Apparent affinity of CFTR for ATP is increased by continuous kinase activity

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Abstract **The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel which is activated by protein phosphorylation and nucleoside triphosphates. We demonstrate here that fusion of the soluble catalytic subunit of cAMP-dependent protein kinase to the membrane protein bacteriorhodopsin yields a constitutively active protein kinase which activates CFTR effectively. As it is membrane-bound it is particularly useful for continuous perfusion of excised inside-out patches. We also tested the effect of a naturally membrane-bound protein kinase, cGMP-dependent protein kinase II, on CFTR. Both kinases, when continuously active, increase apparent affinity of CFTR to ATP about two-fold emphasizing the role of phosphorylation in modulating the interaction of ATP with the nucleotide binding domains.**
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Key words: Cystic fibrosis transmembrane conductance regulator; Phosphorylation site; Membrane-bound phosphatase; ATP dependence; Phosphatase

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR [1]) is an anion channel of low, linear conductance, found in epithelial, cardiac and other cells (for review see [2–4]). The gating of CFTR is tightly regulated by protein phosphorylation and nucleotide concentration (reviewed in e.g. [5–7]). CFTR exhibits two nucleotide binding sites and nucleoside triphosphates are necessary for CFTR channels to open. The published apparent affinities for ATP to open CFTR vary in a considerable range ($\sim$30 to $\sim$300 μM), depending on experimental conditions.

CFTR may be phosphorylated at 10 dibasic cAMP-dependent protein kinase catalytic subunit (PKA) consensus sites and at additional monobasic sites [8,9], with no indication for one essential site. The degree of phosphorylation is reflected in the extent of activity [10,11]. Previously it was suggested that the degree of phosphorylation also influences binding of ATP to CFTR, especially at nucleotide binding domain B [7,12]. For the ATPase activity of purified CFTR it was shown that phosphorylation by PKA enhances this activity and that this enhancement is related to a reduction in apparent K_m for ATP from 1 to 0.3 mM [13]. It was therefore speculated that phosphorylation increases the affinity of CFTR for ATP. Travis et al., however, in experiments with [α-^{32}P]8-N$_3$ATP observed no difference in ATP binding to CFTR under phosphorylating or non-phosphorylating conditions [14] which might underline the well-known difference between binding affinity and apparent affinities determined from the action of an enzyme.

To measure the influence of phosphorylation on interaction with ATP in a continuously superfused excised patch of membrane, taking into account the continuous activity of membrane-bound phosphatases, we decided to study the effect of continuous phosphorylation of CFTR by membrane-bound protein kinase. We show that a natural (cGMP-dependent) membrane-bound protein kinase and an engineered (constitutively active) membrane-bound catalytic subunit of cAMP-dependent protein kinase each lead to strong activation of CFTR in excised membrane patches. The presence of these kinases increases apparent affinity of CFTR for ATP when compared to CFTR only once exposed to kinase. The ATP concentration needed for half-maximal channel activity is decreased to about half its value by the continuous activity of a membrane-bound kinase.

2. Materials and methods

2.1. Chemicals
Buffer chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany), MgATP (equine muscle), cAMP, and cGMP were from Sigma, protein kinase inhibitor peptide was from Life Technologies, PKA catalytic subunit was from Promega (Madison, WI, USA). Tricaine was purchased from Sandoz (Basel, Switzerland) and collagenase A from Roche Molecular Pharmaceuticals (Karlsruhe, Germany).

2.2. RNA preparation and expression in Xenopus oocytes
Human CFTR cRNA was prepared as described in [15] using pSP64 vector. DNA for cGMP-dependent protein kinase II (cGK-II) was a kind gift from Dr. Suzanne Lohmann, Würzburg, Germany. DNA was linearized and cRNA was prepared as described previously. The plasmid with human catalytic subunit of cAMP-dependent protein kinase was a kind gift of Prof. Enrico Avvedimento, University of Naples, Italy. It was used as a template for a polymerase chain reaction with Pfu polymerase and primers introducing *Not*I at the beginning and *Hin*dIII after the stop signal. After digestion with *Not*I and *Hin*dIII it was subcloned into *Not*I- and *Hin*dIII-digested pGEMHE-bop, containing the gene encoding bacterioopsin [16], in this way removing the last few C-terminal amino acids of bacterio-

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rhodopsin (bR) and fusing PKA to bR (bR-PKA). RNA was transcribed in vitro (Ambion, Mmessage Mmachine) as previously for expression of bR [16]. Functional activity of the expressed bR-PKA was confirmed, with respect to bR, by measurement of light-activated proton pumping in oocytes. Female *Xenopus laevis* frogs were anesthetized in 0.2% Tricaine and remained on ice during surgery and parts of the ovary were removed. Oocytes were separated by 2–3 h digestion (18°C) in modified Ringer's solution (without Ca) containing 1–2 mg/ml collagenase A. After washing stage V–VI oocytes were selected for injection (Drummond Nanoject) with 15–25 ng of each RNA. The cells were incubated for 2–5 days at 15–19°C in modified Ringer's solution containing 9.5 mg/l penicillin and 10 mg/l streptomycin.

2.3. Solutions

For oocyte storage a modified Ringer's solution was used (in mM: NaCl 110; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; MOPS 5; pH 7.6 with NaOH; ca. 220 mosmol/l).

2.4. Patch-clamp experiments

For patch-clamp experiments the vitelline membrane of the oocytes was removed using watchmaker's forceps and cells were carefully placed in a small Petri dish (35 mm) filled with pipette solution to avoid contamination of the pipette. The dish was mounted on an inverted microscope (Zeiss Televal 31, Carl Zeiss, Jena, Germany). For detailed experimental set-up, see [17,18]. The micropipettes were prepared from borosilicate glass and heat-polished leading to a tip diameter of 16–22 µm. The pipette was attached to the plasma membrane of the oocyte and gentle suction (2–5 mm H_2O) was applied to accelerate seal formation (1–10 GΩ) which occurred after several minutes. Inside-out configuration was performed by pulling the pipette away from the cell membrane. The pipette was transferred to the recording chamber which was continuously perfused by the different bath solutions which contained the particular substrates. All experiments were performed at room temperature (20–23°C). The standard holding potential was 0 mV.

2.5. Patch-clamp solutions

To obtain considerable outward currents at 0 mV, a chloride concentration gradient of 4 mM/156 mM between bath (in mM: NMG 160; HEPES 10; EGTA 5; $MgCl_2$ 2; titrated to pH 7.4 with D,L-aspartate) and pipette (in mM: NMG 150; $MgCl_2$ 2; HEPES 10; pH 7.4 with HCl) was applied. As described in [17], the velocity of deactivating endogenous Ca-dependent Cl channels by removal of calcium from the bath was used to determine the speed of the solution exchange. Alternatively it was possible to calculate the time resolution by varying the chloride concentration of the referring bath solutions (aspartate was replaced by HCl, resulting in [Cl]$_{inside}$ of ca. 146 mM) and continuous ATP presence using CFTR itself. The observable spikes in Figs. 2C,D and 5A were the result of such temporary Cl jumps. The average time constant for solution exchange was between 50 and 350 ms.

2.6. Data acquisition and analysis

20 Hz low pass-filtered data (sampling rate 50 Hz) were acquired using a personal computer containing KAN1 software for continuous recording. A separate computer was used to control the command potential and to record currents induced by short voltage pulses using pClamp 6/8 software (Axon Instruments, filter: 100 Hz low pass; sampling frequency 250 Hz). Data analysis was performed by Microcal Origin 5/6 and ClampFit (Axon Instruments).

3. Results

When activating CFTR in excised inside-out membrane patches by addition of ATP and PKA it is immediately obvious that phosphatases counteract the phosphorylation by PKA. Fig. 1 shows the recording of electrical current from an excised giant patch of oocyte plasma membrane, expressing human CFTR. As basal cAMP concentration in non-stimulated oocytes is low, the degree of CFTR phosphorylation is low and therefore ATP-induced CFTR chloride current is marginal before application of soluble PKA. Application of
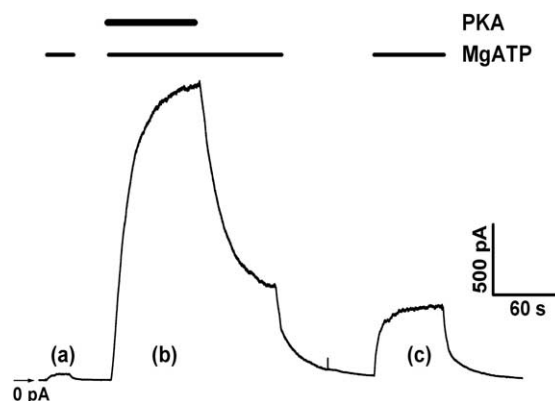

Fig. 1. CFTR is activated by soluble PKA catalytic subunit. Chloride currents evoked by ATP, before, during, and after PKA application in excised inside-out patches from *Xenopus* oocytes injected with CFTR RNA. Membrane potential = 0 mV; [Cl]$^-_{pipette}$ = 156 mM; [Cl]$^-_{bath}$ = 4 mM. Upper bars indicate superfusion with MgATP (500 µM) and PKA catalytic subunit (100 U/ml), respectively. a: Current induced by application of ATP alone preceding phosphorylation; b: addition of PKA; c: ATP-induced signal after removal of PKA. Arrow indicates zero current.

PKA with ATP, however, activates a large CFTR chloride current. Fig. 1 also shows that a large proportion of this chloride current decays quickly upon removal of PKA. The most likely explanation for this observation is a presence of membrane-bound phosphatases in oocyte plasma membrane which rapidly dephosphorylate CFTR to a partially phosphorylated CFTR chloride channel with lower open probability.

In order to test the effect of a higher, and more constant, phosphorylation level we set out to engineer membrane-bound PKA by fusing human cAMP-dependent protein kinase catalytic subunit to a membrane anchor. We chose the archaeal membrane protein bR, a light-activated proton pump. Previously we showed, by demonstration of light-activated electrical current, that bR can be expressed functionally in the plasma membrane of *X. laevis* oocytes [16,19]. Light-activated current was also observed in whole oocytes after injection of cRNA encoding bR-PKA (data not shown) indicating functional expression of bR. If bR-PKA was coexpressed with CFTR a large chloride conductance was measured in whole oocytes (not shown) indicating functional expression of constitutively active PKA. Surprisingly, oocytes with constitutively active PKA, and therefore continuously activated CFTR, survived well for several days so that no measures had to be taken to inhibit the PKA or the chloride conductance.

Fig. 2 shows recordings from patches coexpressing CFTR and bR-PKA. Large chloride currents can be activated without addition of soluble PKA, simply by application of MgATP. Fig. 2A demonstrates the larger proportion of pre-phosphorylated CFTR channels in a membrane patch, seen by ATP-induced current before protein phosphorylation. To reveal prephosphorylation, i.e. ATP-induced current before activation of bR-PKA in the excised patch, MgATP was added together with the inhibitor of PKA, PKI. Removal of PKI then leads to a steady increase of CFTR chloride current demonstrating the phosphorylating activity of the membrane-bound bR-PKA. Fig. 2B shows another patch with CFTR chloride conductance continuously increasing during addition

of ATP, only interrupted at a steady-state level during concomitant addition of PKI. Fig. 2C shows an example of a constant current in the presence of ATP, which might indicate that maximal phosphorylation of CFTR is reached or a steady state between phosphorylation by bR-PKA and dephosphorylation by phosphatases. The latter seems more appropriate as application of the PKA-specific inhibitor PKI together with ATP induces a fast current decline, resembling the current decline after withdrawal of soluble PKA in Fig. 1. Comparison of Fig. 2B and C reveals differences in density of membrane-bound phosphatases in oocyte membrane patches: in 2B an addition of PKI only interrupts the increase of current (i.e. increasing phosphorylation) whereas the same procedure resulted in a decrease of ATP-evoked chloride current in 2C, presumably due to dephosphorylation. Finally, in Fig. 2D we test the effect of soluble PKA on CFTR which is already phosphorylated by bR-PKA. A transient increase of current density of ∼30% is observed, which might indicate the phosphorylation of sites on CFTR by soluble PKA which are inaccessible to membrane-bound bR-PKA. These sites, however, are readily dephosphorylated after PKA washout which means they must be accessible to membrane-bound phosphatases.

The ATP dependence of CFTR chloride channels, phosphorylated by a transient treatment with soluble PKA, was previously determined to 84 ± 9 μM MgATP [17] which we confirmed again in this study (data not shown). When the ATP dependence of CFTR was measured in the presence of bR-PKA we obtained a K_m value of 41 ± 3 μM assuming a simple Michaelis–Menten function (Hill coefficient = 1; $n = 8$), see Fig. 3.

CFTR was shown to be stimulated by cGK-II [20]. This isoform is naturally membrane-bound, due to its N-terminal myristoylation [21], which seems to be necessary for activation of CFTR by cGMP-dependent kinase [22]. After coinjection of mRNA encoding CFTR and cGK-II, a kind gift of Dr. Lohmann, University of Würzburg, functional expression of cGK-II was confirmed by increased chloride conductance of oocytes (with the two-electrode voltage-clamp technique, data not shown) when applying the membrane-permeable dibutyr-
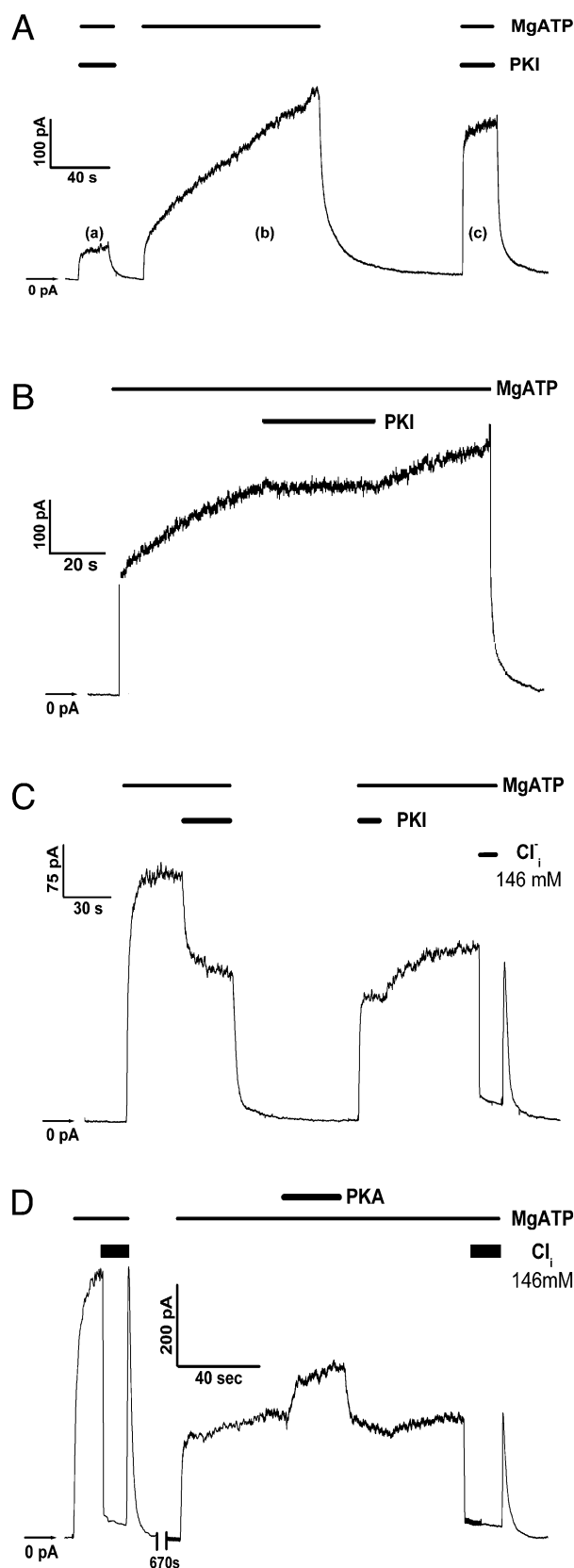
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Fig. 2. bR-PKA-mediated CFTR phosphorylation can be suppressed by kinase inhibitor PKI. A: Removing PKI produces a drastic (six-fold) increase in current. ATP-induced Cl currents prior to (a) and after (c) phosphorylation by bR-PKA (b) are shown. Trace was recorded directly after patch excision. [MgATP] = 500 μM; [Cl$^-_{pipette}$]/[Cl$^-_{bath}$] = 156/4 mM; $V_{holding}$ = 0 mV, [PKI] = 2 μM. B: Addition of PKI (2 μM) interrupts current increase. Same conditions as in A but after phosphorylation of the channels by perfusion with MgATP (500 μM) in absence of PKI. C: Example of current decline by exposure to PKI, reflecting variability of phosphatase activity in excised patches. PKI addition reveals fast phosphatase activity, causing decline of Cl current. The observed spike in the right part of the trace is due to fast changes in intracellular chloride concentration (from 4 mM to 146 mM [Cl]$_i$ and back, see Section 2) which were performed to determine the velocity of the solution exchange ($\tau \approx 80$ ms). Phosphatase-unrelated channel rundown is indicated by the difference in stationary currents before and long after PKI addition. D: Chloride currents evoked by perfusion with soluble PKA in patches already activated via bR-PKA. Left part of the trace represents initial CFTR activation by ATP via bR-PKA. Current rise induced by soluble PKA catalytic subunit (100 U/ml) is maintained only as long as PKA is present. Note break of 8 min at the beginning of the graph. [MgATP] = 500 μM.

yl-cGMP. Alternatively, CFTR was activated by cGMP after additional coexpression of the rat organic anion transporter rOAT1 [23] which permeabilized oocytes for cGMP.

Fig. 4A shows that in a patch where 500 μM MgATP alone

activates a current of about 20 pA, addition of 10 μM cGMP to the ATP solution increases the current to more than 1.5 nA. Phosphorylation by cGK-II seemed rather stable as not much rundown was observed upon a second activation of CFTR with only MgATP, however cGMP dissociates slowly, see below. It is again noticeable that current decay upon ATP removal is rather slow in the beginning ($\tau = 30$ s) whereas it becomes faster during the time course of the experiment, see Figs. 4B and 5A.

As in experiments with oocytes coexpressing bR-PKA we investigated the effect of soluble PKA catalytic subunit on patches which were already phosphorylated to a great extent by the membrane-bound kinase (now cGK-II). As shown in Fig. 4B, cGMP-induced CFTR activation led to chloride currents of more than 1 nA. Nevertheless, PKA addition enhanced this current more than two-fold. When comparing the ATP-induced chloride currents directly after phosphory-

Fig. 4. CFTR is activated by membrane-bound cGMP-dependent protein kinase II in inside-out patches. A: The stationary chloride current elicited by 500 μM MgATP (a) was 500 times smaller prior to the perfusion with cGK-II-activating cGMP (b) than afterwards (c). Cells injected with 18 ng cRNA, each of CFTR and cGK-II. [Cl$_{pipette}$]/[Cl$_{bath}$] = 156/4 mM; holding potential = 0 mV. B: Soluble PKA catalytic subunit increases CFTR current even after preceding phosphorylation with cGK-II. Same conditions as in A. Note the break of 20 min in the time scale.
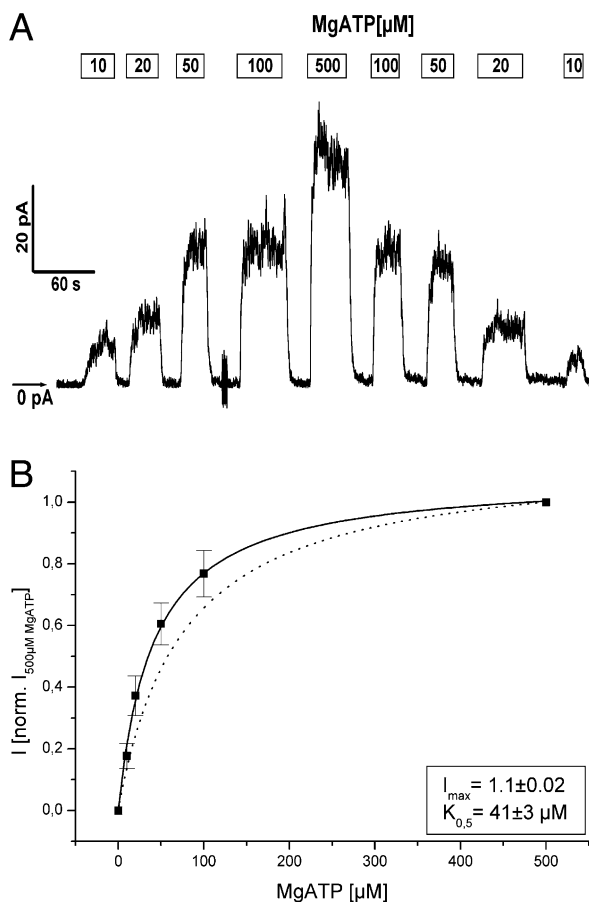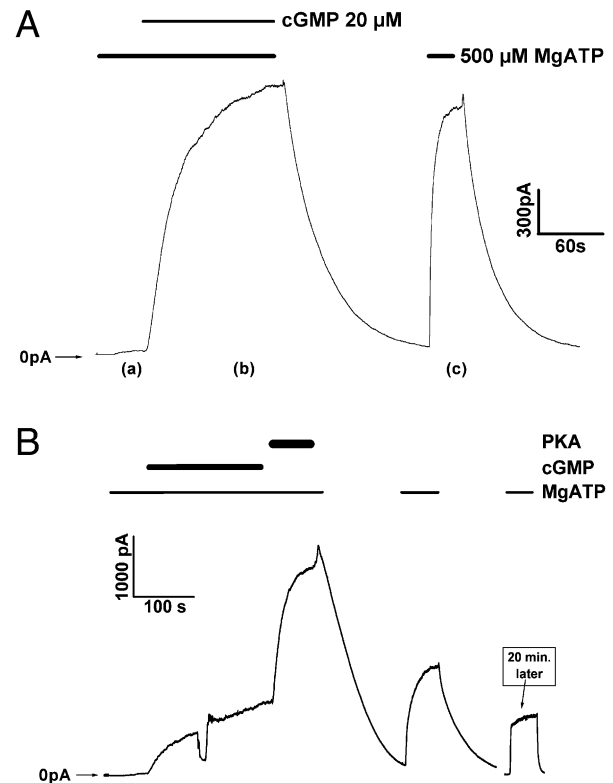
Fig. 3. Concentration dependence of ATP-induced chloride currents of bR-PKA-phosphorylated CFTR. A: Applications of MgATP to an inside-out patch coexpressing CFTR and bR-PKA leading to ATP concentration-dependent stationary chloride currents. [Cl$_{pipette}$]/[Cl$_{bath}$] = 156/4 mM; holding potential = 0 mV. Bars indicate applications of the different ATP concentrations. Rundown is marginal during this measurement. B: Fit of ATP-evoked currents to the Michaelis–Menten equation yields a K_M of 41 ± 3 μM. Data points represent means ± S.E.M. of eight independent patches. Dotted line indicates ATP dependence of CFTR channels transiently prephosphorylated by soluble PKA catalytic subunit ($K_M = 74$ μM, Hill coefficient = 1). The currents were normalized to the signal obtained during perfusion with 500 μM ATP. The K_M for ATP using oocytes that coexpress bR-PKA does not change noticeably within an experiment of duration up to 60 min.

lation and long after perfusion with cGMP, a significant difference in opening and closing times was observed, as previously described for CFTR activated by soluble PKA [17]. Fig. 4B also shows a slow rundown of ATP-induced current, in this case to 50% after 20 min.

We compared the apparent affinities of CFTR for ATP in cGK-II-coexpressing cells (Fig. 5) and determined the K_M both after stimulation of CFTR by a transient activation of cGK-II and during permanent activation of CFTR by cGK-II. The latter conditions were expected to maintain a high degree of CFTR phosphorylation. Measurements with significant rundown during acquisition were not considered, e.g. in Fig. 5A rundown is initially strong (to 60% in 200 s) but during ATP titration acceptable (to 88% in 400 s). Both dependences could be fitted best by a simple Michaelis–Menten function (Hill = 1) revealing K_M values of 64 ± 7 μM (transient perfusion with cGMP) and 28 ± 2 μM (continuous application of cGMP).

4. Discussion

The regulation of CFTR activity by phosphorylation and dephosphorylation is very complex and variable among different tissues or cell types. In this work we studied the effect of coexpressed membrane-bound protein kinases on CFTR in excised patches to obtain a better control over phosphory-
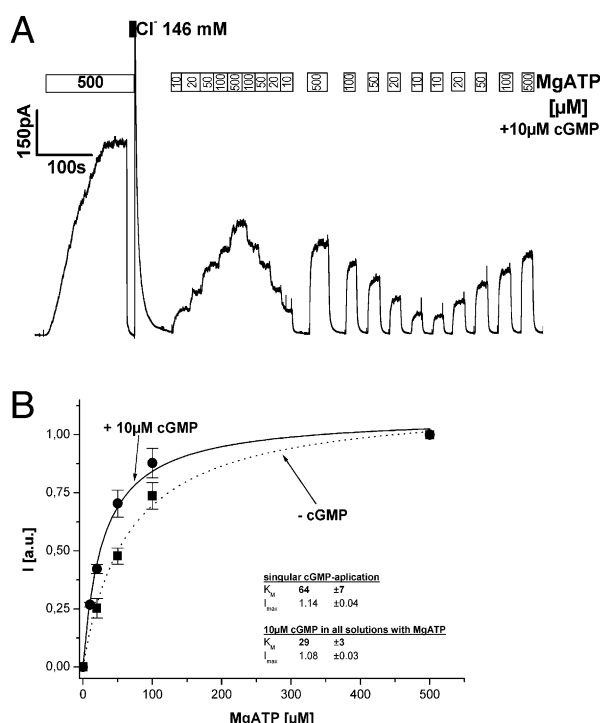
Fig. 5. ATP dependence of chloride currents is modulated by continuous activity of cGK-II. Comparison of apparent ATP affinity after transient and during continuous phosphorylation by cGK-II in oocytes injected with CFTR and cGK-II cRNA. A: Stationary chloride currents obtained using different ATP concentrations, with cGMP (10 μM) in all ATP solutions. There is little rundown during this experiment. B: Dotted curve: fit to the Michaelis–Menten equation of the ATP concentration of patches only initially exposed to cGMP reveals a K_M of 64 ± 7 μM (four independent experiments ± S.E.M.). If cGMP is present in all ATP solutions the K_M decreases to 28 ± 2 μM (straight line, 16 measurements, eight patches). The curve can still be fitted best by a simple Michaelis–Menten function. Currents are normalized to current at 500 μM MgATP.

lation conditions. In an attempt to prevent the rundown of CFTR activity, we first tested the ATP analogue ATPγS together with PKA catalytic subunit to obtain stable phosphorylation (data not shown). In contrast to previous publications [31], under our experimental conditions the loss of signal upon PKA withdrawal was not significantly changed compared to phosphorylation with MgATP. Therefore we did not use ATPγS for further studies of the influence of phosphorylation on CFTR.

Previously it was shown that the myristoylated cGK-II is an effective activator of CFTR [20] and from biochemical experiments it was deduced that the phosphorylation kinetics of cGK-II is slower compared to PKA [24]. So far, a functional comparison of CFTR phosphorylation by PKA and cGK-II was lacking. We show that activation by cGK-II is at least as effective as activation by PKA, as judged from the effect of continuously active cGK-II on apparent affinity of CFTR to ATP in excised inside-out patches from *Xenopus* oocytes.

Moreover, we engineered an artificial membrane-bound protein kinase by fusion of the membrane protein bR with the catalytic subunit of PKA and obtained an active protein which is constitutively active in the presence of ATP providing a novel approach to phosphorylate CFTR.

Phosphorylation was recognized early on as a prerequisite for activation of CFTR by nucleotides [25–27]. Previously it

was already suggested that the degree of phosphorylation modulates interaction of CFTR with nucleotides [12,28,29]. We demonstrate, by comparing the apparent ATP affinities of stationary CFTR chloride currents for different degrees of phosphorylation, that an enhancement of the level of phosphorylation can be achieved by continuously active, membrane-bound protein kinases and that this leads to an increased apparent affinity for ATP. Interestingly, in contrast to ATPase measurements on CFTR [13], no sigmoidal ATP dependence was observed, either after transient kinase addition or with continuous kinase activity. This difference might reflect a loose coupling between ATPase activity and channel opening.

One might argue that the obtained effects on K_M for ATP are influenced by the K_M for ATP of the respective kinase and not only by CFTR itself. The K_M for ATP is 75 μM when CFTR is transiently perfused with high concentrations of PKA catalytic subunit and 64 μM when transiently activated by 500 μM ATP, cGK-II, and 10 μM cGMP. These values represent CFTR-specific properties. The observed decrease of the K_M for ATP when using membrane-bound kinases cannot be due to ATP-limited kinase activity. But the values determined could reflect upper limits of the real apparent ATP affinity of CFTR. Can we exclude that under saturating kinase activity an even lower K_M for ATP of CFTR would be obtained? A lower K_M for ATP of fully phosphorylated CFTR is unlikely in the case of bR-PKA. Assuming that the K_M of bR-PKA (3–10 μM for PKA catalytic subunit, see [28–30]) is not influenced significantly by the fusion to bR then its K_M for ATP is much lower than the K_M of CFTR (41 μM) obtained in experiments with bR-PKA. In contrast to PKA, the K_M for ATP of the cGK-II in the presence of high concentrations (10 μM) of cGMP seems to be much higher (400 μM) [20]. Therefore it is likely that indeed we titrated not only CFTR but also cGK-II activity with ATP. Interestingly, the K_M value obtained with phosphorylation by cGK-II (28 μM) was even lower than that obtained with phosphorylation by bR-PKA (41 μM). It is possible that with saturating cGMP-dependent kinase II activity an even lower K_M for ATP of CFTR would be obtained.

Activation of CFTR is poorly understood, in part because opening of CFTR seems variable and to vary with time. One of the reasons to study the effect of continuous phosphorylation by membrane-bound kinases was the aim to study kinetic constants of CFTR under stable conditions, i.e. to counteract the rundown of CFTR activity in excised membrane patches. This rundown was previously attributed to endogenous phosphatases [17,27]. Rundown may be classified into a slow rundown, occurring over minutes, and a fast rundown, as visible immediately after withdrawal of soluble PKA, see Fig. 1, or after addition of PKI, see Fig. 2C. As demonstrated in Fig. 2C, fast rundown may be reversed by renewed kinase activity and membrane-bound kinases are an economical tool to achieve this. The effects of membrane-bound kinases let us suggest that they may provide continuous phosphorylation at a continuously superfused excised membrane patch. Our experiments also show, however, that dephosphorylation is only one factor causing rundown of channel activity as slow rundown still exists, even with continuously phosphorylated CFTR, see Fig. 2C (where removal of PKI only restores 70% of the initial signal after ∼3 min), Fig. 3A (where the current induced by 10 μM ATP runs down in 8 min to 50%),

or Fig. 5A (where the current induced by 500 μM ATP runs down in 13 min to 40%). Other, as yet unidentified, factors must therefore contribute to CFTR rundown in excised patches. Continuous phosphorylation by membrane-bound kinases, on the other hand, had a clear effect on CFTR as demonstrated by the increased apparent affinity for ATP.

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