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Functional Interaction Between CFTR and Cx45 Gap Junction Channels Expressed in Oocytes

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Abstract. The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride (Cl^-) channel known to influence the function of other channels, including connexin channels. To further study potential functional interactions between CFTR and gap junction channels, we have co-expressed CFTR and connexin45 (Cx45) in *Xenopus* oocytes and monitored junctional conductance and voltage sensitivity by dual voltage clamp electrophysiology. In single oocytes expressing CFTR, an increase in cAMP caused by forskolin application induced a Cl^- current and increased membrane conductance; application of diphenylamine carboxylic acid (CFTR blocker) readily blocked the Cl^- current. With co-expression of CFTR and Cx45, application of forskolin to paired oocytes induced a typical outward current and increased junctional conductance (G_j). In addition, the presence of CFTR reduced the transjunctional voltage sensitivity of Cx45 channels without affecting the kinetics of junctional current inactivation. The drop in voltage sensitivity was further enhanced by forskolin application. The data indicate that CFTR influences cell-to-cell coupling mediated by Cx45 channels.

Key words: Chloride channels — CFTR — Cell communication — Connexins — Gap junctions — Channel gating — *Xenopus* oocytes

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a low-conductance chloride (Cl^-) channel involved in fluid transport across the luminal membrane of several epithelial cells (Sheppard & Welsh, 1999). Mutations in the CFTR gene cause cystic fibrosis, an autosomal recessive disease characterized by defective regulation of CFTR Cl^- channels by adenosine 3′,5′-cyclic monophosphate (cAMP). In normal subjects, CFTR phosphorylation by cAMP-dependent protein kinases activates CFTR mediated Cl^- transport by increasing the CFTR affinity for ATP (Gadsby & Nairn, 1999). In cystic fibrosis patients, absence of CFTR activation due to CFTR mutations impairs the normal function of absorbing and secreting epithelia, resulting in multiple organ failure (Greger et al., 2001).

Interestingly, the mechanisms by which CFTR mutations result in cystic fibrosis may not depend entirely on altered Cl^- transport, as there is evidence that CFTR is capable of influencing the function of other ion channels and transporters (*reviewed* in Wang & Li, 2001). Recently, Chanson, Scerri & Suter (1999) have reported that in pancreatic duct cells, CFTR activation by cAMP increases the Cl^- current as well as the electrical conductance of gap junctions made of connexin45 (Cx45), suggesting that CFTR influences the function of connexin channels. This is quite significant because gap junctions are highly developed in tissues most severely affected by cystic fibrosis. Thus, understanding the relationship between intercellular communication and epithelial cell function may provide novel insights into the pathogenesis of cystic fibrosis.

For studying in more detail the functional interaction between CFTR and Cx45 channels, we have co-expressed CFTR and Cx45 in *Xenopus* oocytes, an expression system easily accessible to genetic manipulation, which has been shown to express well exogenous CFTR channels (Bear et al., 1991; Drumm et al., 1991; Cunningham et al., 1992). The data reported in this study indicate that activation of CFTR channels by cAMP induces a typical Cl^- outward

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current and simultaneously increases junctional conductance (G_j). In addition, the presence of CFTR reduces the transjunctional voltage (V_j) sensitivity of Cx45 channels — a phenomenon that is further enhanced by cAMP activation of CFTR.

Materials and Methods

Oocyte Preparation and Microinjection

Oocytes were prepared as previously described (Peracchia, Wang & Peracchia, 1996). Briefly, adult female *Xenopus laevis* frogs were anesthetized with 0.3% tricaine (MS-222) and the oocytes were surgically removed from the abdominal incision. The oocytes were placed in ND96 medium, containing (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.6 with NaOH). Oocytes at stages V or VI were subsequently defolliculated in 2 mg/ml collagenase (Sigma Chemical St. Louis, MO) for 80 min at room temperature in nominally Ca^{2+}-free OR2 solution, containing (in mM): NaCl 82.5, KCl 2, $MgCl_2$ 1, HEPES 5 (pH 7.6 with NaOH). The defolliculated oocytes were injected with 46 nl (0.25 μg/μl) of antisense oligonucleotide complementary to endogenous *Xenopus* Cx38: 5′-GCTTTAGTAATTCCCATCCTGCCATGTTTC-3′ (commencing at nt −5 of Cx38 cDNA sequence; Barrio et al., 1991), by means of a Drummond nanoject apparatus (Drummond, Broomall, PA). The antisense oligonucleotide blocks the endogenous junctional communication within 24 h. 24–72 h later 46 nl of human CFTR cRNA (∼0.2 μg/μl) were injected into oocytes at the vegetal pole. 6–24 h later the oocytes were reinjected with 46 nl of mouse Cx45 cRNA (∼0.4 μg/μl) and incubated overnight at 18°C. Control oocytes were injected with Cx45 cRNA only. The oocytes were mechanically stripped of their vitelline layer in hypertonic medium (Peracchia et al., 1996) and paired at the vegetal poles in conical wells of culture dishes (Falcon Products, Becton Dickinson Labware, Franklin Lakes, NJ) filled with ND96. All oocyte pairs were studied electrophysiologically 2–3 h after pairing. The human CFTR cDNA used in this study was provided by Dr. D.C. Devor (Department of Cell Biology and Physiology, University of Pittsburgh).

Measurement of Junctional Conductance

The oocyte chamber was continuously perfused at a flow rate of 0.6 ml/min by a peristaltic pump (Dyamax Mod. RP-1, Rainin Instrument, Woburn, MA). The superfusion solution was ejected by a 22 gauge needle placed near the edge of the conical well containing the oocyte pair. The level of the solution in the chamber was maintained constant by continuous suction. All of the experiments were performed using the standard double voltage-clamp procedure for measuring G_j (Spray, Harris & Bennett, 1981). Following the insertion of a current and a voltage microelectrode in each oocyte, both oocytes were individually clamped by two oocyte clamp amplifiers (OC-725C, Warner Instrument Corp., Hamden, CT) to the same holding potential, $V_{m1} = V_{m2}$ (usually −20 mV), so that no junctional current (I_j) would flow at rest ($I_j = 0$). For measuring junctional conductance (G_j) in the presence and absence of forskolin (20 μM), a V_j gradient was created by imposing a −40 mV voltage step (V_1) of 12 s duration at 30 s intervals to oocyte #1, while maintaining V_2 at V_m, thus, $V_j = V_1$. The negative feedback current (I_2), injected by the clamp amplifier in oocyte #2 for maintaining V_2 constant at V_m, was used for calculating G_j as it is identical in magnitude to the junctional current (I_j), but of opposite sign ($I_j = -I_2$); $G_j = I_j/V_j$. Pulse generation and data acquisition were performed by means of pCLAMP v. 8.2.0.232 software (Axon Instruments, Foster City, CA) and DigiData 1322A interface (Axon). Ij and Vj were measured with Clampfit (Axon) and the data were plotted with SigmaPlot (SPSS Inc., Chicago, IL). The time constant (Tau) of G_j decay and the ratio G_j steady-state ($G_{j\,ss}$) over G_j peak ($G_{j\,ss}/G_{j\,peak}$), in the presence and absence of forskolin, were calculated by fitting each I_j curve to a single exponential function, following baseline correction (Clampfit, Axon). $G_{j\,ss}$ was obtained from the exponential fit (parameter "C" of Clampfit, Axon).

For studying voltage dependence of G_j, a standard V_j protocol was used. Each oocyte was first voltage clamped at −20 mV. Voltage steps of −5 mV (80 mV maximum V_j) and 25 s duration were applied every 45 s to either oocyte of the pair, while maintaining the other at −20 mV. To illustrate the relationship between G_j steady-state and V_j, the ratio $G_{j\,ss}/G_{j\,max}$ was plotted with respect to V_j. The curve was fitted to a two-state Boltzmann distribution of the form: $(G_{j\,ss}-G_{j\,min})/(G_{j\,max}-G_{j\,ss}) = \exp[-A(V_j-V_0)]$, where V_0 is the V_j value at which G_j is one half the value of $G_{j\,max}-G_{j\,min}$, $G_{j\,max}$ is G_j at $V_j = 0$ mV and $G_{j\,min}$ is the theoretical minimum normalized G_j. $A = \eta q/kT$ is a constant expressing voltage sensitivity in terms of number of equivalent gating charges, η, moving through the entire applied field, where q is the electron charge, k is the Boltzmann constant and T is the temperature in degrees K.

Measurement of Membrane Conductance in Single Oocytes

Single oocytes injected 24 h earlier with CFTR cRNA were placed in conical wells of culture dishes (Falcon Products) and continuously supervised with ND96. Following the insertion of a current and a voltage microelectrode the oocytes were clamped to V_m = –60 mV. For measuring membrane conductance (G_m), voltage steps of +60 mV were applied at 30 s intervals in the presence and absence of forskolin (20 μM; Alomone Laboratories, Jerusalem, Israel; and Sigma, St. Louis, MO) and/or diphenylamine-2-carboxylate (DPC; 250 μM; Sigma), a Cl^- channel blocker (DiStefano et al., 1985). Both forskolin and DPC were diluted in dimethyl sulfoxide (DMSO) and added to the ND96 supervision solution just before use.

Results

Single Oocytes Expressing CFTR

CFTR activation was induced by application of forskolin, an adenylyl cyclase activator. In single oocytes voltage clamped at −60 mV and subjected to +60 mV square pulses of 12 s duration, application of 20 μM forskolin causes a significant and reversible increase in membrane conductance (G_m, Fig. 1*A* and *B*), which is not observed in oocytes not expressing CFTR (Fig. 1*B*). CFTR channels are characterized by their sensitivity to block by arylaminobenzoates such as diphenylamine-2-carboxylate (DPC), which block CFTR channels by a simple pore-block mechanism (Schultz et al., 1999; Zhang, Zeltwanger & McCarty, 2000; Reddy & Quinton, 2002). When forskolin is applied together with 250 μM DPC, the forskolin-induced increase in G_m is not observed (Fig. 1*C*). DPC also reverses the forskolin-induced increase in G_m (Fig. 1*D*). Application of DPC alone has no effect on G_m (Fig. 1*C*).

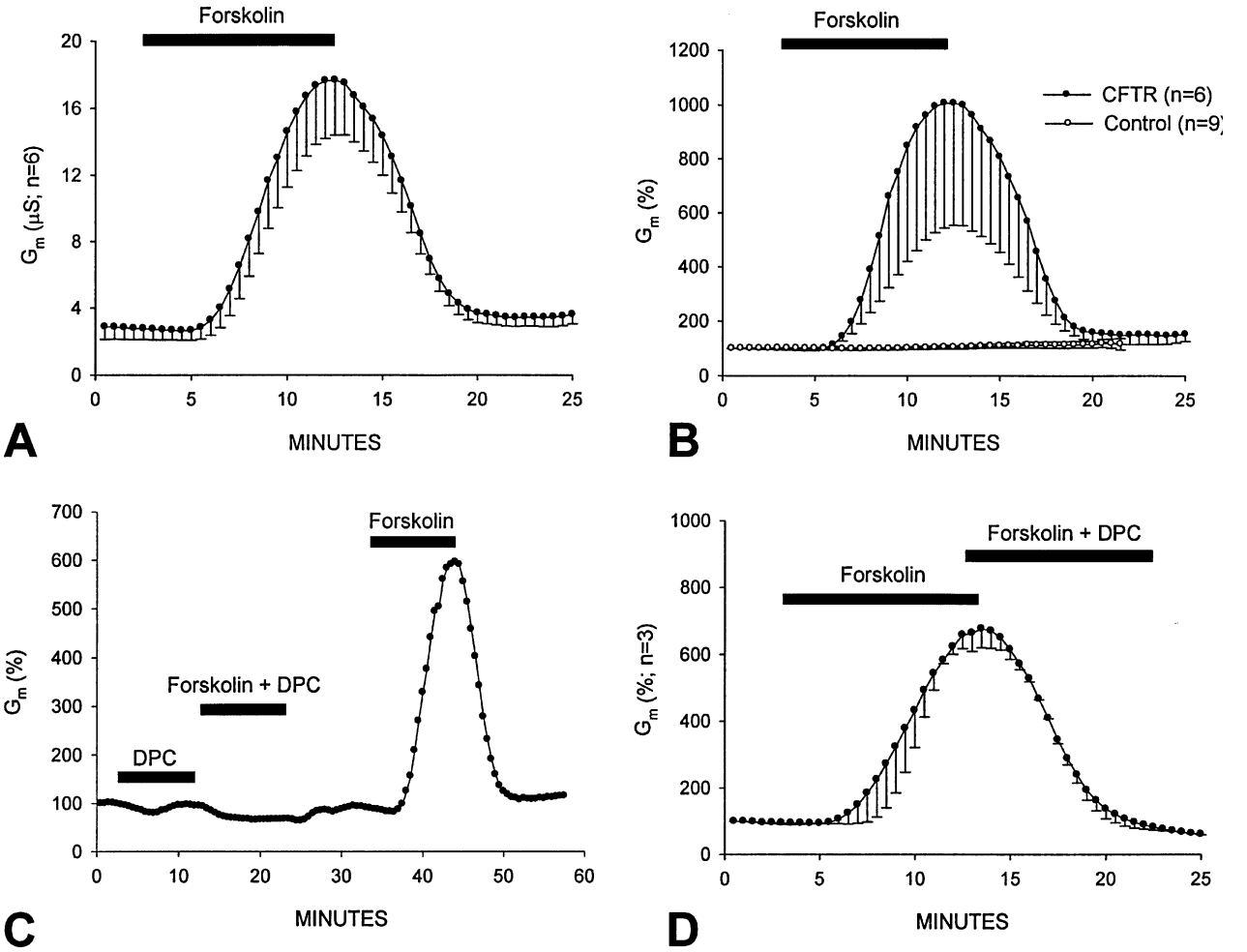

Fig. 1. Changes in membrane conductance (G_m) induced by forskolin application in single *Xenopus* oocytes expressing CFTR. The oocytes were voltage clamped at −60 mV and subjected to +60 mV square pulses of 12 s duration. Application of 20 μM forskolin causes a significant and reversible increase in membrane conductance (*A* and *B*), which does not occur in oocytes not expressing CFTR (*B*). The increase in G_m is not observed with application of forskolin in conjunction with 250 μM DPC (CFTR blocker; *C*). DPC is also able to reverse the forskolin-induced increase in G_m (*D*). DPC alone has no effect on G_m (*C*).

Transjunctional Voltage Sensitivity of Oocyte Pairs Expressing CFTR and Cx45

In oocyte pairs expressing CFTR and Cx45 the sensitivity of junctional conductance (G_j) to transjunctional voltage (V_j) was significantly lower than in controls (oocytes expressing Cx45 alone). V_j sensitivity was determined by applying a conventional V_j protocol: V_j steps of −5 mV, 80 mV maximum, 25 s duration, repeated every 45 s. A comparison of junctional current (I_j) records between CFTR and control oocytes is shown in Fig. 2 (*A* and *B*). Note that the extent of I_j inactivation is considerably reduced in CFTR-expressing oocytes. This is clearly demonstrated by plotting the ratio of steady state over maximum G_j ($G_{j\,ss}/G_{j\,max}$) versus V_j and the two-state Boltzmann distribution (Fig. 2*C*). The Boltzmann values are V_0 = 19.4 mV, $G_{j\,min}$ = 0.20, $G_{j\,max}$ = 1.26, η = 1.74 (n = 11), for CFTR-Cx45 channels, and V_0 = 9.7 mV, $G_{j\,min}$ = 0.08, $G_{j\,max}$ = 1.47, η = 2.8 (n = 4), for Cx45 channels alone.

Effect of Forskolin on Junctional Conductance and Voltage Sensitivity in Oocyte Pairs Expressing CFTR and Cx45

In oocyte pairs co-expressing CFTR and Cx45, application of 20 μM forskolin significantly increased G_j and decreased V_j sensitivity. This is clearly seen in low-speed chart records of currents elicited by the application of −40 mV square voltage pulses (12 s duration) to one oocyte of the pair (Fig. 3). The oocytes were individually voltage-clamped at V_m = −20 mV. Application of 20 μM forskolin results in a reversible increase in I_1 and I_j. The increase in I_1 reflects an increase in both membrane current (I_m) and junctional current (I_j), as $I_1 = I_m + I_j$. The increase in I_m is due to cAMP-induced activation of

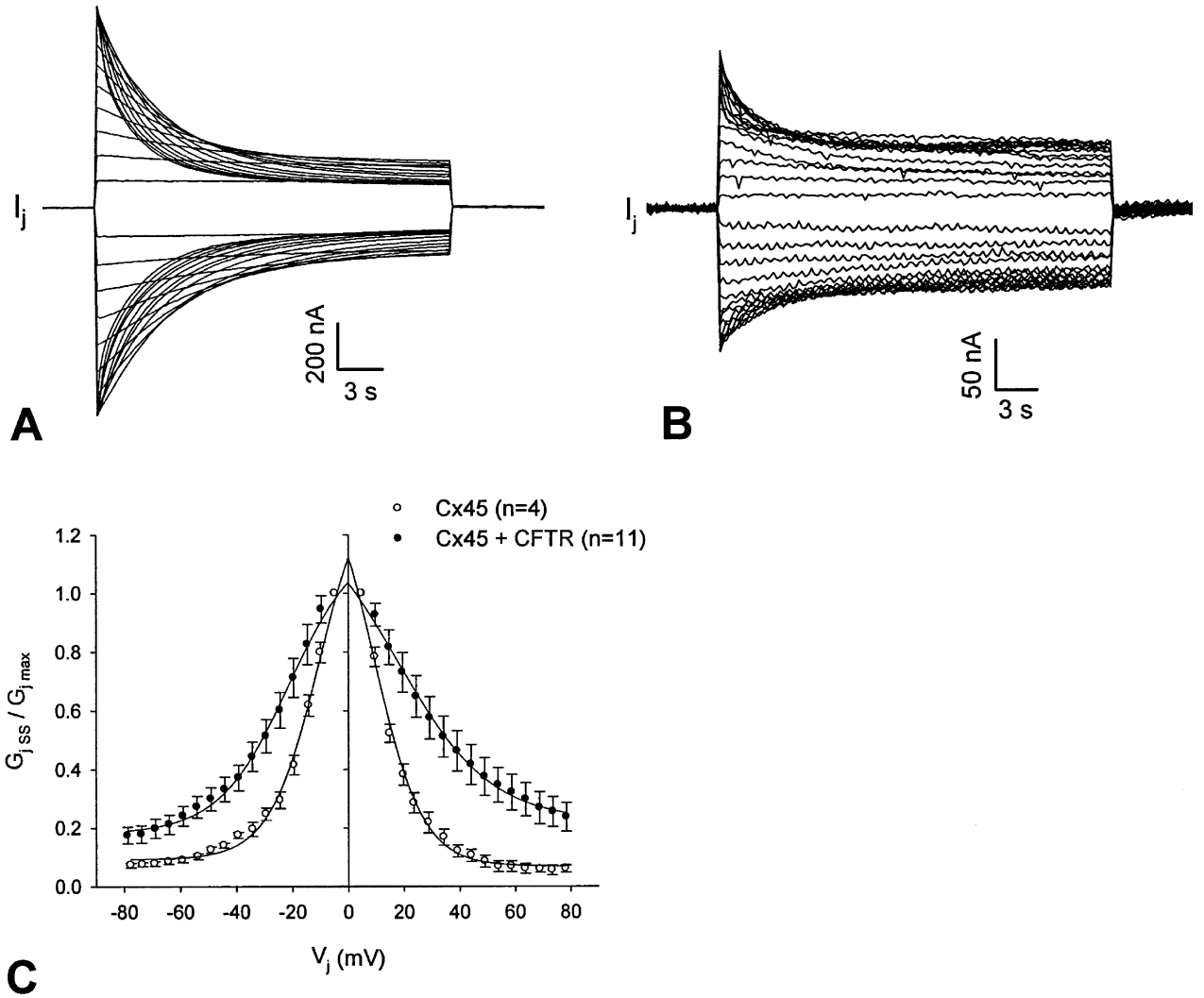

Fig. 2. Expression of CFTR decreases the transjunctional voltage (V_j) sensitivity of Cx45 channels. V_j sensitivity was determined by applying V_j steps of −5 mV (80 mV maximum) and 25 s duration, every 45 s. In the presence of CFTR, the extent of I_j inactivation is considerably reduced (*B*) with respect to controls (*A*). The difference is demonstrated by plotting $G_{j\,ss}/G_{j\,max}$ versus V_j (*C*), and the two-state Boltzmann distribution (*C*), whose values are: V_0 = 19.4 mV, $G_{j\,min}$ = 0.20, $G_{j\,max}$ = 1.26, η = 1.74 (n = 11), for CFTR-Cx45 channels, and V_0 = 9.7 mV, $G_{j\,min}$ = 0.08, $G_{j\,max}$ = 1.47, η = 2.8 (n = 4), for Cx45 channels alone.

CFTR Cl^- channels in the pulsed oocyte (Fig. 3, upper trace), whereas the increase in I_j reflects an increase in G_j (Fig. 3, lower trace). Significantly, one also notices a reversible increase in the ratio of $I_{j\,ss}/I_{j\,PK}$, which reflects a sizable drop in V_j sensitivity (Fig. 3, lower trace). This is more clearly demonstrated in representative traces of junctional current normalized to peak amplitude (Fig. 3, *inset*) and sampled before (*sweep #1*), during (*sweep #26*) and after (*sweep #55*) forskolin application (marked by asterisks in Fig. 3, lower trace).

The effect of forskolin on I_m and G_j are summarized in Fig. 4. In oocyte pairs co-expressing CFTR and Cx45, forskolin application increases I_m by 144.4 ± 46.7% (mean ± SE, n = 13), and both peak and steady-state junctional conductances ($G_{j\,PK}$ and $G_{j\,ss}$, respectively) by 51.8 ± 12.5% and 90.34 ± 17.2% (mean ± SE, n = 15), respectively (Fig. 4*A*). These changes are not observed in oocytes expressing just Cx45 (Fig. 4*B*). In oocytes co-expressing CFTR and Cx45, $G_{j\,ss}$ increases by a greater fraction than $G_{j\,PK}$ (Fig. 4*A*), resulting in significant increase in $G_{j\,ss}/G_{j\,PK}$ (Fig. 4*C*); this is not observed in oocytes expressing just Cx45 (Fig. 4*D*). In contrast, the time constant (Tau) of I_j decay at the pulse is not affected by forskolin application either in oocytes co-expressing CFTR and Cx45 or in those expressing just Cx45 (Fig. 4*C* and *D*, respectively).

Discussion

This study demonstrates that in *Xenopus* oocyte pairs co-expressing CFTR and Cx45 channels, the

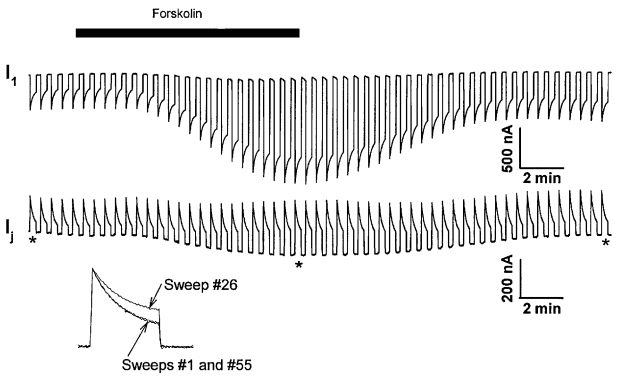

Fig. 3. In oocytes expressing CFTR, forskolin application increases G_j and decreases V_j sensitivity of Cx45 channels. This figure shows a low-speed chart recording of junctional current (I_j) generated by applying −40 mV V_j pulses (12s duration) to oocyte #1, while maintaining oocyte #2 at −20 mV. Application of 20 μM forskolin reversibly increases both I_1 and I_j. The increase in I_1 partially reflects an increase in I_m caused by activation of CFTR channels in the pulsed oocyte (upper trace). The increase in I_j reflects an increase in G_j (lower trace). In addition, the ratio $I_{j\,ss}/I_{j\,PK}$ increases reversibly, reflecting a drop in V_j sensitivity (lower trace). This is clearly shown in representative I_j traces (*inset*) sampled before, during and after forskolin application and normalized to peak amplitude (sweeps #1, 26 and 55, respectively, marked by asterisks in the lower trace).

forskolin-induced activation of CFTR causes a significant increase in junctional conductance (G_j), confirming the results of an earlier study in pancreatic duct cells (Chanson et al. 1999). In oocytes, however, CFTR expression also causes a drop in transjunctional voltage (V_j) gating sensitivity, which is further enhanced by forskolin-induced CFTR activation. Significantly, the drop in V_j gating sensitivity is not accompanied by changes in the time constant (Tau) of I_j decay.

The increase in $G_{j\,PK}$ is likely to reflect a reversible increase in open-channel probability, as shown in small cells studied by dual whole-cell patch clamp (Chanson et al. 1999). This, however, could not be evaluated in oocytes because, due to their large size, the activity of single gap junction channels cannot be detected.

Cx45 channels are unusual among connexin channels because a significant fraction of them are in a closed state even in the absence of chemical uncouplers or V_j gradients (Bukauskas et al., 2002). This explains why in the relationship between $G_{j\,ss}/G_{j\,max}$ and V_j the value of $G_{j\,ss}/G_{j\,max}$ at $V_j = 0$, extrapolated by the Boltzmann fit, is greater than one. Therefore, CFTR activation is likely to affect one of the gating mechanisms of Cx45 channels. Gap junction channels are known to be gated by V_j gradients and increased $[Ca^{2+}]_i$ or $[H^+]_i$,via molecular mechanisms still largely unclear (*reviewed* in Harris, 2001; Peracchia, 2004). Changes in G_j induced by cytosolic acidification are more closely related to $[Ca^{2+}]_i$ than to $[H^+]_i$ (Peracchia, 1990a, b; Lazrak & Perachia, 1993), and there is evidence that in some cells gating is sensitive to near physiological $[Ca^{2+}]_i$, probably via calmodulin (CaM) activation (*reviewed* in Peracchia, 2004). At least two V_j sensitive gates have been identified: fast and slow. The fast V_j gate and chemical gate are believed to be distinct, as the former closes the channel rapidly (< 1ms) but incompletely, leaving a 20–30% residual conductance, whereas the latter closes the channel slowly (8–10 ms) but completely (Bukauskas & Peracchia, 1997). Slow V_j gate and chemical gate are likely to be the same (Bukauskas & Peracchia, 1997; Peracchia, Wang & Peracchia, 1999, 2000). Slow and fast V_j gates are in series and each hemichannel appears to have both gates. The slow gate closes at the negative side of V_j in all connexin channels, whereas the polarity of fast V_j gating varies among connexin channels (*reviewed* in Harris, 2001).

In contrast to the behavior of most connexin channels, in Cx45 channels the gate activated by V_j appears to be preferentially the slow gate, as demonstrated at the single-channel level by evidence for slow and complete channel closure in response to small V_j gradients (Elenes et al., 2001; Bukauskas et al., 2002). Therefore, it is reasonable to believe that CFTR activation somehow influences the behavior of the slow/chemical gate of Cx45 channels. There is evidence that CaM is involved in the function of this gate in Cx45 channels (Peracchia et al., 2003), but there is no indication that CFTR influences CaM function.

Evidence for decreased V_j sensitivity in oocytes co-expressing CFTR and Cx45 even in the absence of forskolin application suggests that the presence of CFTR channels is sufficient to affect the gating behavior of Cx45 channels. Significantly, the drop in V_j sensitivity, manifested by an increase in $G_{j\,ss}/G_{j\,PK}$, is not accompanied by a change in time constant of single exponential I_j decay. This may indicate that CFTR influences the slow gating mechanism of some of the Cx45 channels, rendering it insensitive to V_j. Perhaps this creates two populations of Cx45 channels: V_j-insensitive and V_j-sensitive.

While it seems clear that CFTR affects the function of Cx45 channels, the mechanism involved is unclear. CFTR, aside from being a Cl^- channel, is also known to be a regulator of other ion channels, such as the outwardly rectifying Cl^- channels (ORCC; Schwiebert et al., 1995), the amiloride-sensitive Na^+ channels (ENaC; Stutts et al., 1995), the ATP channels (Sugita, Yue & Foskett, 1998) and the inward rectifier K^+ channels (Kirk 1.1, KCNJ1; Yoo et al., 2004), among others. There is evidence that CFTR interacts with other proteins via PDZ domains (*reviewed* in, Kunzelmann, 2001; Wang & Li, 2001, Haggie, Stanton & Verkman, 2004), as it contains a classic PDZ1 binding domain at its COOH-terminus

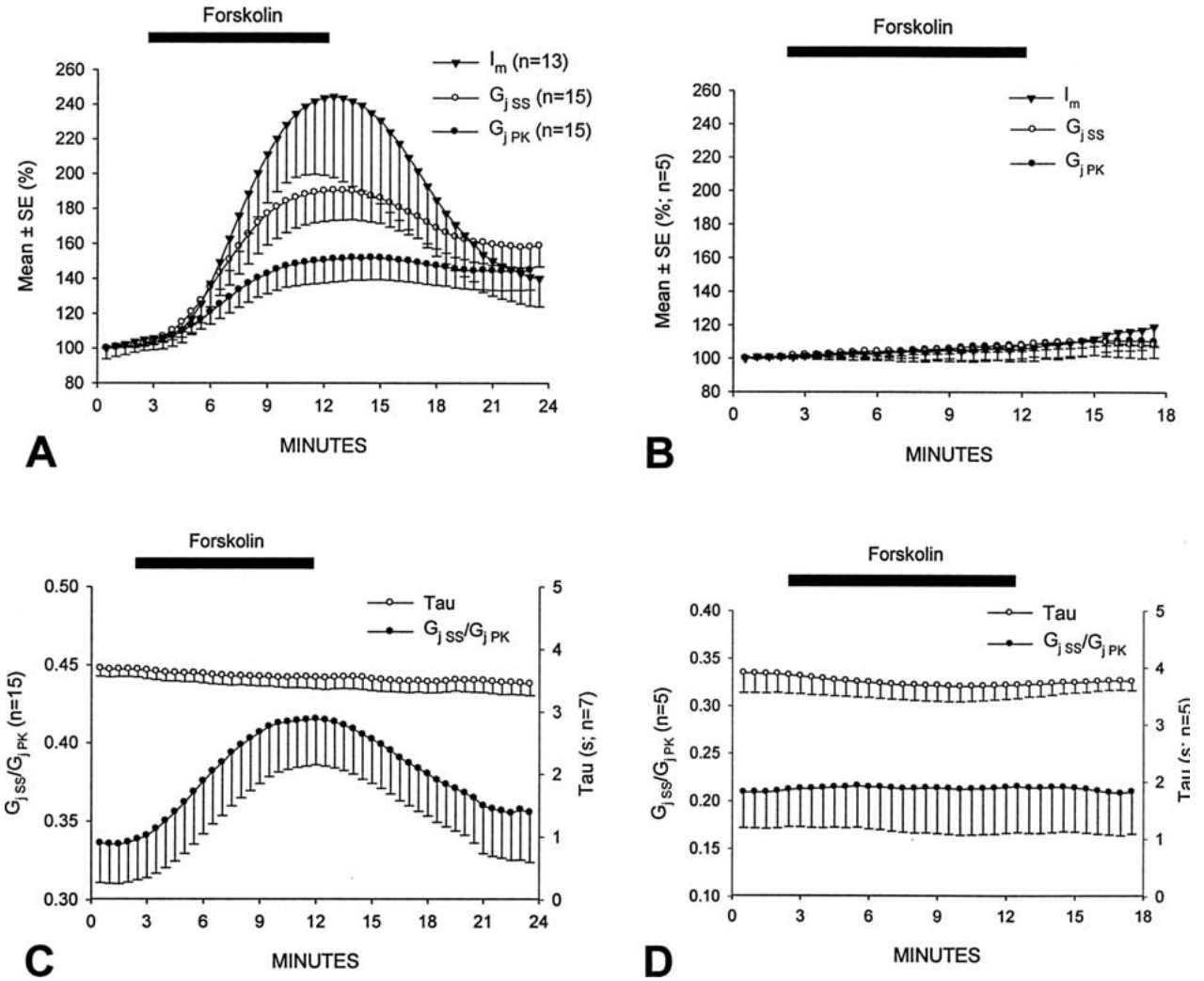

Fig. 4. Effect of forskolin application on junctional and membrane currents. Application of 20 μM forskolin increases I_m by 144.4 ± 46.7% (mean ± SE, n = 13), and both $G_{j\,PK}$ and $G_{j\,ss}$ by 51.8 ± 12.5% and 90.34 ± 17.2% (mean ± SE, n = 15), respectively, in oocyte pairs co-expressing CFTR and Cx45 (*A*), but it has no effect in pairs expressing Cx45 alone (*B*). In oocytes co-expressing CFTR and Cx45, the percent increase of $G_{j\,ss}$ is greater than that of $G_{j\,PK}$ (*A*), resulting in an increase in $G_{j\,ss}/G_{j\,PK}$ (*C*), which is not observed in oocytes expressing Cx45 alone (*D*). In contrast, forskolin application does not change the time constant (Tau) of I_j inactivation at the pulse, either in oocytes co-expressing CFTR and Cx45 (*C*) or in oocytes expressing Cx45 alone (*D*).

whose consensus sequence is conserved among vertebrates (Wang et al., 2000). Significantly, the COOH-terminus of Cx45 also contains a classic PDZ binding motif—the sequence SVWT (residues 293–296)—which has been shown to interact with the PDZ domain of ZO-1, a protein of the zonula occludens (Wang et al., 2000) that colocalizes with Cx45 at sites of cell-cell contact and co-immunoprecipitates with Cx45 (Kausala, Reichert & Hunziker, 2001; Laing et al., 2001).

The functional interaction between CFTR and other channels and transporters appears to be mediated by scaffolding proteins containing multiple PDZ domains, such as the Na^+/H^+ exchanger regulatory factor (NHE-RF), also known as EBP50 (ezrin binding protein of 50 kDa; Reczek, Berryman & Bretscher, 1997), the CFTR associated protein (CAP70; Wang et al., 2000) and the CFTR-associated ligand (CAL; Cheng & Guggino, 1998; Cheng, Wang & Guggino, 2004). Therefore, one may ask: does CFTR interact with Cx45 via PDZ-endowed scaffolding proteins? Presently, ZO-1 is the only PDZ containing protein known to bind to Cx45, but there is no evidence that it binds to CFTR as well. However, CFTR and Cx45 may indirectly interact via cytoskeletal components, because ZO-1 is known to bind actin and CFTR is believed to interact with actin, probably via intermediate cytoplasmic proteins (Haggie, Stanton, Verkman, 2004).

In conclusion, the data show that forskolin-induced activation of CFTR expressed in oocytes cau-

ses a significant increase in the electrical conductance of gap junctions made of Cx45 and decreases the transjunctional voltage-gating sensitivity of Cx45 channels. The drop in voltage sensitivity is not accompanied with changes in the kinetics of voltage-dependent inactivation of junctional current. The data suggest a functional interaction between CFTR and gap j unction channels.

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