The Interaction of Na^+ and K^+ in the Pore of Cyclic Nucleotide-Gated Channels

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ABSTRACT The permeability ratio between K^+ and Na^+ ions in cyclic nucleotide-gated channels is close to 1, and the single channel conductance has almost the same value in the presence of K^+ or Na^+. Therefore, K^+ and Na^+ ions are thought to permeate with identical properties. In the α-subunit from bovine rods there is a loop of three prolines at positions 365 to 367. When proline 365 is mutated to a threonine, a cysteine, or an alanine, mutant channels exhibit a complex interaction between K^+ and Na^+ ions. Indeed K^+, Rb^+ and Cs^+ ions do not carry any significant macroscopic current through mutant channels P365T, P365C and P365A and block the current carried by Na^+ ions. Moreover in mutant P365T the presence of K^+ in the intracellular (or extracellular) medium caused the appearance of a large transient inward (or outward) current carried by Na^+ when the voltage command was quickly stepped to large negative (or positive) membrane voltages. This transient current is caused by a transient potentiation, i.e., an increase of the open probability. The permeation of organic cations through these mutant channels is almost identical to that through the wild type (w.t.) channel. Also in the w.t. channel a similar but smaller transient current is observed, associated to a slowing down of the channel gating evident when intracellular Na^+ is replaced with K^+. As a consequence, a rather simple mechanism can explain the complex behavior here described: when a K^+ ion is occupying the pore there is a profound blockage of the channel and a potentiation of gating immediately after the K^+ ion is driven out. Potentiation occurs because K^+ ions slow down the rate constant K_{off} controlling channel closure. These results indicate that K^+ and Na^+ ions do not permeate through CNG channels in the same way and that K^+ ions influence the channel gating.

INTRODUCTION

Cyclic nucleotide-gated (CNG) channels from photoreceptors and olfactory sensory neurons (for review Menini, 1995; Zimmerman, 1995; Kaupp, 1995; Biel et al., 1995; Finn et al., 1996; Zagotta and Siegelbaum, 1996; Zagotta, 1996) have a high degree of homology with voltage-gated channels, such as Na^+, K^+, and Ca^{2+} channels (Hille, 1992) and are thought to belong to the same superfamily of ionic channels (reviewed in Jan and Jan, 1990, 1992; Catterall, 1994). Nonetheless voltage-gated channels and CNG channels have different selectivity properties: voltage-gated channels are highly selective among alkali monovalent cations (Hille, 1992), while CNG channels are poorly selective among these cations (Kaupp et al., 1989; Menini, 1990; Picco and Menini, 1993; Sesti et al., 1996).

Na^+ and K^+ have the same permeability and conductance through CNG channels and currents carried by these two ions have almost identical—both macroscopic and microscopic—properties. This feature of CNG channels is rather remarkable, for two reasons: first, CNG channels have a high homology with voltage-gated channels, which usually distinguish quite well between Na^+ and K^+. Second, the thermodynamics of Na^+ and K^+ (Hille, 1992; Laio and Torre, 1999), i.e., hydration free energy, mobility, are rather different. In order to clarify these puzzling observations, we decided to analyze in more details the permeation of Na^+ and K^+ ions through CNG channel.

The pore region of CNG channels is characterized by a loop of three prolines which in the α-subunit of the CNG channel from bovine rods are at positions 365 to 367. These three prolines are found in all known α-subunits of CNG channels, but not in voltage gated channels. In this manuscript it is shown that mutation of proline 365 in the α-subunit of the CNG channel from bovine rod to a threonine, a cysteine or an alanine, leads to mutant channels with unusual properties. Mutant channels P365T, P365C, and P365A are powerfully blocked by K^+, Rb^+, and Cs^+ and a complex interaction between K^+ and Na^+ ions is observed in them. In mutant P365T the presence of K^+ ions in the intracellular medium affects the channel gating and activates an inward transient current carried by Na^+ ions at negative membrane potentials. Similarly, K^+ ions in the extracellular medium activate an outward transient current carried by Na^+ ions at positive potentials. This current transient is caused by a transient increase of the open probability, which in the presence of low cGMP concentrations may be extremely large, even over 20 times larger than the steady-state current.

The results observed in mutant P365T suggested to analyze in greater details the permeation of Na^+ and K^+ ions in the wild type (w.t.) CNG channel. Also in the w.t. channel a similar but smaller transient current is observed, associated to a slowing down of the channel gating observed when intracellular Na^+ is replaced with K^+. At large membrane voltages the inward current carried by Na^+ in the w.t. CNG channel is influenced by the presence of K^+ at the opposite

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side of the membrane, but the inward current carried by K^+ does not significantly differ in the presence of either Na^+ or K^+ in the intracellular medium. Thus also in the w.t. channel Na^+ and K^+ do not have the same permeation properties.

The experiments here described can be explained, at least in part, by a simple mechanism: in mutant P365T K^+ ions in the pore block it but cause a potentiation of gating immediately after they are driven out. Potentiation occurs because K^+ ions slow down the rate constant Koff controlling channel closure. These results show that K^+ ions affect the gating of CNG channels and provide a basis for understanding why Na^+ and K^+ ions, which have rather different hydration properties, have the same selectivity and single channel conductance in CNG channels.

MATERIALS AND METHODS

Molecular biology and mutagenesis

In vitro site-directed mutagenesis was performed on the α-subunit of the CNG channel from bovine rods (Kaupp et al., 1989) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutations were checked by sequencing of the region of interest (LI-COR DNA sequencer 4000L, LI-COR, inc., Lincoln, NE) using the SequiTherm EXCEL II Long-Read DNA Sequencing Kits-LC (Epicentre Technologies, Madison, WI). The DNA of the mutated channels was subsequently prepared in larger scale using the Plasmid Midi Kit from QIAGEN (QIAGEN GmbH, Hilden, Germany) to allow the complete sequencing of the mutants as well as their corresponding cRNA synthesis. In vitro mRNA synthesis was primed with the m7G(5′)ppp(5′)G cap analog using the mCAP RNA Capping Kit (Stratagene).

Dissection and recording apparatus

Recordings from homomultimeric channels composed of the α-subunit of the bovine retinal CNG channel and channel mutants were obtained from *Xenopus laevis* oocytes injected with the cRNA encoding for these channels. The cRNA was injected into *X. laevis* oocytes (Centre National de la Recherche Scientifique, Montpellier, France) that were treated as described in Nizzari et al. (1993). Mature *X. laevis* were anaesthetized with 0.2% tricainemethanesulfonate (Sigma) and ovarian lobes were removed surgically. The vitelline membrane was removed under visual control in a hyperosmotic medium. Currents activated by cyclic GMP were recorded under voltage clamp conditions from membrane patches, excised from oocytes, in the inside out configuration (Hamill et al., 1981). The recording apparatus was the same as that described in Sesti et al. (1995). In order to properly resolve the brief current transient shown in Fig. 2, currents were recorded at a bandwidth up to 10 kHz and were sampled at 30 kHz. If a lower bandwidth up to 2 kHz was used, current transients were significantly distorted. Each trace was obtained from at least 5 distinct trials and was computed as the current in the presence of cGMP minus the current in its absence. Approximately 50 nl of mRNA was injected into each oocyte and membrane patches containing a single channel could be obtained after about 18 h of incubation. When the incubation time was longer than 36 h, single channel recordings were very rare and large macroscopic currents were detected.

Solutions

The solution filling the patch pipette was composed of 110 mM of NaCl or KCl, 0.2 mM EDTA and 10 mM HEPES buffered to pH 7.6 with tetramethylammonium hydroxide (TMAOH). Neutralizing the solution with TMAOH or NaOH or KOH did not affect either the macroscopic or the single channel current. Similarly using 2 or 10 mM HEPES did not appreciably modify cGMP activated currents. Solutions bathing the intracellular side of the membrane had the same composition as that filling the patch pipette, but could contain also micromolar amounts of cGMP. EDTA was omitted from solutions containing Ni^{2+}.

Data analysis

Permeability ratios relative to Na^+ P_xP_{Na} were computed from the reversal potential V_{rev} obtained with experiments with 110 mM Na^+ in the extracellular medium and 110 mM of ion X in the intracellular medium. Measured V_{rev} was not corrected for junction potentials, as these quantities are not larger than a few mV (Menini, 1990). P_xP_{Na} was calculated from the equation:

$$\frac{P_x}{P_{Na}} = \exp\left(-\frac{FV_{rev}}{RT}\right) \tag{1}$$

where F is the Faraday constant, R is the gas constant and T is the absolute temperature.

The dependence of the current I/I_{max} on the cGMP concentration was fitted with the Hill equation

$$\frac{I}{I_{max}} = \frac{g^n}{g^n + K^n} \tag{2}$$

where g is the cGMP concentration and K is the cGMP concentration activating half of the maximal current. In allosteric models it is assumed that the liganded state C_1 and the open state C_0 are in a thermodynamic equilibrium with an equilibrium constant $L = K_{on}/K_{off}$ (Li et al., 1997; Sunderman and Zagotta, 1999; Tibbs et al., 1997) where:

$$C_1 \underset{K_{off}}{\overset{K_{on}}{\rightleftharpoons}} C_o \tag{3}$$

The value of L controls the maximal open probability p_{max} in the presence of a saturating cyclic nucleotide concentration and we have the relation:

$$p_{max} = \frac{L}{(L+1)} \tag{4}$$

The quantity K present in the Hill Eq. (2) is related to the dissociation constant K_d of cGMP with the channel, usually used in allosteric models (Li et al., 1997; Sunderman and Zagotta, 1999; Tibbs et al., 1997), by the equation:

$$K = \frac{K_d}{(1+L)^{1/n}} \tag{5}$$

Single channel analysis

Single channel properties were analyzed from current recordings obtained at a high gain and at a bandwidth up to 5 kHz sampled at 20 kHz. As membrane patches usually contained more than one channel gated by cGMP, it was difficult to measure gating properties reliably and only the single channel current was determined. As shown in Fig. 3 these amplitude histograms had well resolved peaks corresponding to the closed state and openings of one, two, three or four channels. The single channel current was determined by measuring the current interval between these peaks.

RESULTS

Several recombinant channels of the α-subunit of the bovine rod CNG channel with mutations in and near the proline loop (Fig. 1) were constructed and expressed in *X. laevis* oocytes.

The proline loop of CNG channels

The amino acid sequences in the pore region of a variety of CNG channels and voltage-gated channels are shown in Fig. 1. All known α-subunits of CNG channels have a conserved sequence of three prolines (shown in bold type) which is not present either in K^+ or in Na^+ channels. The second proline of this loop is replaced by an aspartate in all known β-subunits of CNG channels. This proline loop is likely to be located in the pore of the CNG channels near a glutamate residue known to play an essential role in ionic permeation (Root and MacKinnon, 1993; Eismann et al., 1994; Sesti et al., 1995; Park and MacKinnon, 1995). A recent study with cysteine scanning mutagenesis (Becchetti et al., 1999) has identified this proline loop as part of the external vestibule of CNG channels.

Properties of mutant P365T

The proline loop in the α-subunit from bovine rods extends from residue 365 to 367. When proline in position 365 was replaced by a threonine, a cysteine or an alanine, mutant channels exhibited an unexpected interaction between Na^+ and K^+ ions.

The cRNA of mutant channels was injected into oocytes and after two or three days it was possible to record currents activated by micromolar amounts of cGMP added to the intracellular medium. Fig. 2 reproduces current recordings measured in voltage clamp mode obtained from membrane patches excised from oocytes injected with the cRNA of mutant P365T. The voltage commands were changed from 0 mV to + or −180 mV in steps of 20 mV. The current activated at the steady state by 500 μM cGMP exhibited a significant outward rectification (Fig. 2 *A*), which was observed also in the presence of 100 and 1000 μM cGMP (Fig. 2 *C*).

Currents activated by 500 and 1000 μM cGMP were almost identical and therefore 500 μM cGMP can be considered as a saturating cGMP concentration. The cGMP concentration activating half of the maximal current was about 200 μM for mutants P365A, P365T and P365C and was larger than the value measured in the w.t. channel, ranging from 50 to 100 μM. The ratio between the current flowing in the steady state at +100 and −100 mV was about 3, while at the higher voltage of +180 mV it was about 5. In the w.t. channel in the presence of saturating cGMP concentrations this ratio is about 1. At positive voltages the outward current was activated with a delay of some milliseconds. This slow activation and the degree of rectification observed in mutant P365T in the presence of saturating cGMP concentrations are reminiscent of similar properties reported in the native CNG channels in the presence of subsaturating cGMP concentrations (Karpen et al., 1988).

When Na^+ ions present in the intracellular medium were substituted by an equimolar amount of K^+ ions, no appreciable outward current was observed, even at membrane voltages larger than +100 mV (Fig. 2 *B*) and at 0 mV no net inward current carried by Na^+ ions was observed. This last observation suggests a blockage of the Na^+ influx by intracellular K^+. The steady state I-V relation in the presence of K^+ in the intracellular medium, as shown in Fig. 2 *D*, is flat at voltages between −20 and +120 mV. In some patches a very small outward current of 1 or 2 pA was observed at membrane voltages larger than +140 mV. Another remarkable feature of current recordings shown in Fig. 2 *B* is the presence of a large current transient, when the voltage command was stepped from 0 mV to membrane voltages more negative than −120 mV. This current transient was reduced or almost absent when K^+ ions were replaced by Na^+ ions in the intracellular medium. The decay of the cGMP gated current at negative voltages in the presence of intracellular K^+ is analyzed in Fig. 2 *E*. At −100 and −180 mV, the current decayed with a single time constant of about 1.8 and 0.6 ms, respectively. This time constant was shorter at larger voltages and varied between 0.8 and 0.3 ms (Fig. 2 *F*).

Single channel properties of mutant P365T

In order to understand the origin of the outward rectification shown in Fig. 2 *A*, single channel properties at positive and negative voltages were analyzed. Fig. 3 *A* illustrates current recordings obtained in a symmetrical Na^+ solution at ±100, ±140, and ±180 mV from a patch containing a small number of mutant channels P365T. The analysis of amplitude histograms (Fig. 3 *B*) obtained at positive voltages indicates the presence of well resolved peaks corresponding to the close state and to current levels with one, two, three and four open channels. At negative voltages the probability of observing two channels open simultaneously was very small, indicating that the open probability increases with voltage.

Fig. 3 *C* reproduces the current-voltage relation corresponding to the single channel recordings from the experiment shown in Fig. 3 *A*. A straight line provides a good fit of the data, indicating a single channel conductance of about 18 pS between −160 and +160 mV. As a consequence, the origin of the outward rectification of the macroscopic current shown in Fig. 2 is due to a voltage-dependent gating.

In order to test the hypothesis of a K^+ blockage of the Na^+ current and to examine if a very small outward current may be carried by K^+ ions at positive voltages, current

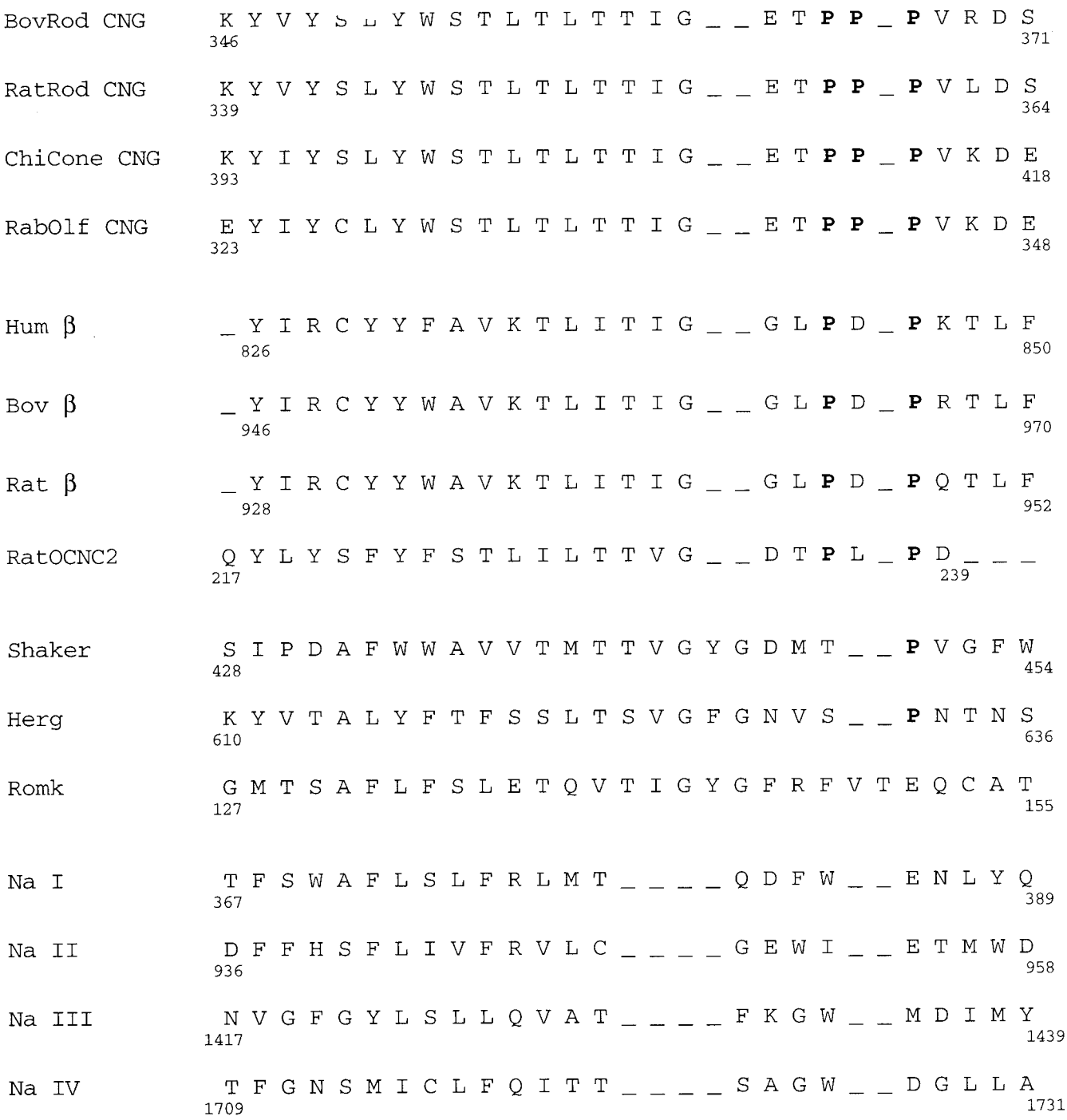

FIGURE 1 Sequence alignment of the pore-forming regions of α and β subunits of CNG channels with selected K^+ channels and the four domains of a Na^+ channel. The sequences are from top to bottom: CNG α-subunit from bovine rods (BovRod CNG, *Bos taurus*, accession number (acc) Swissprot Q00194); CNG α-subunit from rat rods (RatRod CNG, *Rattus norvegicus*, acc Swissprot Q62927); CNG α-subunit from chick cones (ChiCone CNG, *Gallus gallus*, acc PIR I50630); CNG α-subunit from rabbit olfactory sensory neurons (RabOlf CNG, *Oryctolagus cuniculus*, acc Swissprot Q28718); CNG β-subunit from human rods (Hum β *Homo sapiens*, acc GenBank AF042498); CNG β-subunit from bovine rods (Bov β *Bos taurus*, acc EMBL x89626); CNG β-subunit from rat rods (Rat β *Rattus norvegicus*, acc GenBank AJ000496); CNG β-subunit from rat olfactory sensory neurons (RatOCNC2, Rattus norvegicus, acc Swissprot Q64359); K^+ channel Shaker (*Drosophila melanogaster*, acc Swissprot P08510); K^+ channel Herg (H. sapiens, acc PIR I38465); K^+ channel Romk (*Rattus norvegicus*, acc Swissprot P35560); Na^+ channel domains I, II, III, IV (*Rattus norvegicus*, acc Swissprot P04774). The sequences of the CNG channels were aligned to the sequences of the potassium channels as suggested in Doyle et al. (1998). The alignment of the four domains of the Na^+ channels was that proposed by Guy and Durell (1995).

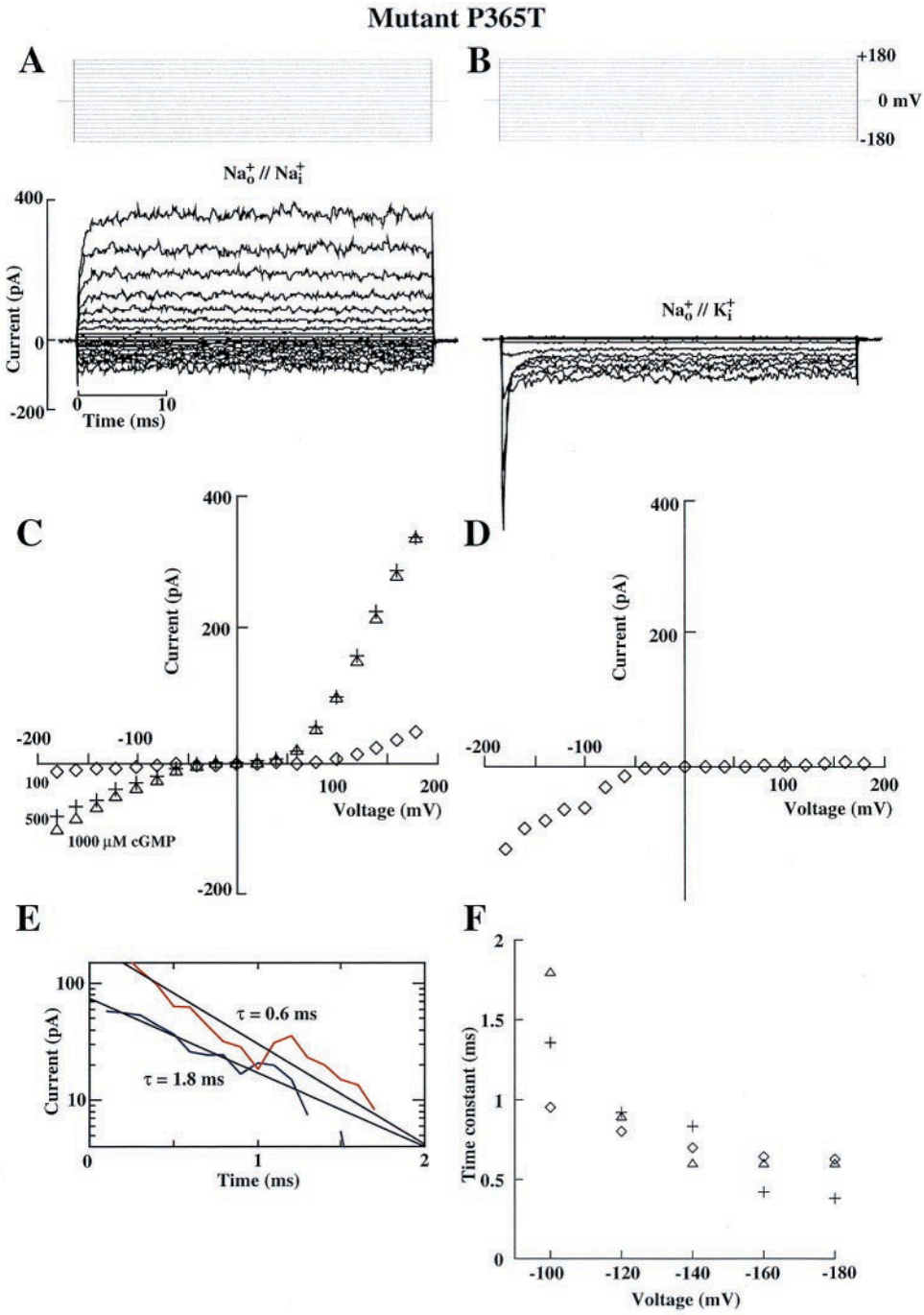

FIGURE 2 Current-voltage relation of mutant P365T. (*A* and *B*) Current traces recorded in voltage clamp conditions in the presence of 500 μM cGMP. 110 mM NaCl in the extracellular medium and 110 mM NaCl (*A*) or KCl (*B*) in the intracellular medium. Holding voltage 0 mV, pulses from −180 to 180 mV in steps of 20 mV. Traces obtained by averaging 20 different trials. (*C*) I-V relations in the steady state in the presence of 100 (◇), 500 (+), and 1000 μM (Δ) cGMP with 110 mM NaCl on both sides of the patch. (*D*) I-V relations in the presence of 500 μM cGMP with 110 mM KCl in the intracellular medium. Data from the experiment illustrated in *B*. (*E*) Decay of the current from the peak to the steady state value in the presence of intracellular K^+ at −100 (*blue line*) and −180 mV (*red line*). The two straight lines through the points indicate time constants of 0.6 and 1.8 ms. (*F*) Dependence of the time constant of the current relaxation to its steady state value on membrane voltage in the presence of KCl in the intracellular medium. Data from three different patches.

fluctuations of membrane patches containing very few channels were studied (Fig. 4 *A–C*). At −40 mV current fluctuations carried by Na^+ ions were almost completely blocked by the presence of intracellular K^+. This blockage progressively decreased at more negative membrane voltages, such as −80 mV and was almost absent at membrane

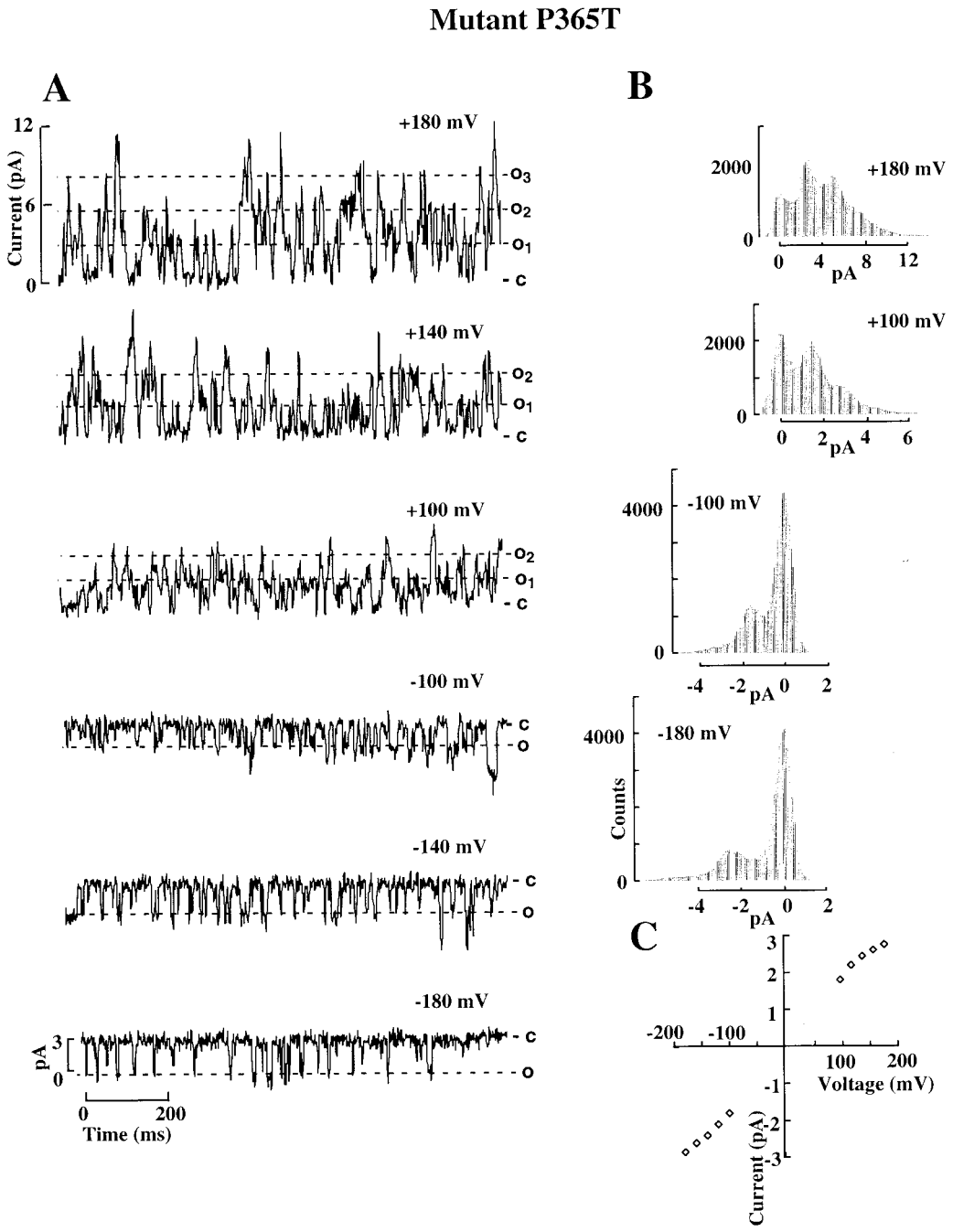

FIGURE 3 (*A*) Single channel current recordings obtained at +180, +140, +100, −100, −140, −180 mV from a patch containing at least four channels of mutant P365T. 500 μM cGMP was present in the intracellular medium. (*B*) Amplitude histograms of current fluctuations shown in *A* at +180, +100, 100 and −180 mV. Histograms obtained from 5 s of continuous recording. The amplitude of the single channel current was obtained by measuring the distance between successive peaks in the amplitude histogram. (*C*) I-V relation of a single channel of mutant P365T. The straight line was computed by assuming a single channel conductance of 18 pS.

voltages more negative than −120 mV. Single channel analysis of channel openings at −100 mV indicated that K^+ blockage was caused by a decrease of both the single channel conductance and the open probability.

At membrane voltages comprised between 0 and +100 mV current recordings obtained with K^+ in the intracellular medium and in the absence or in the presence of 500 μM cGMP were indistinguishable. At +120 mV and at more positive voltages very brief outward transients carried by K^+ ions were observed (upper traces in Fig. 4 *B*). These transients were very rare and in the patch recordings shown in Fig. 4 *B* not more than 20 of them on average could be

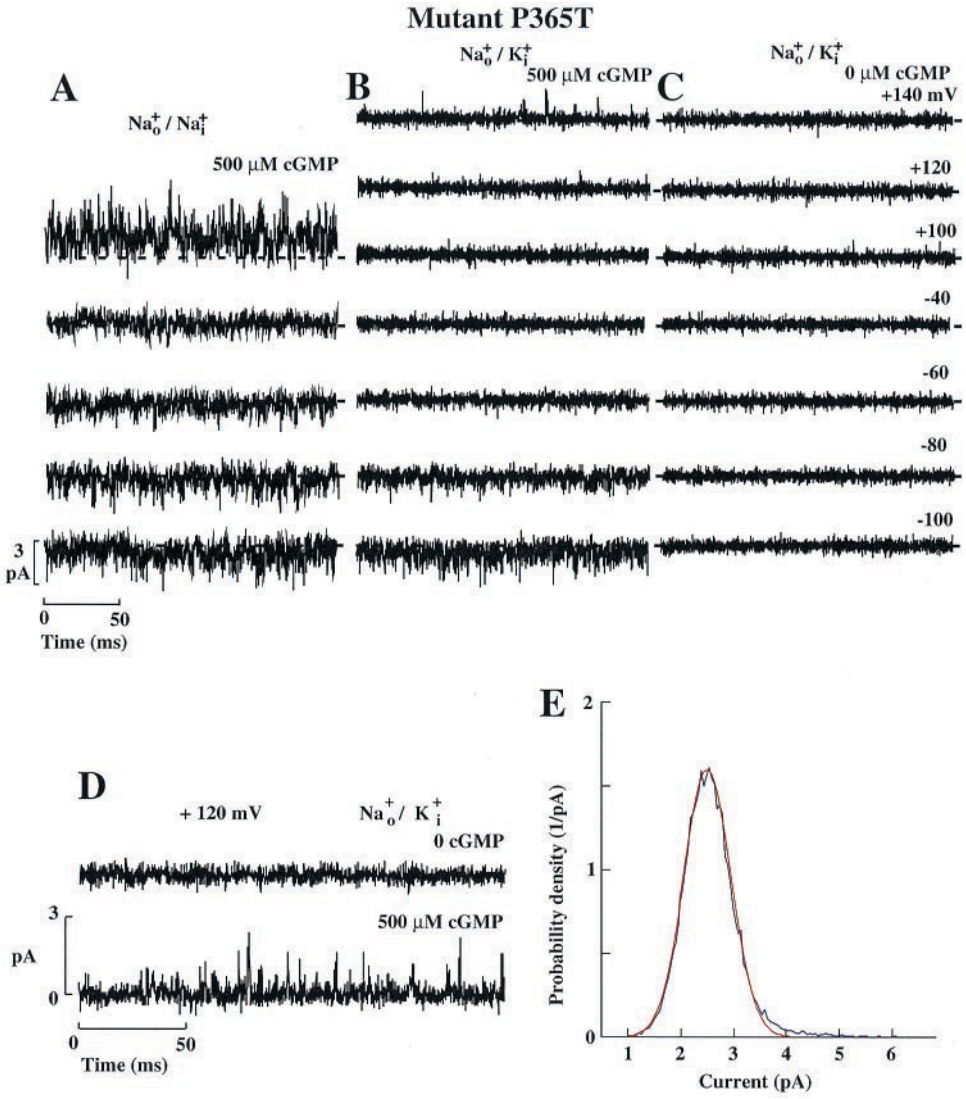

FIGURE 4 K^+ blockage and permeation. (*A–C*) Current recordings obtained in the presence of 110 mM Na^+ in the extracellular medium and in the presence of 110 mM Na^+ (*A*), 110 mM K^+ (*B* and *C*) in the intracellular medium at the membrane voltages indicated. Recordings in *A* and *B* were obtained in the presence of 500 μM cGMP, while those in *C* in the absence of cGMP. (*D*) A current recording from a membrane patch containing mutant channels P365T at +120 mV in the presence of 110 mM Na^+ in the extracellular medium and 110 mM K^+ in the intracellular medium in the absence (*upper trace*) and in the presence (*lower trace*) of 500 μM cGMP in the intracellular medium. In the presence of 110 mM Na^+ in the extracellular medium a macroscopic current of about 55 pA was observed at +120 mV. (*E*) Amplitude histogram of current fluctuations during 5 s of recording in the presence of 110 mM K^+ and 500 μM cGMP in the intracellular medium at +120 mV. The red line is a Gaussian distribution with a RMS of 0.3 pA corresponding to the noise observed in the absence of cGMP. The area of the histogram corresponding to channel openings was about 0.03, providing a net mean current of 0.06 pA carried by K^+.

observed in one second. In order to have a better characterization of these outward transients a patch containing many P365T channels was analyzed at a high gain and in the presence of K^+ in the intracellular medium. In the absence of cGMP the current trace at +120 mV was quiet with a variance σ^2 of 0.08 pA^2 (Fig. 4 *D*). When 500 μM cGMP was added to the intracellular medium brief current transients were detected and the current variance increased to 0.1313 pA^2. These current transients never lasted longer than 1 ms and had a variable amplitude up to 3 pA. The amplitude histogram of the current recording in the presence of cGMP (Fig. 4 *E*) indicates that the probability of observing these openings was lower than 0.02 and that the net average current carried by K^+ ions was about 0.026 pA. The single channel current of these events was estimated from the ratio of the variance (0.1313 − 0.08 pA^2) and the average current (0.026 pA) giving an estimate of 1.9 pA corresponding to a single channel conductance of 16 pS, almost identical to that observed for Na^+ ions (Fig. 3). As the macroscopic current carried by Na^+ in the same patch at the same voltage was 25 pA, the ratio between the current carried by Na^+ and K^+ was about 1000. The same exper-

iment was repeated in another four patches and the estimate of the single channel conductance varied between 15 and 18 pS and the ratio between the current carried by Na^+ and by K^+ varied between 250 and 1100.

The estimate of the single channel conductance of about 16 pS must be taken with caution given the brief duration of current transients and the very small size of the mean current and variance. These results, however, clearly show three basic properties of K^+ ions in the pore of mutant P365T: K^+ ions block the permeation of Na^+ ions in a voltage dependent way; K^+ ions can permeate through the mutant P365T at very positive voltages; K^+ ions modify channel gating in a complex way.

The effect of Ni^{2+} on mutant P365T

The rectification of the I-V relations of mutant channel P365T reported in Figs. 2 and 3 may be caused by a voltage dependence, similar to that of usual voltage-gated channels (Hille, 1992), acquired by the mutant channel or by the fact that a saturating cGMP concentration activates mutant channels P365T only partially (Li et al., 1997). This last possibility is consistent with the observation that the degree of rectification and the time constant of current activation in the presence of saturating cGMP concentrations in mutant P365T (Fig. 2) are similar to what observed in native CNG channels but in the presence of subsaturating cGMP concentrations (Karpen et al., 1988). In order to understand the origin of the rectification observed in Fig. 2, Ni^{2+} ions were added to the intracellular medium. Indeed this compound is known to fully activate CNG channels in the presence of a partial agonist (Gordon and Zagotta, 1995a,b). If the rectification of the I-V relation shown in Fig. 2 is a consequence of cGMP being only a partial agonist, Ni^{2+} is expected to remove this rectification.

10 μmol of Ni^{2+} was added to the intracellular medium, after the current recordings shown in Fig. 2 were obtained. In the presence of Ni^{2+} the current recordings presented in Fig. 5, *A* and *B* were measured. As shown by the steady state I-V relations in Fig. 5 *C*, the addition of Ni^{2+} abolished the outward rectification previously observed. This result indicates that, in agreement with the single channel recordings shown in Fig. 3, a saturating cGMP concentration does fully activate mutant P365T and that cGMP is only a partial agonist.

At membrane voltages between 0 and +160 mV the addition of Ni^{2+} increased by 3 to 4 times the outward current carried by intracellular Na^+ ions. In contrast, even in the presence of Ni^{2+}, no clear outward current carried by intracellular K^+ ions (Fig. 5, *B* and *D*) was detected. Ni^{2+} had a different effect on the inward current carried by Na^+ ions at the peak and at the steady state: on average the peak and the steady state current increased by about 3 and 12 times, respectively. Therefore the relative amplitude of the transient current previously described in the absence of Ni^{2+} (Fig. 2 *B*) was significantly reduced when Ni^{2+} was added. Single channel recordings obtained in the presence of 10 μM Ni^{2+} showed that the single channel conductance was unaltered and that the maximal open probability in the presence of saturating cGMP concentrations did not approach the maximal value of 1, but the lower value of 0.9.

The results reported in Fig. 5 offer some explanations for understanding the properties of mutant P365T. The rectification observed in mutant P365T (Fig. 2, *A* and *C*) is due to the fact that the cGMP does not fully activate the P365T mutant but behaves like a partial agonist for this channel. As a consequence in the presence of a saturating cGMP concentration, the maximal open probability of mutant channel P365T is significantly less than 1. As the potentiation induced by Ni^{2+} at the steady state is about 12, the maximal open probability of mutant P365T in the absence of Ni^{2+} is not larger than 0.08, as also evident from the single channel data shown in Fig. 3.

The transient current is a transient potentiation

The large transient current shown in Fig. 2 *B* can be caused by a transient increase of the single channel conductance and/or by a transient potentiation, i.e., an increased sensitivity of mutant channels P365T to cGMP. Therefore the effect of cGMP concentration on the amplitude of the transient current was analyzed. Panel *A* in Fig. 6 reproduces current recordings obtained in the presence of either Na^+ or K^+ in the intracellular medium when V_c was stepped from 0 to −180 mV. In the presence of 20 μM cGMP no appreciable current was observed in the presence of intracellular Na^+, but a brief current transient was measured in the presence of intracellular K^+. Increasing the cGMP concentration led to the appearance of a steady state current, which was always larger in the presence of K^+ in the intracellular medium. The dependence of I_{peak} on the cGMP concentration in the presence of intracellular K^+ (+) and Na^+ ($\diamond$) for the experiment shown in panel *A* is reproduced in panel *B*. The dependence of $I_{peak}/I_{peak,\ sat}$ and of $I_{ss}/I_{ss,\ sat}$ on cGMP concentration is shown in Fig. 6, *C* and *D,* respectively in the presence of intracellular K^+ (filled symbols) and Na^+ (open symbols). In panel *C* the solid line through the filled symbols was obtained with Eq. 2 with $n = 2$ and $K = 50$ μM, while the line through the open symbols was obtained with $K = 150$ μM cGMP. The data at the steady state (*D*) were fitted with the same equation (with $n = 2$) but with $K = 150$ and 240 μM cGMP for the filled and open symbols, respectively.

When K^+ ions were present in the intracellular medium, Na^+ ions at very negative voltages carried a larger current also at the steady state as clearly shown in Fig. 6 *A*. This steady state potentiation was consistently observed in all patches, but its amplitude varied: at −180 mV the increase of Na^+ current when intracellular Na^+ was replaced by K^+ varied by 20 and 80%.

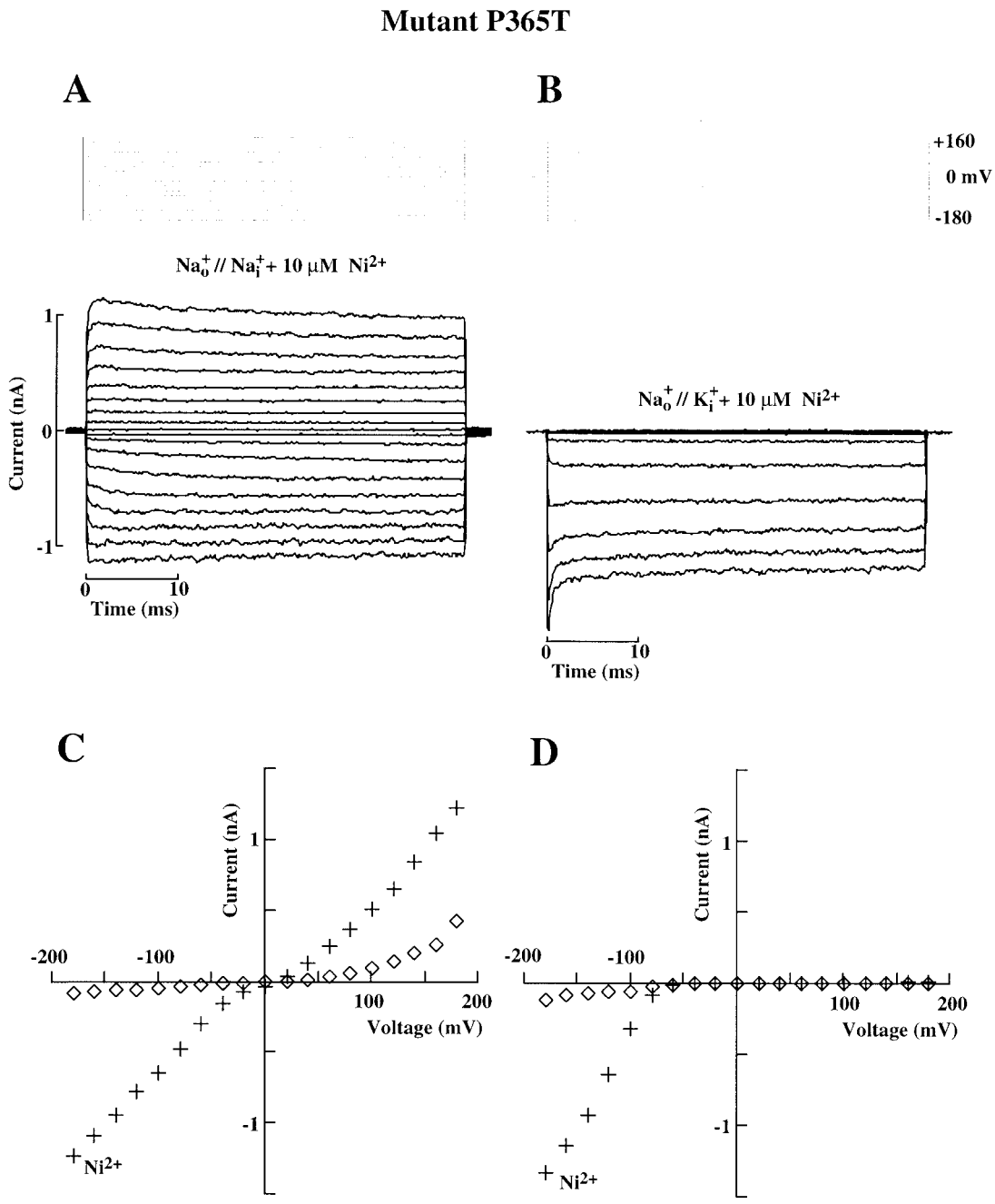

FIGURE 5 Current-voltage relation of mutant P365T in the presence of 10 μM Ni^{2+}. (*A* and *B*) Current traces recorded in voltage clamp conditions in the presence of 500 μM cGMP and of 10 μM Ni^{2+}. 110 mM NaCl in the extracellular medium and 110 mM NaCl (*A*) and KCl (*B*) in the intracellular medium. Traces obtained by averaging 20 different trials. (*C* and *D*) I-V relations in the steady state from the experiments shown in *A* and *B,* respectively. + and ◇ refer to data obtained in the presence and absence of Ni^{2+}. Data from the same patch as in Fig. 2.

When the extracellular Na^+ was replaced with K^+, current recordings shown in Fig. 7 *A* were obtained. Under these conditions when Na^+ was present at the intracellular side only a macroscopic outward current was observed and no macroscopic inward current was detected. In the presence of 20 μM cGMP only transient outward currents were measured. When the cGMP concentration was raised a large outward current transient was followed by a smaller steady state current. The current carried by Na^+ at positive voltages increased steeply with voltage and the current flowing at +120 mV was about 10 times the current flowing at +60 mV. At 0 mV, no appreciable net inward current was observed. In the presence of K^+ on both sides of the patch no appreciable macroscopic current was flowing in either direction.

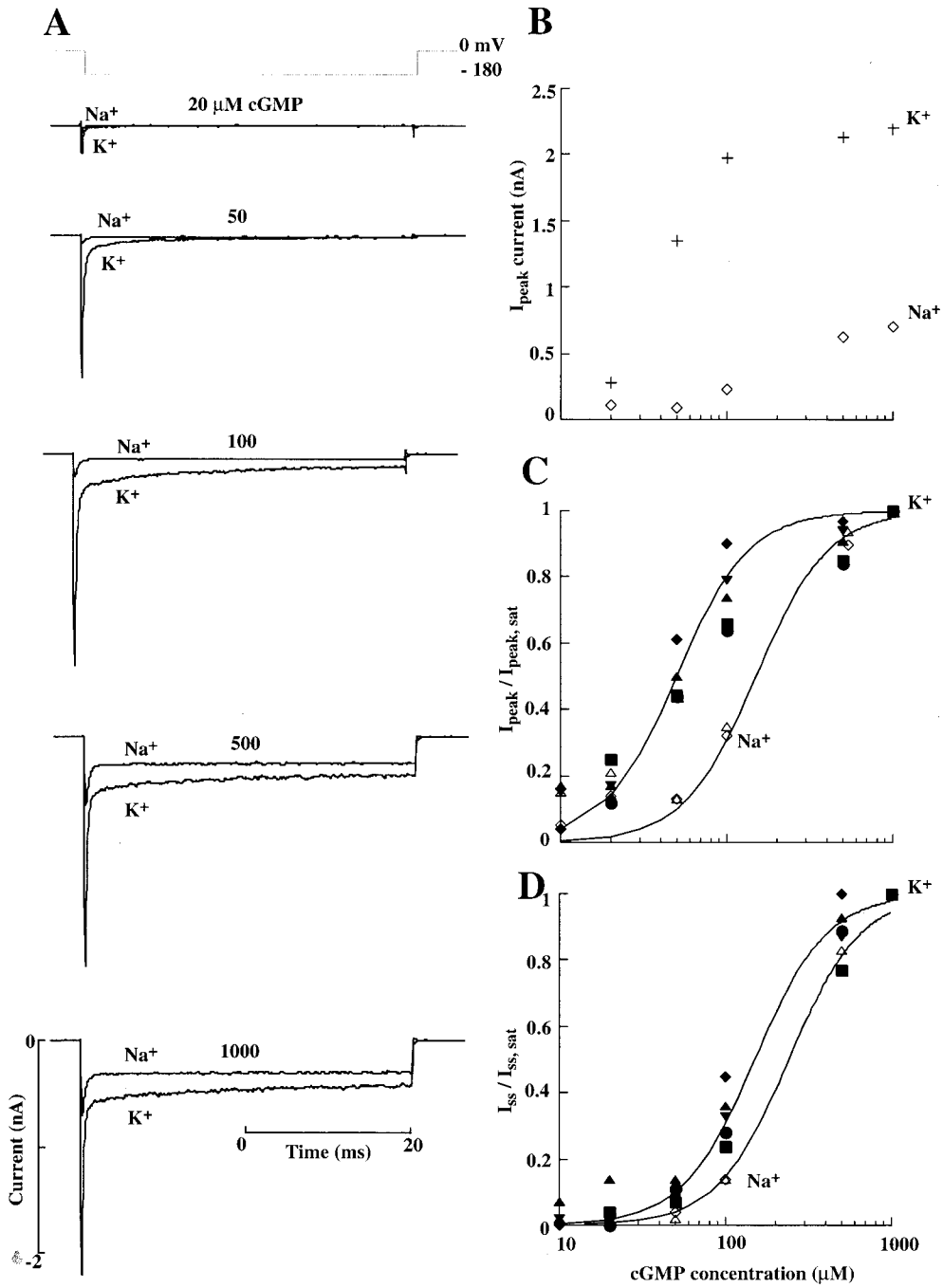

FIGURE 6 The effect of cGMP concentration on the amplitude of the transient current in mutant P365T. (*A*) Current recordings in the presence of Na^+ and K^+ in the intracellular medium with different cGMP concentrations. 110 mM NaCl was present in the extracellular medium. Voltage command from 0 to −180 mV. (*B*) Dependence of I_{peak} on cGMP concentration in the presence of K^+ (+) and Na^+ (◇) in the intracellular medium. Data from the experiments shown in *A*. (*C*) Dependence of $I_{peak}/I_{peak,\ sat}$ on cGMP concentration. Open and filled symbols obtained in the presence of Na^+ and K^+, respectively, in the intracellular medium. $I_{peak,\ sat}$ is the peak current recorded in the presence of a saturating cGMP concentration. Data from 5 different patches. Solid lines computed from Eq. 2 with $n = 2$ and $K = 50$ and 150 μM. (*D*) Dependence of $I_{ss}/I_{ss,\ sat}$ on cGMP concentration. Open and filled symbols obtained in the presence of Na^+ and K^+, respectively, in the intracellular medium. $I_{ss,\ sat}$ is the steady state current recorded in the presence of a saturating cGMP concentration. Data from five different patches. Solid lines computed from Eq. 2 with $n = 2$ and $K = 150$ and 240 μM.

The dependence of I_{peak} and I_{ss} measured at +180 mV on the cGMP concentration is shown in Fig. 7 *B*. Panel *C* represents the ratio I/I_{max} calculated for the peak (*open symbols*) and for the steady-state current (*filled symbols*) as a function of the cGMP concentration for three different patches. The solid line through the open and filled symbols

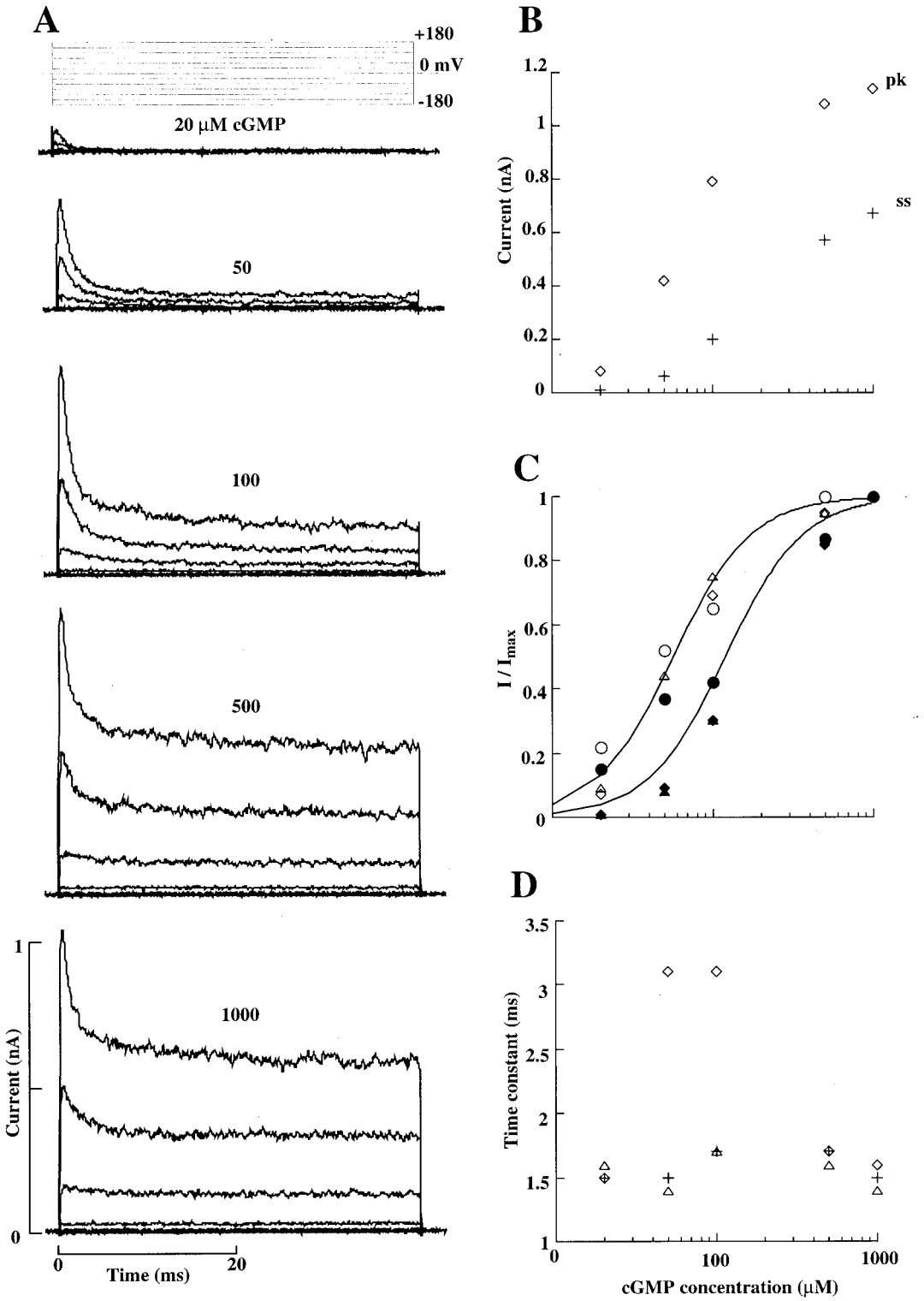

FIGURE 7 Current recordings in the presence of K^+ in the extracellular medium. (*A*) Current traces recorded in voltage clamp conditions in the presence of different cGMP concentrations. 110 mM KCl in the extracellular medium and 110 mM NaCl in the intracellular medium. Holding voltage 0 mV, pulses from −180 to 180 mV in steps of 30 mV. Traces obtained by averaging 10 different trials. (*B*) Dependence of I_{peak} and I_{ss} measured at + 180 mV on cGMP concentration. Data from the experiments shown in panel *A*. (*C*) Dependence of $I_{peak}/I_{peak,\ sat}$ (*open symbols*) and $I_{ss}/I_{ss,\ sat}$ (*filled symbols*) on cGMP concentration. Data from three different patches. Solid lines computed from Eq. 2 with $n = 1.8$ and $K = 57$ and 120 μM. (*D*) Dependence of the time constant of the current transient on cGMP concentration. Data from three different patches.

were obtained from Eq. 2 with $n = 1.8$ and $K = 57$ and 120 μM, respectively. The concentration activating half of maximal current was larger at the steady state than at the peak. The time constant measuring the decay of the peak current at +180 mV was evaluated and plotted as a function of the cGMP concentration (Fig. 7 *D*). The value of the time constant was not significantly dependent on the cGMP concentration and was around 1.6 ms (values obtained for 3 different patches). These results indicate that during the development of the transient current mutant channels P365T are more sensitive to cGMP, thus suggesting a transient potentiation.

Ionic selectivity of mutants P365T and P365A

The ionic selectivity of mutant channels P365T, P365A and P365C to alkali monovalent cations was investigated by measuring the reversal potential V_{rev} of the macroscopic current under bi-ionic conditions. The extracellular medium contained 110 mM NaCl and currents activated by 500 μM cGMP were studied in the presence of equimolar amounts of Na^+ (Fig. 8 *A*), Li^+, K^+, Rb^+ and Cs^+ in the intracellular medium.

As shown in Fig. 8, an outward current was measured in the mutant P365T only in the presence of either Li^+ or Na^+ at the intracellular side of the membrane. In the presence of Rb^+ and Cs^+ no outward macroscopic current was ever n even at membrane voltages up to +100 mV. In two patches a small transient outward current of 1–2 pA carried by large alkali cations such as Rb^+ and Cs^+ was observed at +160 mV. The reversal potential in the presence of Li^+ was +9.5 mV, 16.2 mV and 14.5 mV for mutants P365A, P365T, and P365C, respectively, indicating a permeability ratio P_{Li}/P_{Na} equal to 0.7, 0.54 and 0.6 for the three mutants, respectively. In the presence of Rb^+ and Cs^+ a large transient current was observed when the voltage command was turned to very negative values. The value of the reversal potential in the presence of K^+, Rb^+ and Cs^+ in the intracellular medium could not be reliably determined, as the I-V relations were flat between −20 and + 100 mV.

The selectivity to organic monovalent cations was analyzed in the experiments shown in Fig. 8 *B*. The mutant channel P365T was permeable to formamidinium, guanidinium, aminoguanidinium and ammonium ions. Some methyl derivatives of ammonium such as methylammonium and dimethylammonium were also permeant. As shown in Table 1 the reversal potential for ammonium was slightly negative, as in wild type CNG channels (Picco and Menini, 1993). Ammonium, however, carried a rather small outward current; rather unexpectedly methylammonium carried a larger current than ammonium at positive voltages, indicating a larger chord conductance for methylammonium.

As shown in Fig. 8 *B*, when the voltage command was stepped to a negative voltage in the presence of intracellular ammonium, a large inward current very similar to the current transient previously described in the presence of K^+, was observed. This current rapidly declined to a smaller level within a few milliseconds. This result was consistently observed in all patches and in both mutants P365T and P365A.

Table 1 summarizes data collected on reversal potentials and on permeability ratios between different cations and Na^+. Permeability ratios of mutants P365T and P365A were almost identical (Table 1) for monovalent alkali cations and similar for organic cations. For organic cations, the permeability of mutant P365A was very similar to that of CNG channels while the permeability of mutant P365T to the same cations was intermediate between that of CNG channels and that of Na^+ channels. The selectivity of mutant P365C to organic compounds was not determined.

Properties of other mutants near the proline loop

We have analyzed a variety of mutants around the proline loop namely: P366T, P366C, P367V, P367C, P365T, and P367V; ΔP mutants where one proline of the loop was deleted; YG mutants where the two amino acids tyrosine and glycine were added between residues 362 and 363 of the w.t. CNG channel. The "YG" containing mutants were designed because an alignment of sequences from the pore region of most K^+ channels showed the presence of a YG motif located between the corresponding residues 362 and 363 of the CNG channels (Heginbotham et al., 1992; Fig. 1). In agreement with Heginbotham et al. (1992), the addition of the YG motif into the CNG channel sequence was fatal in all tested mutants. This result suggests that the YG addition disrupts the organization of the pore in CNG channels.

The alignment of the pore region sequences from both the K^+ channels and the CNG channels indicates that the third proline (P367) of the proline loop in CNG channels is highly conserved also in the K^+ channels. Indeed, all mutants with the P367C or P367V mutation were not functional. When proline 366 was mutated to a cysteine or a threonine, mutant channels had a voltage sensitivity and an ionic selectivity very similar to those of the wild type.

Deleting one proline from the P365T mutant surprisingly conferred a significant permeability to K^+ to the resulting mutant P365T and ΔP. In this double mutant similar outward currents were observed in the presence of Na^+ and K^+ in the intracellular medium. In addition the voltage dependence clearly present in the single mutant P365T disappeared in the double mutant P365T and ΔP.

Na^+ and K^+ permeation in the w.t. channel

The unusual interaction between Na^+ and K^+ observed in channel mutant P365T suggests that also in the w.t. CNG channel these two ions do not permeate in the same way.

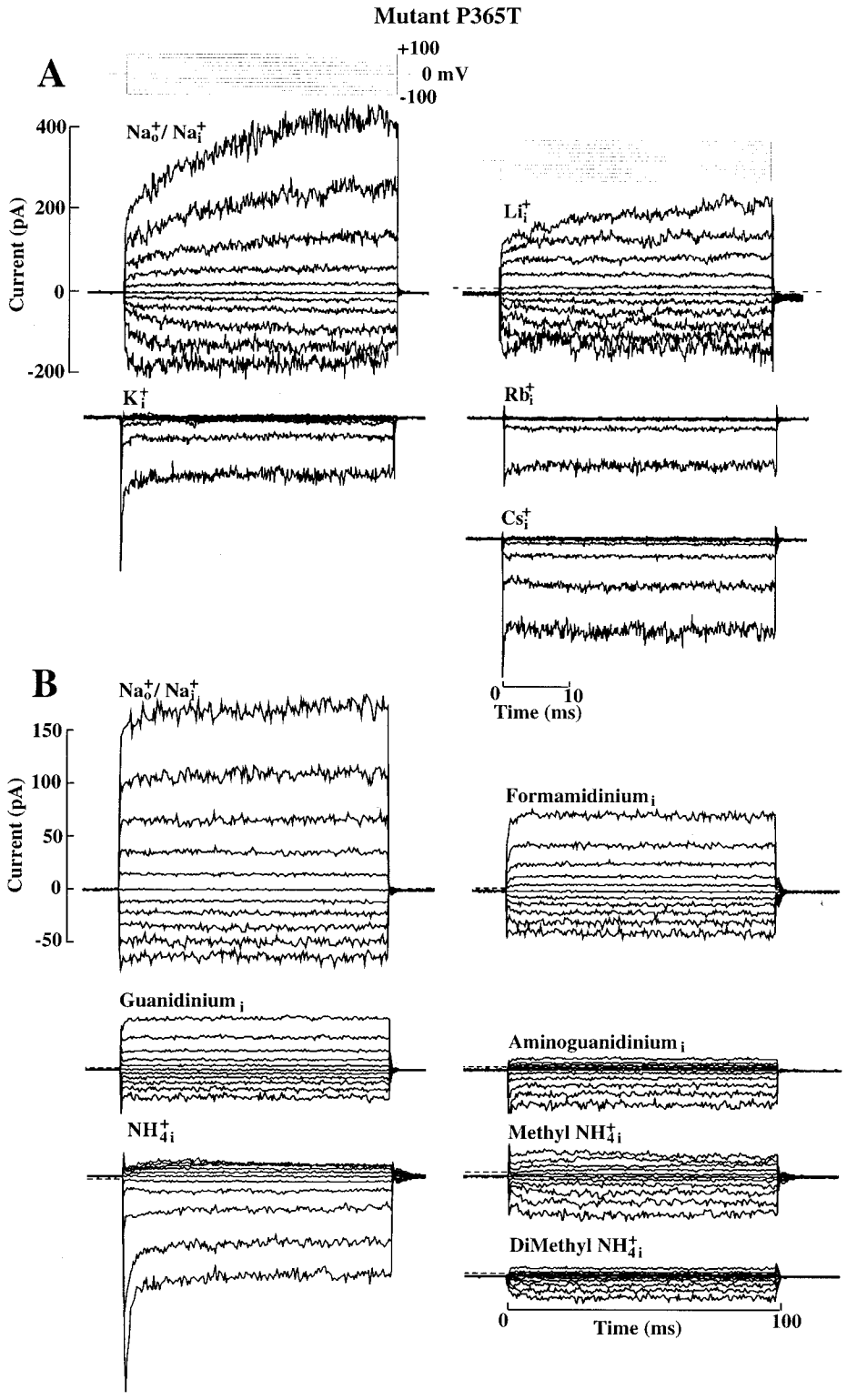

FIGURE 8 Ionic selectivity of mutant P365T to alkali monovalent (*A*) and organic (*B*) cations. (*A*) Current recordings in the presence of 110 mM Na^+, Li^+, K^+, Rb^+ and Cs^+ at the intracellular side of the membrane. 110 mM Na+ was present in the extracellular medium. The reversal potential for Li^+ was 9.5 mV. (*B*) Current recordings in the presence of 110 mM Na^+, formamidinium, guanidinium, aminoguanidinium, ammonium, methylammonium and dimethylammonium at the intracellular side of the membrane. 110 mM Na^+ was present in the extracellular medium. Holding voltage 0 mV and voltage commands from −100 to 100 mV in steps of 20 mV. All data obtained in the presence of 500 μM cGMP in the intracellular medium.

TABLE 1 Permeability ratios (P_x/P_{Na}) of mutant channels P365T and P365A

Cation	P365T V_{rev} (P_x/P_{Na}) n (mV)	P365A V_{rev} (P_x/P_{Na}) n (mV)	cGMP P_x/P_{Na}	Na^+ P_x/P_{Na}
Ammonium	−6 ± 2 (1.2) 3	−16 ± 3.5 (1.8) 4	2.8	0.16
Guanidinium	8 ± 2.5 (0.74) 3	−6 ± 2 (1.2) 3	1.12	0.13
Formamidinium	8 ± 3.3 (0.74) 3	−2 ± 0.8 (1.08) 4	1	0.14
Aminoguanidinium	20 ± 4.5 (0.46) 4	10 ± 2.7 (0.68) 3	0.63	0.06
Methylammonium	22 ± 5.3 (0.4) 4	13 ± 2 (0.6) 3	0.6	<0.007
Dimethylammonium	50 ± 12 (0.14) 3	45 ± 5 (0.18) 2	0.14	<0.007
Li^+	16 ± 4 (0.54) 5	9 ± 3.5 (0.7) 5	1.14	0.93
Na^+	1	1	1	1
K^+	n.d.	n.d.	0.98	0.086
Rb^+	n.d.	n.d.	0.84	<0.012
Cs^+	n.d.	n.d.	0.54	<0.0013

First and second columns reproduce the value of the reversal potential V_{rev} and of the permeability ratio (in parentheses) computed from Eq. 1. V_{rev} was measured under bi-ionic conditions with 110 mM Na^+ in the extracellular medium and 110 mM cation X in the intracellular medium. n is the number of different patches. Third and fourth columns report the permeability ratios of the native cGMP channel from tiger salamander rods (Menini, 1990; Picco and Menini, 1993) and of the Na^+ channel (Hille, 1992). n.d., not determined.

Na^+ and K^+ ions have almost the same permeability and single channel conductance through w.t. CNG channels (Kaupp et al., 1989; Nizzari et al., 1993). Therefore differences in their permeation properties are likely to be small and visible only under special conditions, such as at large membrane voltages.

Membrane patches were excised from an oocyte expressing w.t. CNG channels and the current activated by cGMP was studied under voltage clamp conditions. When the extracellular medium contained 110 mM NaCl and at the voltage commands shown at the top of panels *A* and *B*, the current recordings shown in Fig. 9 were obtained. In the presence of Na^+ on both sides of the membrane, currents recorded when the voltage command was turned to −180 mV were flat both in the presence of 100 (*A*) and 500 μM (*C*) cGMP. When K^+ replaced Na^+ in the intracellular medium, the steady state inward current carried by Na^+ at −180 mV was identical, but it was preceded by a clear transient current, which was larger in the presence of 100 (*B*) than of 500 μM (*D*) cGMP. This current transient had a time constant of about 4 ms. This transient current was not observed when 10 μM Ni^{2+} was added to the extracellular medium, similarly to what observed in mutant P365T (*B, inset*).

In the presence of 100 μM cGMP, the time constant of the current activation when the voltage command was stepped from 0 to + 150 mV was 0.8 ± 0.2 ms in the presence of intracellular Na^+, but became 1.9 ± 0.9 ms when Na^+ was replaced by K^+ (Fig. 9 *E*). At the larger voltage command of +180 mV the time constant of current activation was 1.4 ± 0.4 and 3.0 ± 0.8 ms in the presence of intracellular Na^+ and K^+, respectively (Fig. 9 *F*). In the presence of intracellular K^+, when the voltage command was stepped from +180 to −180 mV (Fig. 9 *B*), the current quickly reached a peak level, which subsequently declined to its steady state value with a time constant of about 4 ms. No slow relaxation was observed in the presence of intracellular Na^+, as shown by the black trace in the inset of panel B. These results, which were repeated in five different patches, show that intracellular K^+ causes a small but statistically significant slowing down of the channel gating.

The value of I_{peak}/I_{ss} at −180 mV varied in different patches, but for voltage prepulses V_{ireo} higher than +50 mV it was always bigger in the presence of 100 μM cGMP than of 500 μM cGMP. The ratio I_{peak}/I_{ss} in the presence of intracellular Na^+ was plotted as a function of the ratio obtained with intracellular K^+ for five different patches (panel *F*). It is evident that the ratio I_{peak}/I_{ss} is always higher in the presence of intracellular K^+ than Na^+.

When the extracellular medium contained 110 mM KCl and the voltage protocol used in Fig. 9 had opposite polarities, current recordings shown in Fig. 10 were obtained.

The inward current carried by K^+ did not show any significant current transient when the voltage was stepped to negative voltages, either in the presence of Na^+ or K^+ in the intracellular medium, whatever cGMP concentration was used (100 or 500 μM). Contrary to what observed in the presence of Na^+ in the extracellular medium (Fig. 9), the outward current carried by Na^+ at +180 mV decayed with a time constant of about 11 ms both in the presence of 100 (*A*) and 500 μM (*C*) cGMP. The peak of the outward current at +180 mV depended on V_{pre} and was larger for very negative prepulses, i.e., when K^+ ions enter the channel. The outward current carried by K^+ at +180 mV had a different kinetics than that carried by Na^+. Indeed its peak was smaller but its steady state was larger, leading to a clear crossing of the current traces carried by Na^+ and K^+. The dependence of the ratio $(I_{peak} - I_{ss})/I_{peak}$ at +180 mV on voltage prepulses in the presence of Na^+ and K^+ is shown in Fig. 10, *E* and *F,* respectively. It is evident that the ratio $(I_{peak} - I_{ss})/I_{peak}$ was consistently larger in the presence of intracellular Na^+. The results shown in Figs. 9 and 10 show that the permeation of Na^+ and K^+ through the w.t. CNG

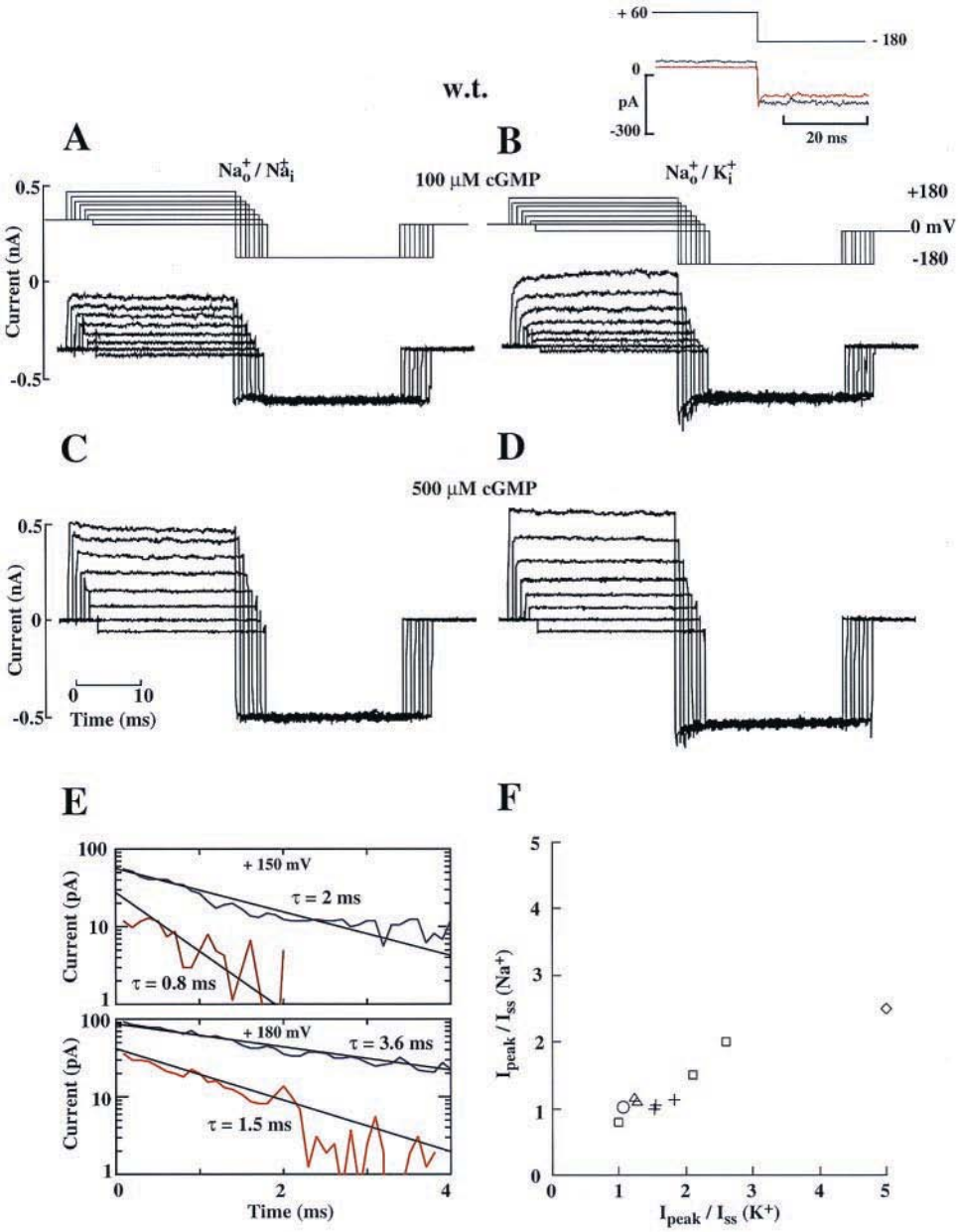

FIGURE 9 Transient K^+-dependent potentiation in w.t. channel in the presence of Na^+ in the extracellular medium. Current recordings obtained with the voltage protocol shown in the upper part of the panel in the presence of 100 (*A* and *B*) and 500 μM (*C* and *D*) and 110 mM NaCl (*A* and *C*) and KCl (*B* and *D*) in the intracellular medium. 110 mM NaCl in the extracellular medium. The *inset* in *B* shows the current relaxation to the steady level when the voltage command was stepped from 60 to -180 mV in the presence of 100 μM cGMP in the absence (*red line*) and in the presence (*black line*) of 10 μM Ni^{2+}. (*E*) Time constants of current activation at $+150$ (*upper panel*) and $+180$ (*lower panel*) mV, respectively, in the presence of intracellular Na^+ (*red lines*) and K^+ (*blue lines*). (*F*) I_{peak}/I_{ss} at -180 mV in the presence of Na^+ versus I_{peak}/I_{ss} at -180 mV in the presence of K^+ in the intracellular medium. 110 mM NaCl in the extracellular medium. Data from 5 different patches and in the presence of different cGMP concentrations.

channel is not identical and that intracellular K^+ makes the channel gating slightly slower.

DISCUSSION

Na^+ and K^+ ions have different hydration properties, but in CNG channels they have a permeability ratio P_{Na}/P_K close to 1 (Kaupp et al., 1989) and carry almost the same macroscopic and single channel current (Nizzari et al., 1993). Therefore these two ions are thought to permeate through CNG channels with identical properties. This manuscript, however, shows that in w.t. CNG channels, the permeation of Na^+ and K^+ ions is not entirely identical.

This manuscript also describes unexpected properties of CNG channels observed changing the proline in position 365 of the α-subunit of the bovine rods into a threonine, an

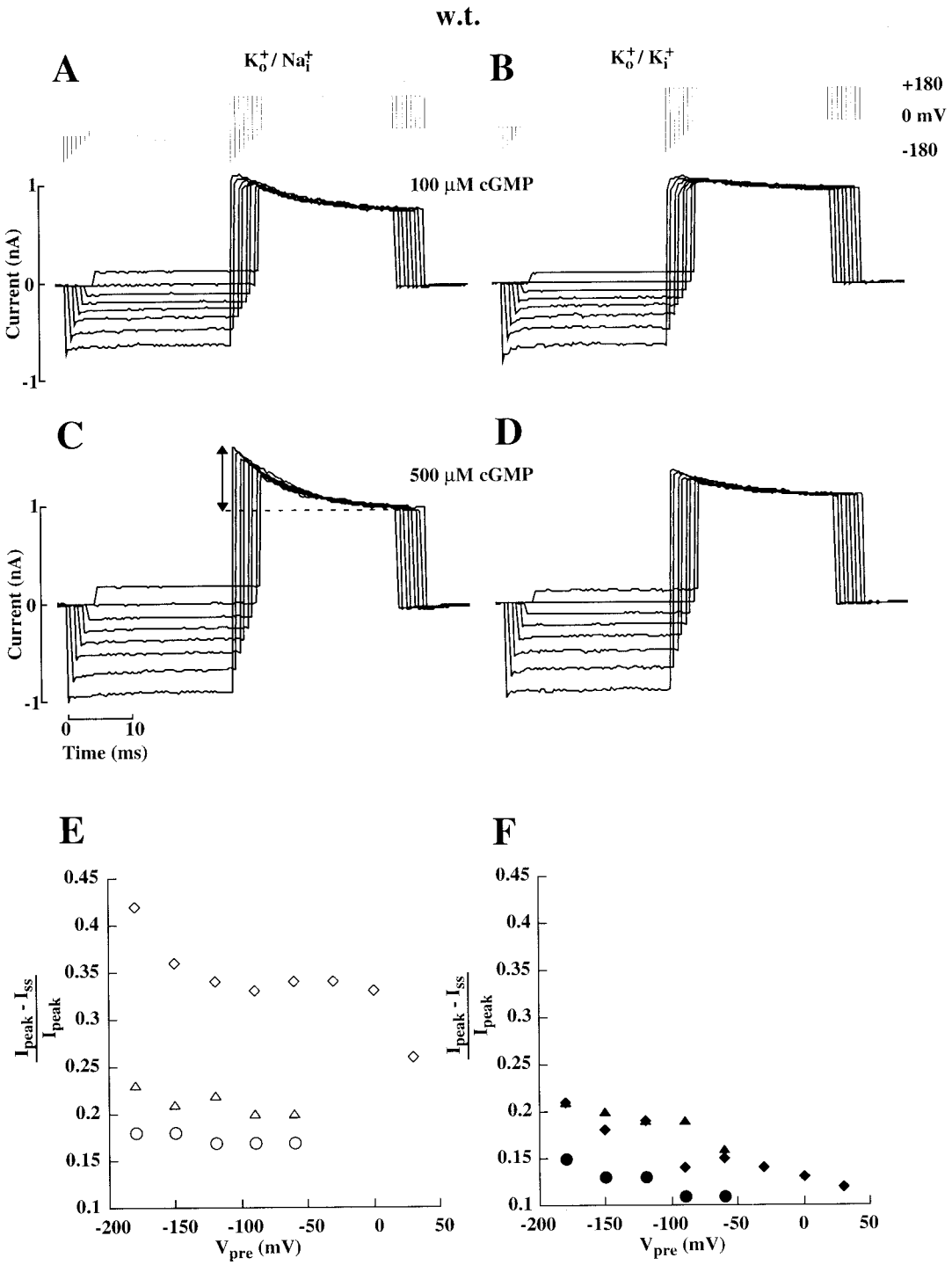

FIGURE 10 Na^+ and K^+ interaction in the w.t. CNG channel. Current recordings obtained with the voltage protocol shown in the upper part of the panel in the presence of 100 (*A* and *B*) and 500 μM (*C* and *D*) and 110 mM NaCl (*A* and *C*) and KCl (*B* and *D*) in the intracellular medium. 110 mM KCl in the extracellular medium. *E* and *F*) Dependence of the ratio $(I_{peak} - I_{ss})/I_{peak}$ at +180 mV on voltage prepulse V_{pre} in the presence of 500 μM cGMP with Na^+ (*E*) and K^+ (*F*) in the intracellular medium. 110 mM KCl in the extracellular medium. Data from 3 different patches.

alanine or a cysteine. In mutant channels P365T, P365C and P365A, K^+ ions powerfully block the channel. In these mutant channels and in the presence of Na^+ and K^+ on opposite sides of the membrane a transient K^+ dependent current was observed. This transient current is caused by a transient potentiation of the channel, i.e., a transient in-

crease of the open probability. In these mutant channels there is a profound blockage of the channel while a K^+ ion is occupying the pore and a potentiation of gating immediately after the K^+ ion is driven out. Potentiation occurs because K^+ ions slow down the rate constant K_{off} controlling channel closure. The behavior observed in these mutants is likely to be an exaggerated version of a complex interaction between Na^+ and K^+ present in the w.t. channel as well.

These results show that: the gating of the Na^+ current is significantly affected by the presence of K^+; permeation and gating in CNG channels are coupled; and Na^+ and K^+ ions permeate differently through the pore of CNG channels.

The transient current is a transient potentiation

The large transient current described in this manuscript is attributed to a transient potentiation, i.e., to a transient increase of the open probability of mutant channels P365T, for two reasons. Firstly, the cGMP concentration evoking half of maximal transient current is about 50 μM (Fig. 7 *C*), while about 200 μM cGMP is necessary to evoke half of the maximal current at the steady state (Fig. 7). Secondly, as shown in Fig. 5, when the channel opening was potentiated by the addition of Ni^{2+} (Gordon and Zagotta, 1995a), the ratio I_{peak}/I_{ss} became close to 1, thus showing that when CNG channels are mostly in the open state, the relative amplitude of transient current is significantly reduced. These observations strongly argue that the origin of transient current is a transient potentiation of CNG channels caused by the presence of K^+ ions inside the channel pore.

Several kinetics schemes have been proposed to describe the channel opening and allosteric models have been considered (Gordon and Zagotta, 1995a; Liu et al., 1996, 1998; Li et al., 1997; Ruiz and Karpen, 1999; Varnum and Zagotta, 1996, 1997). In allosteric models it is assumed that the liganded state and the open state are in a thermodynamic equilibrium with an equilibrium constant $L = K_{on}/K_{off}$ (Li et al., 1997; Sunderman and Zagotta, 1999; Tibbs et al., 1997), which controls the maximal open probability p_{max} and the relation $p_{max} = L/(L + 1)$ holds.

The experiments here described suggest that in mutant P365T intracellular K^+ modulates the equilibrium constant L of the allosteric transition to the open state, therefore coupling gating and permeation. This transient potentiation associated to an increase of the allosteric equilibrium constant L, can be caused by an increase of K_{on} and/or a decrease of K_{off}. As the time constant of the channel gating, in the presence of saturating cGMP concentrations, is inversely proportional to the sum of K_{on} and K_{off} (Karpen et al., 1988), the slowing down of the channel gating shown in Fig. 9 suggests a decrease of K_{off} in the presence of K^+. As a consequence the transient potentiation is likely to be caused by a transient decrease of K_{off}.

Blockage and permeation of K^+ ions

The analysis of current fluctuations in patches containing few mutant channels (Fig. 4) and of macroscopic currents (Figs. 2 and 5) indicates that K^+ ions block the single channel current carried by Na^+ ions in a voltage dependent way. Indeed the strong blockage observed at -40 mV was almost abolished at membrane voltages more negative than -100 mV. As shown in Fig. 4 brief transient outward currents carried by K^+ ions were observed at membrane potentials larger than 100 mV. An estimate of their single channel conductance, based on noise analysis, indicates a value of about 16 pS, similar to that measured with Na^+ ions (Fig. 3). Therefore, at $+100$ mV Na^+ and K^+ ions appear to have almost the same single channel conductance, but with a different gating: in mutants P365T the open probability is very low while a K^+ ion is occupying the pore.

Ionic selectivity and permeation in mutants P365T, P365C, and P365A

The w.t. CNG channel is poorly selective among monovalent alkali cations (Kaupp et al., 1989; Menini, 1990), but mutant channels P365T, P365A, and P365C are significantly permeable only to Na^+ and Li^+. When large cations such as K^+, Rb^+ and Cs^+ were present in the intracellular medium no macroscopic outward current was observed and no appreciable inward current was observed at 0 mV, indicating that these cations blocked the permeation of Na^+. Therefore these ions m to reach the inner core of the channel but then they remain trapped in the pore and block it (Fig. 4).

Similarly to w.t. CNG channels and, at some extent, also to Na^+ channels, mutant channels P365T and P365A are appreciably permeable to large organic cations, such as aminoguanidinium, which can permeate through a pore with a dimension of at least 3.8×5 Å. Therefore the diameter of the inner pore of these mutant channels is not very narrow and the blockage by K^+ ions requires specific molecular mechanisms. Contrary to Na^+ channels, but similarly to the w.t. CNG channel (Picco and Menini, 1993; Goulding et al., 1993; Sesti et al., 1996), mutant channels P365T and P365A are significantly permeable to methyl compounds such as methylammonium and dimethylammonium (Table 1). These results indicate that the molecular mechanisms controlling the permeation of monovalent alkali and organic cations are distinct.

Na^+ and K^+ interactions in the w.t. CNG channel

As shown in Fig. 9 a transient current is also observed in the w.t. CNG channel. This current transient is abolished by the addition of Ni^{2+} in the extracellular medium, as in mutant P365T. This transient current is much smaller than the one observed in mutant P365T and at -180 mV the ratio $I_{peak}/$

I_{ss} is about 1.6 and 1.3 in the wild type in the presence of 100 and 500 μM, respectively. The channel gating is slightly but significantly slowed down in the w.t. channel when intracellular Na^+ is replaced with K^+. This effect was best *n* at very large positive membrane potentials (Fig. 9 *E*) where the time constant of the current activation increased from about 1–2 to about 3–4 ms. When the voltage command was quickly turned from +180 to −180 mV in the presence of intracellular K^+ a transient current appeared which decayed with a similar time constant of about 4 ms (Fig. 9).

In the w.t. channel the inward current carried by extracellular Na^+ is influenced by the presence of K^+ (Fig. 9), but the inward current carried by extracellular K^+ is not significantly different in the presence of Na^+ or K^+ in the intracellular medium (Fig. 10). Thus the interaction between Na^+ and K^+ in the w.t. channel is asymmetric: K^+ ions influence the gating of the current carried by Na^+, but Na^+ ions do not significantly affect the current carried by K^+.

These results indicate that although the permeability ratio P_K/P_{Na} is close to 1 (Kaupp et al., 1989; Menini, 1990) and the single channel conductance of Na^+ and K^+ is almost identical in the wild type. CNG channel (Nizzari et al., 1993), Na^+ and K^+ ions do not permeate in the same way. This conclusion is not really surprising given the significant differences of their hydration thermodynamics (Hille, 1992; Laio and Torre, 1999). Therefore Na^+ and K^+ might have the same permeability and single channel conductance through CNG channels as the result of two distinct mechanisms: their different hydration thermodynamics and a complex interaction with the channel itself. This possibility is reminiscent of a recent theory of ionic selectivity (Laio and Torre, 1999) through CNG channels: in order to explain the low selectivity of CNG channels it is necessary to assume that the pore walls are flexible. In this view large alkali cations, such as K^+, Rb^+ and Cs^+, permeate only when the pore becomes wider, while small alkali cations, such Li^+ and Na^+, do not need a larger fluctuation for their permeation. The flexibility necessary to explain ionic selectivity may originate from the interaction of permeating ions with the channel pore.

The proline loop of CNG channels

The mutation substantially reducing the K^+ permeation and dramatically enhancing the transient current affects the first proline of a loop of three prolines located at positions 365 to 367 in the α-subunit of the bovine rod CNG channel. These three residues are localized in the extracellular vestibule (Becchetti et al., 1999) very close to the narrowest section of the CNG pore. The three prolines may be arranged in a polyproline II helix (Creighton, 1993; Adzhubei and Sternberg, 1993), leading to a rigid stick of 9.3 Å, which has few main chain hydrogen bonds with the rest of the protein (Adzhubei and Sternberg, 1993). This rigid stick is likely to confer unusual structural properties to the pore of CNG channels. The exact molecular mechanisms by which this proline loop acts and the molecular interactions between K^+ ions and the CNG channels will be better understood when the molecular structure of the inner pore of the CNG channel is determined.

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REFERENCES

Adzhubei, A. A., and M. J. E. Sternberg. 1993. Left-handed polyproline II helices commonly occur in globular protein. *J. Mol. Biol.* 229:472–493.

Becchetti, A., K. Gamel, and V. Torre. 1999. Cyclic nucleotide-gated channels: pore topology studied through the accessibility of reporter cysteines. *J. Gen. Physiol.* 114:377–392.

Biel, M., X. Zong, and F. Hofmann. 1995. Molecular diversity of cyclic nucleotide-gated cation channels. *Naunyn Schmiedebergs Arch. Pharmacol.* 353(1):1–10.

Catterall, W. A. 1994. Molecular properties of a superfamily of plasma-membrane cation channels. *Curr. Opin. Cell Biol.* 6:607–615.

Creighton, T. E. 1993. Proteins: Structures and Molecular Properties. New York, Freeman Company, Inc.

Doyle, D. A., J. M. Cabral, R. A. Pfuetzner, A. Kuo, J. M. Gulbis, S. L. Cohen, B. T. Chait, and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of the K^+ conduction and selectivity. *Science.* 280:69–77.

Eismann, E., F. Müller, S. Heinemann, and B. Kaupp. 1994. A single negative charge within the pore region of a cGMP-gated channel controls rectification, Ca^{2+} blockage and ionic selectivity. *Proc. Natl. Acad. Sci. U.S.A.* 91:1109–1113.

Finn, J. T., M. E. Grunwald, and K-W. Yau. 1996. Cyclic nucleotide-gated ion channels: an extended family with diverse functions. *Annu. Rev. Physiol.* 58:395–426.

Gordon, S. E., and W. N. Zagotta. 1995a. Localization of regions affecting an allosteric transition in cyclic nucleotide-activated channels. *Neuron.* 14:857–864.

Gordon, S. E., and W. N. Zagotta. 1995b. Subunit interactions in coordination of Ni^{2+} in cyclic nucleotide-gated channels. *Proc. Natl. Acad. Sci. USA.* 92:10222–10226.

Goulding, E. H., G. R. Tibbs, D. Liu, and S. A. Siegelbaum. 1993. Role of H5 domain in determining pore diameter and ion permeation through cyclic nucleotide-gated channels. *Nature.* 364:61–64.

Guy, H. R., and J. Durell. 1995. Structural models of Na^+, Ca^{2+}, and K^+ channels. *In* Ion Channels and Genetic Diseases. New York, Rockfeller University Press. 1–16.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100.

Heginbotham, L., T. Abramson, and R. MacKinnon. 1992. A functional connection between the pores of distantly related ion channels as revealed by mutant K^+ channels. *Science.* 258:1152–1155.

Hille, B. 1992. Ionic Channels of Excitable Membranes. Sunderlands, MA, Sinauer Associates.

Jan, L. Y., and Y. N. Jan. 1990. A superfamily of ion channels. *Nature.* 345: 672–675.

Jan, L. Y., and Y. N. Jan. 1992. Tracing the roots of ion channels. *Cell.* 69:715–718.

Karpen, J. W., A. L. Zimmerman, L. Stryer, and D. A. Baylor. 1988. Gating kinetics of the cyclic GMP-activated channel of retinal rods: flash photolysis and voltage-jump studies. *Proc. Natl. Acad. Sci. USA.* 85:1287–1291.

Kaupp, U. B., T. Niidome, T. Tanabe, S. Terada, W. Bönigk, W. Stühmer, N. Cook, K. Kangawa, H. Matsuo, T. Hirose, and S. Numa. 1989. Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. *Nature.* 342: 762–766.

Kaupp, U. B. 1995. Family of cyclic nucleotide-gated channels. *Curr. Opin. Neurobiol.* 14:434–442.

Laio, A., and V. Torre. 1999. Physical origin of selectivity in ionic channels of biological membranes. *Biophys. J.* 76:129–148.

Li, J., W. N. Zagotta, and H. A. Lester. 1997. Cyclic nucleotide-gated channels structural basis of ligand efficacy and allosteric modulation. *Q. Rev. Biophys.* 30:177–193.

Liu, D. T., G. R. Tibbs, and S. A. Siegelbaum. 1996. Subunit stoichiometry of cyclic nucleotide-gated channels and effects of subunit order on channel function. *Neuron.* 16:983–990.

Liu, D. T., G. R. Tibbs, P. Paoletti, and S. A. Siegelbaum. 1998. Constraining ligand-binding site stoichiometry suggests that a cyclic nucleotide-gated channel is composed of two functional dimers. *Neuron.* 21:235–248.

Menini, A. 1990. Currents carried by monovalent cations through cyclic GMP-activated channels in excised patches from salamander rods. *J. Physiol.* 424:167–185.

Menini, A. 1995. Cyclic nucleotide-gated channels in visual and olfactory transduction. *Biophys. Chem.* 55:185–196.

Nizzari, M., F. Sesti, M. T. Giraudo, C. Virginio, A. Cattaneo, and V. Torre. 1993. Single channel properties of a cloned channel activated by cGMP. *Proc. R. Soc. Lond.* 254:69–74.

Park, C. S., and R. MacKinnon. 1995. Divalent cation selectivity of an ion binding site in the pore of a cyclic nucleotide-gated channel. *Biochemistry.* 34:13328–13333.

Picco, C., and A. Menini. 1993. The permeability of the cGMP-activated channel to organic cations in retinal rods of the tiger salamander. *J. Physiol.* 460:741–758.

Root, M. J., and R. MacKinnon. 1993. Identification of an external divalent binding site in the pore of a cGMP-activated channel. *Neuron.* 11: 459–466.

Ruiz, M. L., and J. W. Karpen. 1999. Opening mechanism of a cyclic nucleotide-gated channel based on analysis of single channels locked in each liganded state. *J. Gen. Physiol.* 113:873–895.

Sesti, F., E. Eismann, U. B. Kaupp, M. Nizzari, and V. Torre. 1995. The multi-ion nature of the cGMP-gated channel from vertebrate rods. *J. Physiol.* 487:17–36.

Sesti, F., M. Nizzari, and V. Torre. 1996. Effect of changing temperature on the ionic permeation through the cyclic CMG-activated channel from vertebrate photoreceptors. *Biophys. J.* 70:2616–2639.

Sunderman, E. R., and W. N. Zagotta. 1999. Mechanisms of allosteric modulation of rod cyclic nucleotide-gated channels. *J. Gen. Physiol.* 113:601–619.

Tibbs, G. R., E. H. Goulding, and S. A. Siegelbaum. 1997. Allosteric activation and tuning of ligand efficacy in cyclic-nucleotide-gated channels. *Nature.* 386:612–615.

Varnum, M. D., and W. N. Zagotta. 1996. Subunit interaction in the activation of cyclic nucleotide-gated ion channels. *Biophys. J.* 70: 2667–2679.

Varnum, M. D., and W. N. Zagotta. 1997. Interdomain interactions underlying activation of cyclic nucleotide-gated channels. *Science.* 278: 110–113.

Zagotta, W. N. 1996. Molecular mechanisms of cyclic nucleotide-gated channels. *J. Bioenerg. Biomembr.* 28:269–278.

Zagotta, W. N., and S. A. Siegelbaum. 1996. Structure and function of cyclic nucleotide-gated channels. *Annu. Rev. Neurosci.* 19:235–263.

Zimmerman, A. L. 1995. Cyclic nucleotide-gated channels. *Curr. Opin. Neurobiol.* 5:296–303.