroscopic currents from ΔNco expressed in *Xenopus* oocytes. The oocyte was pulsed continuously (500 ms pulses to +50 mV; holding potential, −90 mV, 0·1 Hz) during application of 10 mM 4-AP which resulted in a decrease and eventual abolition of current. Note the appearance of inactivation-like behaviour. *B*, block of ΔNco occurs very slowly in the absence of channel activation. Continuous application of 10 mM 4-AP while holding at −90 mV for 3 min reduced the peak current of the first 500 ms pulse to +50 mV by only 69 ± 7% ($n = 6$); thereafter, the current rapidly decreased in subsequent pulses applied at a frequency of 0·1 Hz. *C*, block of the early phase of activation of ΔNco. The protocol of *B* was repeated using the cut-open oocyte voltage clamp technique (no off-line leakage or capacitance subtraction was applied to the data). Application of 10 mM 4-AP to the intracellular, guard, and extracellular chambers while holding at −90 mV for 3 min reduced the peak current of the first 500 ms pulse to +50 mV at the earliest time points during activation. The block reached steady state for subsequent pulses applied at a frequency of 0·1 Hz.

& Strauss, 1994), a phenomenon which is indicative of obligatory drug unbinding from the open channel prior to deactivation. However, such behaviour was not observed for ΔNco tail currents in the presence of 0·2 or 1·0 mM 4-AP (data not shown). This suggests either that the kinetic rates of open channel binding and unbinding of 4-AP are such that the tail current cross-over cannot be resolved or that a pure open-channel binding scheme (e.g. Yao & Tseng, 1994) may be inappropriate for describing the binding of 4-AP to this channel.

In order to elucidate more fully the mechanism of 4-AP block we examined its voltage dependence using a two-pulse protocol. A 500 ms pulse to varying potentials (holding potential, −90 mV; one protocol per minute) was immediately followed by a second 500 ms pulse to +50 mV. Figure 3*A* shows the current recorded under control conditions for the ΔNco channel using this protocol. ΔNco activation threshold was approximately −50 mV (Comer *et al.* 1994) and some slow (presumably 'C-type') inactivation developed during the first 500 ms pulse for potentials positive to −60 mV. Figure 3*B* shows currents recorded from the same oocyte using the same voltage clamp protocol after application of 0·2 mM 4-AP. The potential dependence of block developed during a prepulse was examined by analysing the decrease in the peak current in 0·2 mM 4-AP relative to control, as a function of the prepulse potential. The results of this analysis are shown in Fig. 3*C*. Block by 4-AP during the prepulse increases with depolarization in the range of activation potentials (−60 to −40 mV; Comer *et al.* 1994), which differs from the effects of potential reported for Kv2·1 and Kv3·1 channels (Kirsch *et al.* 1993). However, for potentials well above the ΔNco activation threshold (> 0 mV), a weak voltage-dependent unblock was observed which was similar to that reported for Kv1·1 (Stephens *et al.* 1994). For an intracellular site of action, this weak voltage dependence corresponds to an effective electrical distance for binding, δ, of $-0{\cdot}077 \pm 0{\cdot}01$ ($n = 4$, $P < 0{\cdot}05$). This δ is oppositely directed from what is expected for a positively charged compound acting from an intracellular site. Thus, the anomalous voltage dependence of open channel block may reflect weak voltage-dependent conformation changes which occur depolarized to the threshold for channel opening; we do not believe that this δ measures penetration into the permeation pathway.

Evidence for closed channel block/trapping

Trapping by deactivation has been shown to be an important high affinity mechanism of 4-AP binding in both Kv2·1 and Kv3·1 channels, with channel affinity for

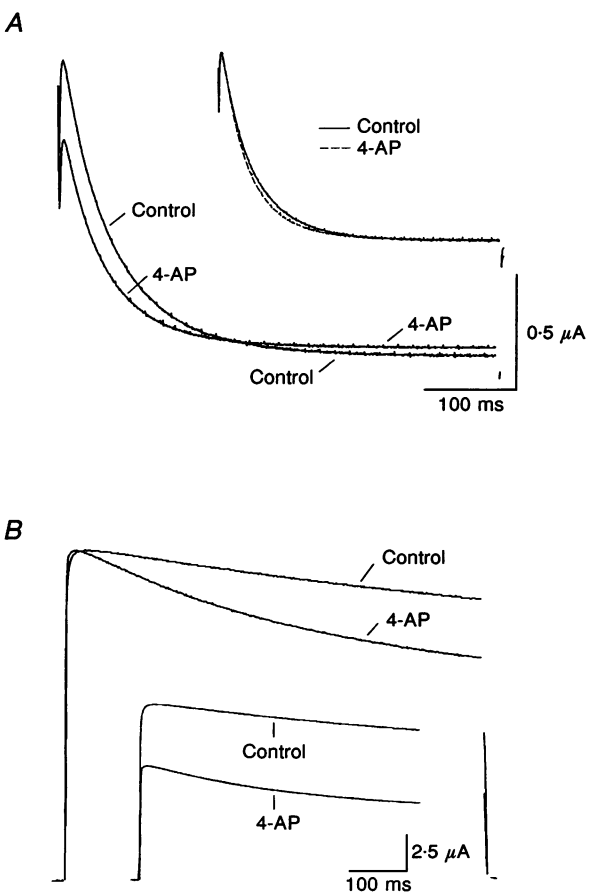


Figure 2. Steady-state block of FK1 and ΔNco

A, 4-AP (1·0 mM) caused a cross-over in the current waveforms (main panel) and only slightly increased the rate of inactivation of FK1 (inset). Although peak currents were reduced by 4-AP, late currents were actually larger in the presence of 4-AP. Holding potential, −90 mV; test potential, + 50 mV for 500 ms; rate, 0·1 Hz. Inset: currents normalized to the same peak in order to emphasize the very small change in apparent inactivation rate. *B*, 4-AP causes inactivation-like behaviour in ΔNco. Main panel: currents normalized to peak to emphasize the increased rate of time-dependent decline of current in the presence of 0·2 mM 4-AP. Holding potential, −90 mV; test potential, +50 mV for 500 ms; frequency, 0·1 Hz. Inset: raw data showing relative reduction in current.

4-AP showing a marked correlation with deactivation rate (Kirsch *et al.* 1993). Deactivation is an intrinsically voltage-dependent process, with rates increasing with increasing hyperpolarization. Consequently, we examined the effect of deactivation potential on the ability of 4-AP to become trapped in the deactivated ΔNco channel. To do so, the following three-pulse protocol was applied. ΔNco channels were first fully activated by a 500 ms pulse (P1) to +50 mV. P1 was then followed by a second 500 ms pulse (P2) to varying potentials (−90 to +50 mV in 10 mV increments). After P2 the membrane was then held at −90 mV for 10 s, after which a third pulse (P3) to +50 mV for 1 s was applied.

Figure 4*A* shows representative results of applying this protocol to ΔNco, firstly in control solution, and secondly after application of 0·2 mM 4-AP (Fig. 4*B*). Under control conditions, the peak current during the P3 pulse was unaffected by the potential of the P2 pulse. However, in the presence of 0·2 mM 4-AP the P3 current amplitude was modulated in a complex manner by the P2 potential, with block first progressively increasing in the P2 potential range of −70 to −30 mV and then progressively decreasing at more depolarized P2 potentials. The relative decrease of current in 4-AP during the final P3 pulse was measured and normalized with control values at the same potential in the absence of 4-AP to give an index of the degree of trapping as a function of P2 potential. The mean results obtained from this protocol for four oocytes are summarized in Fig. 4*C*. The mean degree of trapping was greatest when the P2 pulse potential was near the threshold for activation (−50 to −30 mV), i.e. where deactivation tail currents display their slowest kinetics but where the intracellular vestibule is still presumably in a conformation that would allow binding of an inactivation 'ball' if it were present.

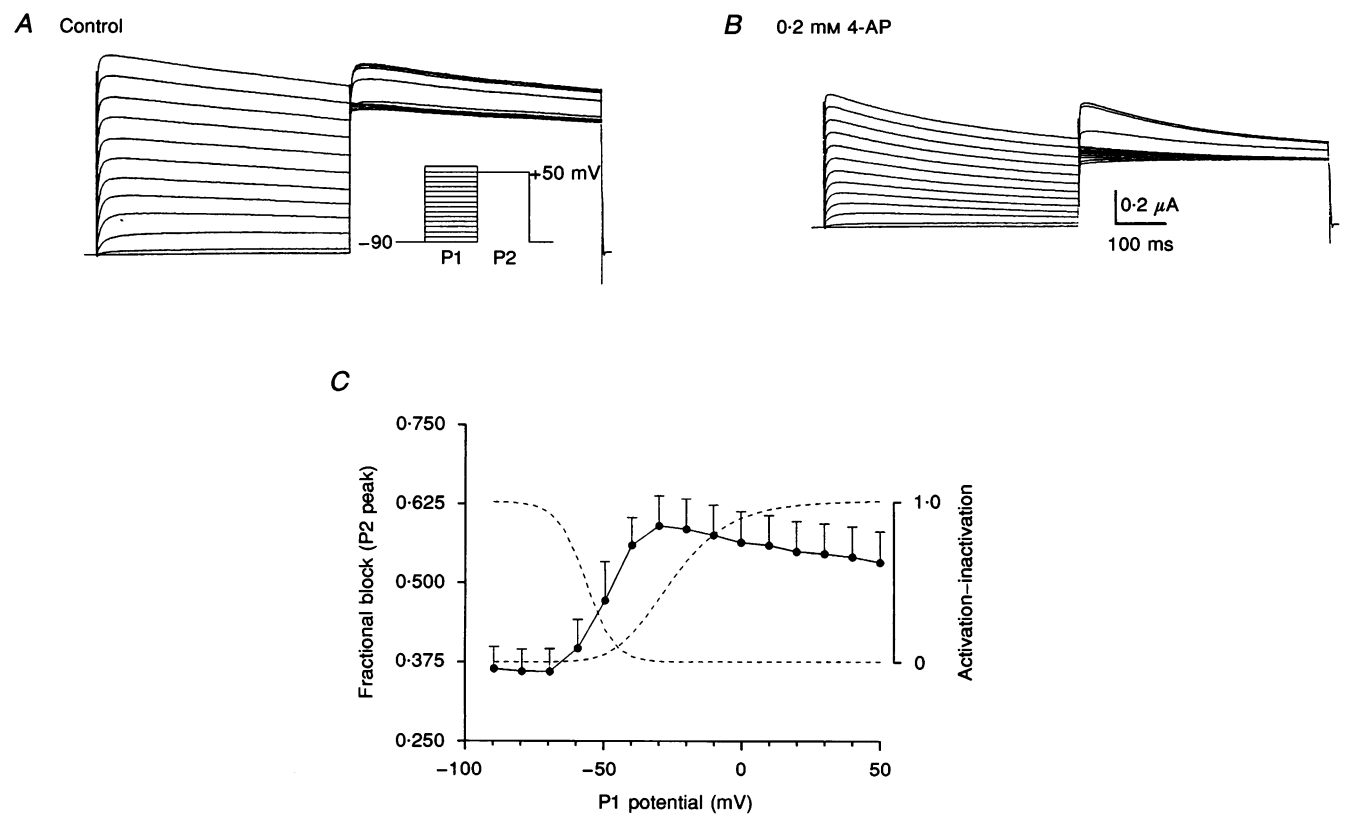


Figure 3. Voltage-dependent development of block of ΔNco during a prepulse

A, currents recorded in control solution. P1 was to varying potentials (−90 to +60 mV in 10 mV increments in ascending order) for 500 ms. P2 was to +50 mV for 500 ms (holding potential, −90 mV; rate, one P1–P2 protocol per minute; a 1 s P3 pulse (not shown) to +50 mV was applied 10 s after the P1–P2 pulses to check for accumulation of block. No block accumulated between protocols). *B*, currents recorded in the same oocyte and using the same protocol as in *A* but in the presence of 0·2 mM 4-AP. *C*, voltage dependence of block during P1. The voltage dependence of block at the end of the 500 ms P1 pulse was measured as the percentage reduction in P2 peak current and plotted as a function of P1 potential. Dashed lines denote FK1 steady-state activation and inactivation relations (Comer *et al.* 1994). Data presented as means ± S.E.M. ($n = 4$). [4-AP], 0·2 mM.

It should be noted that although ΔNco has its NH_2-terminus deleted, and therefore shows no rapid 'N-type' inactivation, the currents recorded during P2 show a significant time-dependent decrease in amplitude. On average, ΔNco showed a 20 ± 4% ($n = 10$) reduction of peak current for a 500 ms pulse to +50 mV. Presumably, this decrease is due to a 'C-type' or slow inactivation mechanism (Hoshi, Zagotta & Aldrich, 1991). Therefore, the currents recorded during P2 result from a complex interaction of both deactivation and 'C-type' inactivation, and the amount of 4-AP bound during P2 may be affected by both of these processes. Consequently, modulation of P3 amplitude might be attributed to either changes in deactivation or to competition of 4-AP binding with 'C-type' inactivation.

If the degree of trapping is modulated by competition with 'C-type' inactivation, then it would be expected that manipulations which increase the degree of 'C-type' inactivation will reduce the degree of trapping. We therefore modulated the degree of 'C-type' inactivation but left the deactivation potential constant by varying the potential of P1 and leaving P2 constant at +50 mV. The degree of modulation of 'C-type' inactivation developed in this protocol was similar to that developed using the protocol in Fig. 4. However, this protocol did not alter 4-AP block of the P3 pulse (data not shown). Thus, the degree of trapping is sensitive to the deactivation potential and not to the degree of 'C-type' inactivation. Furthermore, the ability of the P2 pulse potential to modulate current amplitude during a pulse

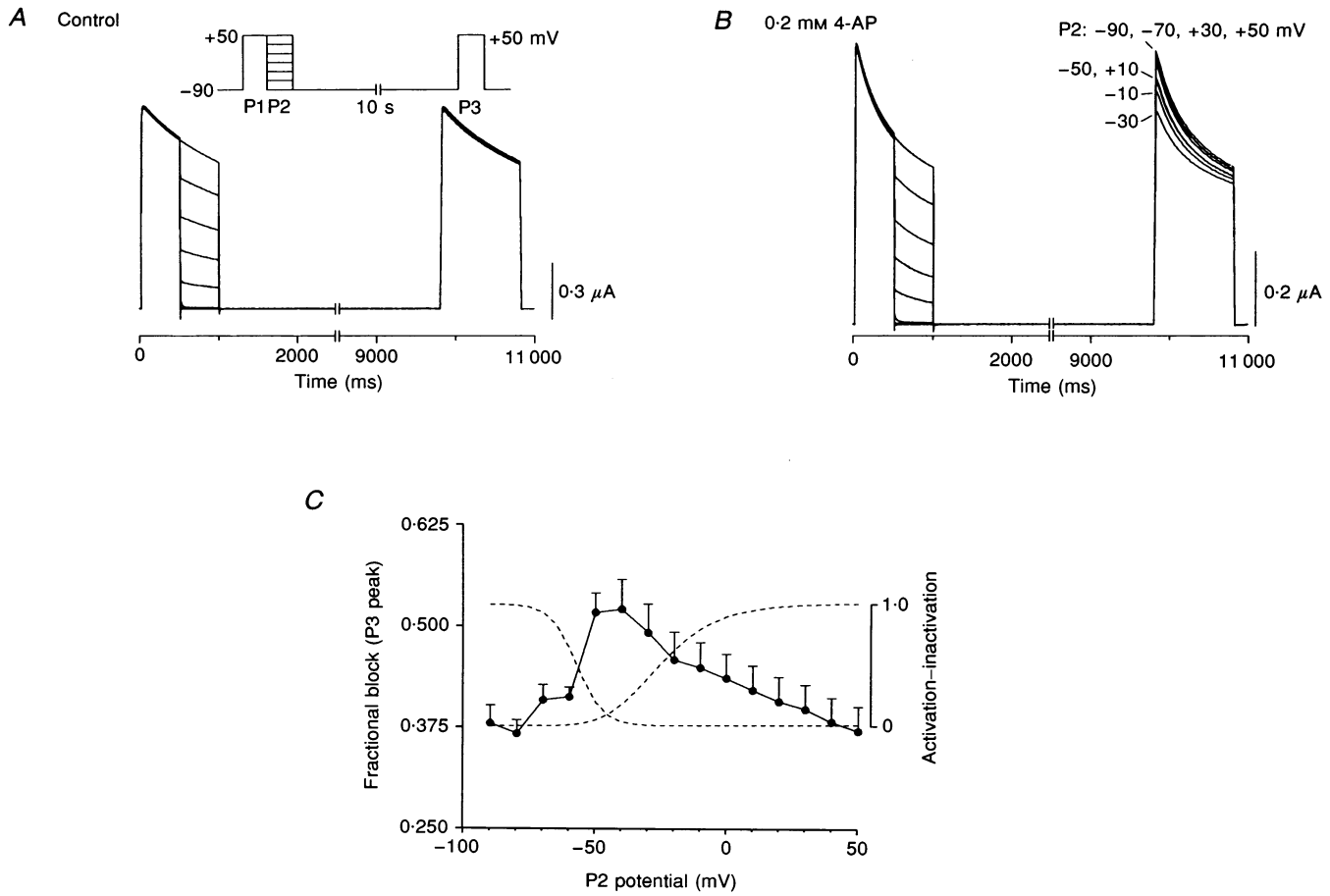


Figure 4. Voltage-dependent trapping of 4-AP by ΔNco

A, currents recorded in control solution. Voltage-clamp protocols are shown above the current traces. P1 was to +50 mV for 500 ms. P2 was to varying potentials (−90 to +50 in 10 mV increments in ascending order, traces shown in 20 mV increments for clarity) for 500 ms. The membrane was then held at −90 mV for 10 s and followed by a P3 pulse to +50 mV for 1 s (holding potential, −90 mV; rate, one protocol per minute). *B*, currents recorded in the same oocyte and using the same protocol as in *A* but in the presence of 0·2 mM 4-AP. *C*, voltage dependence of 4-AP trapping during P2. The voltage dependence of 4-AP trapping achieved at the end of the 500 ms P2 pulse was measured by determining the percentage reduction in P3 peak current and plotted as a function of P2 potential. Dashed lines denote FK1 steady-state activation and inactivation relationships (Comer *et al.* 1994). Data presented as means ± S.E.M. ($n = 4$). [4-AP], 0·2 mM.

which occurs 10 s later (Fig. 4*B*) is clear evidence of the ability of 4-AP to become trapped within the closed or deactivated channel, and is qualitatively consistent with the slow access time of 4-AP for the resting native I_{TO} channel in ventricular myocytes (e.g. Campbell *et al.* 1993) and the previously reported ability of Kv2·1 and Kv3·1 channels to trap 4-AP (Kirsch *et al.* 1993; Kirsch & Drewe, 1993).

The results presented in Fig. 4 suggest that closed-state trapping might be an important mechanism of 4-AP interaction within the channel and that the relative availability of channels in different deactivating conformations might alter 4-AP binding. The transient nature of open channel block of ΔNco suggests that the amount of 4-AP bound to the channel at the end of the pulse is not 100% trapped during the process of deactivation, otherwise successive pulses would already be at equilibrium and would therefore not show time-dependent inactivation-like behaviour.

Pulse duration modulates the ratio of open *vs.* closed channel block

The ability of 4-AP trapping to be modulated by the voltage range of deactivation suggests the possibility that deactivation may remove some or all of the open-state block. To test this possibility the following voltage clamp protocol was applied to both FK1 and ΔNco in the presence of 1 mM 4-AP. From a holding potential of −90 mV a series of short (25 ms) voltage clamp pulses to +50 mV were applied at a frequency of 0·1 Hz (protocol shown in inset to Fig. 5*A*). After steady-state block had been reached (16–32 pulses, depending upon oocyte) the pulse duration was then changed to 500 ms (schematic in Fig. 5*B*).

Upon application of this protocol, block of both FK1 and ΔNco elicited by the short 25 ms pulse train took many pulses to reach steady state (Fig. 5*A* and *C*). In the case of FK1, for the particular oocyte illustrated in Fig. 5*A*, a steady-state peak current reduction of 66% was reached

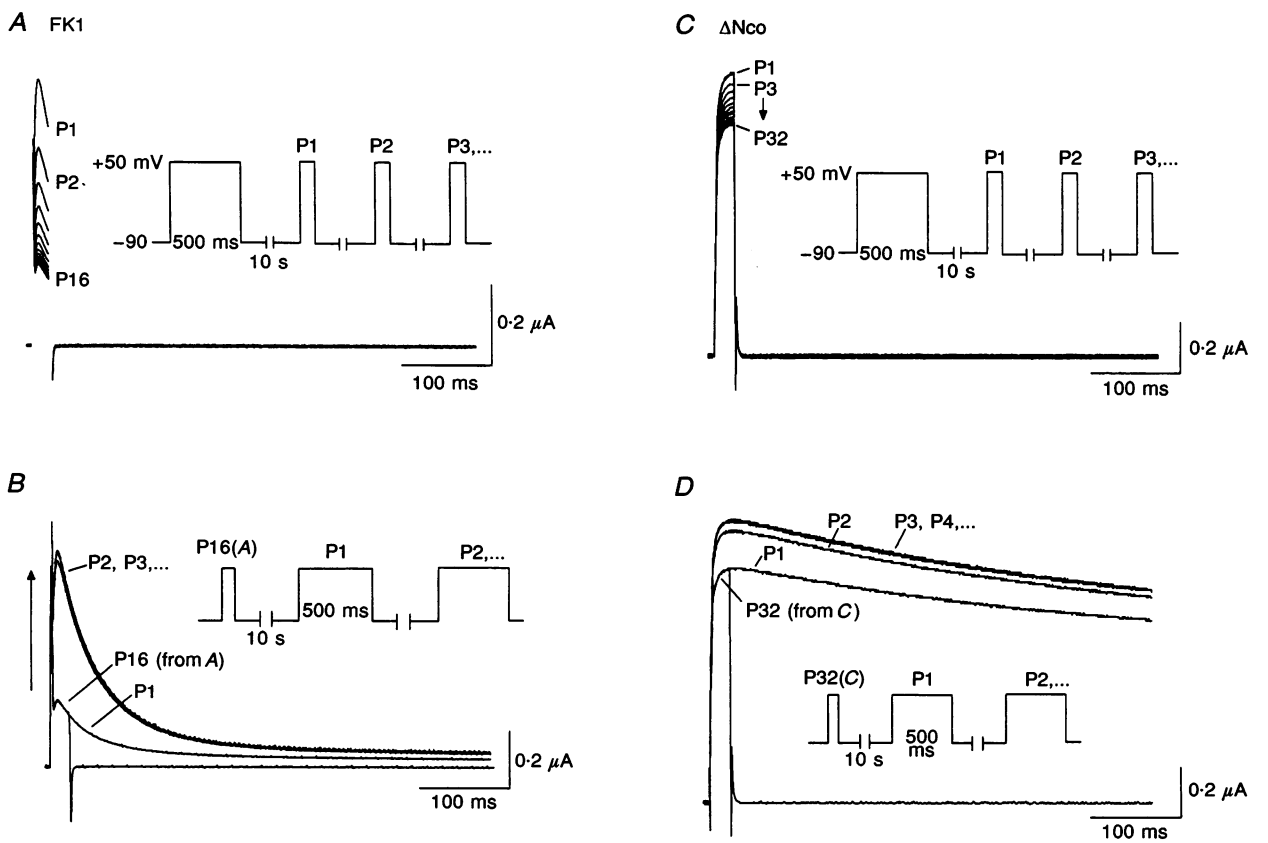


Figure 5. Pulse-duration dependence of 4-AP block

A, development of 4-AP block of FK1 by a long train of brief pulses. An oocyte expressing FK1 was pulsed at 0·1 Hz to + 50 mV for 500 ms from a holding potential of −90 mV in the presence of 1 mM 4-AP until steady-state current was achieved. Pulse width was then reduced to 25 ms and the oocyte was pulsed continuously at 0·1 Hz to +50 mV until a new steady state was reached. *B*, relief of brief-pulse duration block by long pulses in FK1. Following attainment of steady-state block using 25 ms duration pulses in *A*, pulse duration was abruptly changed to 500 ms and the reduction in current was reversed. *C*, development of block of ΔNco by a long train of brief pulses using the same protocol as in *A*. *D*, relief of brief-pulse duration block by long pulses in ΔNco using the same protocol as in *B*.

by the 16th 25 ms pulse, corresponding to an increase in block of 200%. However, after the 16th pulse, increasing the pulse duration to 500 ms caused the peak current height to rapidly increase back to the original steady-state value within three pulses (Fig. 5*B*). One possible explanation for the slow reduction in peak current height with short duration pulses would be that fast N-type inactivation (Hoshi *et al.* 1991) might obstruct the binding site for 4-AP (e.g. Yao & Tseng, 1994), and that the increased block observed during the series of short pulses was due to the increased availability of non-inactivated deactivating channels (Russell *et al.* 1994). To test this possibility we applied the same pulse train protocol to ΔNco. Although fast N-type inactivation is missing in ΔNco, results similar to those observed for FK1 were obtained: a long series of very short pulses resulted in a very slow accumulation of block (Fig. 5*C*), while block was rapidly removed upon increasing pulse duration to 500 ms and reached steady state within three pulses (Fig. 5*D*). Block of FK1 was much more sensitive than that of ΔNco to changes in pulse duration, consistent with a competition between N-type inactivation and 4-AP binding (Russell *et al.* 1994; Yao & Tseng, 1994). However, deletion of the NH_2-terminus failed to completely remove the sensitivity of the channel to pulse duration, indicating that other factors may be involved.

In summary, block of both peak FK1 and ΔNco currents by short duration pulses took many pulses to reach steady state (Fig. 5*A* and *C*). This is consistent with relatively high affinity binding to a short-lived state which occurs during deactivation. However, unbinding of 4-AP during long duration pulses was rapid and took at most three pulses to return to steady state (Fig. 5*B* and *D*). Such relatively rapid relief of block suggests that unbinding of 4-AP from the deactivated state occurs during a prolonged depolarizing pulse. This would account for the difference between the time course of the ΔNco currents between the first and subsequent 500 ms pulses illustrated in Fig. 5*D*. These results therefore indicate that pulse duration modulates the ratio of open to closed channel block.

An example of the modulation of steady-state currents for trains of pulses of varying duration in the presence of 0·1 mM 4-AP applied to ΔNco are shown in Fig. 6*A*. The time-dependent nature of open channel block was also manifested by the appearance of inactivation-like behaviour of ΔNco for the 250 and 500 ms pulses. It is important to note that reduction of initial current for 10 and 25 ms pulses was suppressed below the level of open channel block achieved for the current at the end of 500 ms pulses. Such pulse-length-dependent behaviour was not observed in control solution for FK1 or ΔNco (data not shown). On average, reducing the pulse duration from 500 to 25 ms in the presence of 0·1 mM 4-AP resulted in a decrease in the calculated apparent K_D for 4-AP by 42 ± 9% for ΔNco (paired data from $n = 6$, mean K_D shifted from 0·15 ± 0·042 to 0·1 ± 0·035 mM). In the presence of 0·2 mM 4-AP the apparent K_D for 4-AP decreased by 41 ± 2 and 53 ± 2% ($n = 3$) for a decrease in pulse duration from 500 ms to 25 and 10 ms, respectively

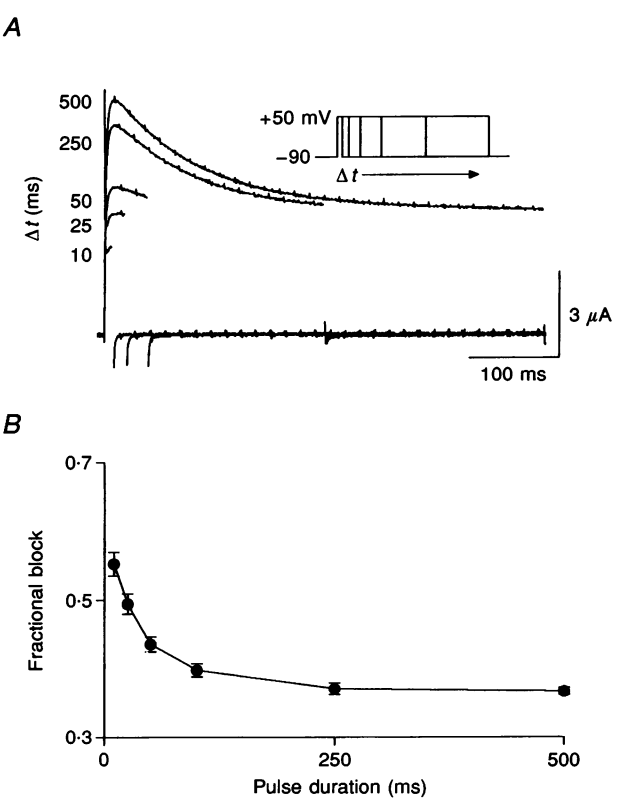


Figure 6. Steady-state block of ΔNco for different duration pulses

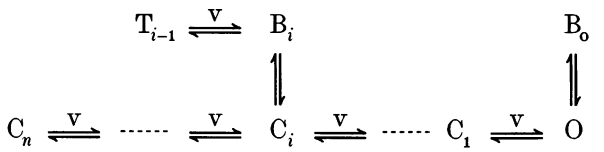
A, the final steady-state ΔNco current for pulse trains (0·1 Hz to + 50 mV; holding potential, −90 mV, [4-AP] = 0·1 mM) of 10, 25, 50, 250 and 500 ms duration pulses. Note that steady-state block for 25 and 10 ms pulse trains is greater than the steady-state open-state block observed at the end of 500 ms pulses. *B*, average degree of block of ΔNco by 0·2 mM 4-AP at steady state for pulse trains (0·1 Hz to + 50 mV; holding potential, −90 mV) of 10, 25, 50, 100, 250 and 500 ms duration. Data points are means ± S.E.M. for $n = 3$.

(Fig. 6*B*). Reducing pulse duration from 500 to 15 ms resulted in a decrease in the calculated apparent K_D for 4-AP by 70 ± 14% for FK1 (paired data from $n = 4$, data pooled from 0·1 and 1·0 mM 4-AP, mean apparent K_D shifted from 2·4 ± 1·0 to 0·3 ± 0·080 mM). Again, the reduced sensitivity of block to pulse duration produced by deletion of inactivation suggests that 4-AP competes for binding in the region in which the NH_2-terminus binds during fast inactivation (Kirsch *et al.* 1993; Russell *et al.* 1994; Yao & Tseng, 1994).

Although the ΔNco mutation removed fast inactivation, a slow component of inactivation remained. This raises the possibility that 4-AP has a low affinity for, or does not block, channels in the 'C-type' inactivated state. For such a scheme, the inactivated state, in a sense, competes with drug molecules for open channels. Upon repolarization, inactivated channels recover fully in 10 s and are ready to reopen, but blocked channels are unable to do so. This scheme is also qualitatively consistent with the data of Fig. 5 in which brief pulses (which favour channel openings but not inactivation) promote more block than longer pulses (which promote channel openings and inactivation). Such an inactivation–competition mechanism also predicts that the relationship between pulse duration and degree of block should reflect the time course of development of 'C-type' inactivation. The time constant of 'C-type' inactivation is greater than 2 s (Comer *et al.* 1994) for ΔNco. The steady-state current (after > 10 pulses) achieved for ΔNco in the presence of 4-AP for pulse durations of 10, 25, 50, 250 and 500 ms (Fig. 6) demonstrates that the relationship between pulse duration and degree of block is sensitive to changes in pulse duration of less than 500 ms. Pulses of greater than 500 ms duration did not result in further relief of block (data not shown). The marked disparity between the time dependence of 'C-type' inactivation (seconds) and pulse duration-dependent modulation of block (tens of milliseconds) provides further evidence that 'C-type' inactivation is not involved in the time and voltage dependence of 4-AP block of ΔNco.

DISCUSSION

Our data indicate that 4-AP blocks both FK1 and its fast inactivation deletion mutant ΔNco in a very complex time- and voltage-dependent manner (i.e. 'use-dependent' block). Possibly the most novel and interesting aspect of 4-AP block of these cloned Kv1·4 channels is their dependence on pulse duration. These complex use-dependent effects arise from the fact that 4-AP can bind to the resting (closed), activated (open) and deactivated ('trapping block') states of these channels. However, the rate at which 4-AP associates with the resting channel is so slow (in the order of minutes) as to be negligible when compared with block of the activated channel. We therefore conclude that none of the following models can adequately account for all of our observations: (i) a pure open channel blocking mechanism (e.g. Yao & Tseng, 1994), (ii) a pure closed-state blocking mechanism (e.g. Campbell *et al.* 1993), or (iii) a pure 'trapping' mechanism where 4-AP bound to the open state becomes trapped upon deactivation (Kirsch & Drewe, 1994). To account for all of our data, both open- and closed-state mechanisms can be combined to form a minimal two-state binding model:



where C_x denotes one of several closed states, O denotes open channels, v denotes voltage-sensitive transitions, B denotes drug-bound channels from which the drug may freely associate and dissociate, and T denotes closed channels in which the drug is trapped by deactivation.

Open channel block and deactivated state trapping are separated by a lower affinity conformation

As drawn above, there is no direct communication between the blocked open state and the blocked or trapped closed states. If intercommunication between these two blocked states was identical to the intervening opening and closing transitions of the unblocked channel, the general behaviour of this would approximate that of an open channel model with trapping, but with a lower threshold for the onset of block. In addition, it would not produce the time-dependent block observed upon depolarization. In other words, 4-AP bound to the open state and 4-AP trapped in the closed channel are separated by transitions through lower affinity conformations associated with activation and deactivation. Although the lack of communication between B_i and B_o probably represents an oversimplification, the following qualitative observation remains: activation and deactivation pass through a transition state which has a lower affinity for 4-AP than binding to the open state or the partially activated/deactivated state.

What sort of physical factors might give rise to such behaviour? Previous studies of the action of 4-AP using segmental exchanges between Kv2·1 and Kv3·1 channels allowed Kirsch *et al.* (1993) to reach several important conclusions: (1) block had highest affinity for the deactivated state of the channel; (2) block potency increased at more negative potentials, consistent with a low affinity of the open channel for 4-AP and trapping in the closed state; (3) mutations which slowed the rate of deactivation reduced 4-AP sensitivity; (4) 4-AP block was competitive with block induced by tetrapentylammonium; (5) block had an effective electrical distance of approximately zero; (6) mutations in the S6 region

altered affinity for 4-AP without altering gating kinetics; and (7) mutations in the intracellular portion of S5 altered affinity for 4-AP but also altered activation kinetics. These properties of Kv2·1 and Kv3·1 channels are qualitatively different from those obtained in FK1 (Kv1·4). In particular, time-dependent open channel block of FK1 and ΔNco by 4-AP was observed and showed a weak voltage dependence. Block of FK1 by 4-AP resulted in a small but measurable cross-over of the current waveforms in response to a depolarizing pulse, possibly due to competition with inactivation. A qualitatively similar cross-over was observed in Kv3·1 after application of tetrapentylammonium, which mimics inactivation (Kirsch *et al.* 1993). Kirsch & Drewe (1993) presented a four-state model which could explain their observations on Kv2·1 and Kv3·1 channels. In their model ('K–D model') it was proposed that the channel could reside in one of two states, activated (A) or resting (R). Both the activated and resting states could bind 4-AP, resulting in drug-bound conformations of the channel (AB or RB). Binding to the resting state occurred at a much slower rate than binding to the activated state. The main distinguishing characteristic of binding in the K–D model is that trapping is governed by the degree of open channel binding at the end of a pulse, the relative rate constants and affinities for the activated and deactivated states, and the deactivation rate (Kirsch *et al.* 1993). Thus, the K–D model fails to predict the combination of time dependence of open-state block for ΔNco and the pulse-duration dependence of block observed for ΔNco and FK1. To illustrate this inconsistency, we can consider the following three possibilities. Firstly, the affinity of 4-AP for the R and A states is identical, and only the rate of interaction is different between these two states. In this case, no time- or voltage-dependent open channel block should be observed once 4-AP binding has reached steady state, which is inconsistent with the observed steady-state time and voltage dependence. Secondly, the affinity of 4-AP for the A state of the channel is higher than that for the R state. In this case, we would be able to reproduce the observed time-dependent development of block during a depolarizing pulse at steady state. However, a train of short pulses should produce a relief of block relative to long pulses, reflecting the ratio of time spent in the low affinity (R) *versus* high affinity (A) states. Just the opposite effect was observed for ΔNco. Thirdly, the affinity of 4-AP for the A state of the channel is lower than for the R state. Such a system might explain the pulse-duration dependence of FK1 and ΔNco. However, this set of parameters predicts that a time-dependent unblock should occur during depolarizing pulses. In fact, a time-dependent development of block was observed for ΔNco. In summary, the K–D model used to describe 4-AP block of Kv2·1 and Kv3·1 channels is inconsistent with our data from Kv1·4 channels because of the lack of a direct correspondence between open channel block developed during a pulse and trapping during a subsequent pulse. In contrast to Kv2·1 and Kv3·1, activation and deactivation of Kv1·4 may involve conformational changes through intermediate states which have a lower affinity for 4-AP than either the fully activated or deactivated channel.

Such bi-stable binding is consistent with a physical interaction either directly coupled to movement of the S4 voltage sensor or with interaction with regions which are energetically coupled to such movement. Because the portion of the membrane-spanning segment S5 near the cytoplasmic side has been implicated in binding of 4-AP in Kv2·1 and Kv3·1 channels and its coupling to activation (Kirsch *et al.* 1993), it is intriguing to speculate that the binding sites may move along the axis of the channel pore in response to movement of the S4 voltage sensor. In contrast to the predictions of the K–D model of no effect of 4-AP on Kv1·2 and Kv3·1 gating currents (Kirsch & Drewe, 1993; Kirsch *et al.* 1994), our results indicate that at least some fraction of Kv1·4 gating currents will be altered. Removal of the slow component of gating current by 4-AP in *Shaker* B channels has recently been observed (McCormack, Joiner & Heinemann, 1994), indicating some interaction between 4-AP and the voltage sensor in at least one K^+ channel clone.

If we assume that 4-AP binding involves simultaneous interaction with four subunits (McCormack *et al.* 1994) similar to that described for TEA binding (Hegginbotham & MacKinnon, 1992), then the unstable intermediate state may reflect an asymmetric arrangement of the activation states of the α-subunits (Tytgat & Hess, 1992). Bi-stable binding may reflect a symmetric arrangement when all subunits are in the fully activated or fully deactivated conformations. Such a putative mechanism provides a potential additional biophysical basis for generating the diversity of state-dependent mechanisms of 4-AP binding. If native K^+ channels are heteromultimeric in nature (Sheng, Liao, Jan & Jan, 1993; Wang, Kunkel, Martin, Schwartzkoin & Tempel, 1993), the different activation properties of the α-subunits comprising the channel may not permit formation of a symmetric binding site in either the activated or deactivated range of potentials. Failure to produce a symmetric binding site in the open state would produce a channel with closed-state 4-AP-binding properties, and one with an asymmetric closed state might produce a channel with open-state 4-AP-binding properties.

Campbell *et al.* (1993) demonstrated closed-state binding but did not find evidence for open channel block by 4-AP of I_{TO} from ferret ventricular myocytes. The lack of observed open channel binding in the native channels, as opposed to the FK1 clone, may simply reflect a relatively

low apparent affinity of 4-AP for the open state in the presence of fast inactivation which severely limits access to the open channel. In addition, FK1 channels do not rapidly (< 1 s) bind or unbind 4-AP in their closed state. Native I_{TO} channels in ferret ventricular myocytes bind 4-AP in their closed states somewhat more rapidly than FK1 but still much more slowly than they unbind 4-AP from the open state, suggesting that the native ventricular I_{TO} 4-AP-binding site may also be at least partially occluded in its stable resting conformation (Campbell *et al.* 1993). The reason for the quantitative difference in access between the cloned and native channels is not clear at present. Assuming that FK1 does represent a component of native I_{TO}, possible reasons include formation of heteromultimers in the native channels (Sheng *et al.* 1993; Wang *et al.* 1993), missing subunits in the oocyte expression system (Scott *et al.* 1994; Rettig *et al.* 1994), or altered metabolic regulation by the intracellular enzymes present in oocytes. Whatever the basis, this difference in access to the 4-AP closed-state binding site between native and cloned channels may suggest that a factor is missing in the cloned channel system which modulates occlusion of the vestibule region in the resting state.

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