UNIVERSIDADE ESTADUAL DE CAMPINAS

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MODULAÇÃO *in vitro* DA FUNÇÃO DE BARREIRA EPITELIAL E INTEGRIDADE DA JUNÇÃO DE OCLUSÃO, EM LINHAGEM CELULAR MDCK

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural na área de Histologia.

Orientadora: **Profa.Dra. Carla Beatriz Collares Buzato**

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Aos meus pais e minha família, por todo amor, por serem a felicidade, a força e a essência da minha vida.

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RESUMO

Os epitélios exercem uma função importante como barreira de permeabilidade, delimitando compartimentos de composições químicas distintas e assegurando, assim, as funções específicas do tecido/órgão. Esta barreira é estabelecida por duas rotas de transporte através do epitélio: a transcelular e a paracelular. A junção de oclusão (JO) é a principal estrutura anatômica que define as propriedades específicas da barreira paracelular nos epitélios e que tem sua permeabilidade regulada por processos intracelulares. A JO não é uma estrutura estática, mas sim dinâmica e prontamente adaptável a uma variedade de circunstâncias fisiológicas e patológicas, podendo ser modulada também em condições experimentais. O comprometimento da função de barreira paracelular está associado a várias doenças como a infecção bacteriana intestinal, inflamação e outras.

O objetivo geral do presente trabalho foi estudar os processos de modulação da função de barreira epitelial e o possível envolvimento da junção de oclusão, utilizando como um modelo "in vitro" de células epiteliais, a cultura da linhagem celular MDCK (Madin-Darby canine kidney). Investigamos duas condições experimentais que potencialmente poderiam afetar a estrutura e a função da barreira epitelial: 1) tratamento com dexametasona (DEX), um glicocorticóide sintético, e prolactina (PRL); e 2) exposição à proteína catiônica, protamina.

Nosso estudo demonstra que o tratamento de monocamadas de células MDCK com DEX e PRL induz aumento da função de barreira. Este efeito parece não envolver diretamente um aumento na expressão das proteínas da JO, mas sim aumento da viabilidade e/ou proliferação celular. Em contrapartida, o tratamento "in vitro" com protamina resultou em ruptura da barreira paracelular e este efeito foi associado a uma desestruturação do citoesqueleto e diminuição da expressão juncional de proteínas associadas à JO.

ABSTRACT

One of the most important attributes of epithelia is that they function as a barrier that separates two different compartments, ensuring the specific functions of the tissue and/or organ. The barrier function is achieved by limiting the transport through two putative pathways: the transcellular route and the paracellular route. The tight junction (TJ) is the major physical structure defining the specific properties of the paracellular barrier. TJ was once regarded as static structures; nevertheless, it is now well-known that this junction is a very dynamic structure that is readily modulated by a variety of physiological, pathological and experimental conditions.

The general objective of this thesis was to investigate the modulation processes of the epithelial barrier function and the possible involvement of TJ, using an "in vitro" model of epithelial cells – the cultured Madin–Darby canine kidney (MDCK) cells. We studied two different experimental conditions that would affect potentially the epithelial barrier structure and function. One of these conditions was the treatment with dexamethasone (DEX), a synthetic glucocorticoid, in combination with the lactogenic hormone prolactin (PRL). The other condition was the treatment with a polycationic protein, the protamine.

We demonstrated herein that DEX and PRL treatment induces enhancement of the epithelial barrier function in cultured MDCK cells. This effect seems not to be result of changes in TJ structure, but may involve increase in cell proliferation and/or viability. On the other hand, the "in vitro" treatment with protamine elicits disruption of the epithelial paracellular barrier that is associated with rearrangement of the cytoskeletal F-actin and decrease in junctional immunoreaction of several TJ-associated proteins.

SUMÁRIO

CAPÍTULO 1

1. INTRODUÇÃO

1.1 O epitélio e a barreira de difusão seletiva

Os epitélios desempenham um papel crucial como barreira de difusão delimitando dois compartimentos de composições químicas distintas, permitindo o transporte transepitelial de nutrientes, eletrólitos e água entre estes compartimentos (Madara, 1990). As funções de barreira e transporte dos epitélios são determinadas por duas rotas: a rota transcelular e a rota paracelular. A rota transcelular constitui uma via de transporte direcional de substâncias através da membrana citoplasmática e do citoplasma (Madara, 1990; Lutz and Siahaan, 1997; Mitic et al., 2000; Anderson, 2001). A membrana celular apresenta uma baixa permeabilidade passiva, principalmente a solutos hidrofílicos, portanto, o transporte transcelular é caracteristicamente realizado por uma gama de transportadores celulares específicos e por canais posicionados nas membranas apical e basolateral das células (Frömter & Diamond, 1972; Diamond, 1977; Mitic et al., 2000; Anderson, 2001). O transporte pela rota transcelular freqüentemente envolve gasto de energia.

A rota paracelular, porém, é composta pelas junções intercelulares e pelo espaço intercelular (Diamond, 1977; Cereijido, 1992; Anderson, 2001). O transporte paracelular é um transporte passivo e resultante da difusão, eletrodifusão ou osmose, regido pelo gradiente eletroquímico gerado pelo mecanismo transcelular (Anderson et al., 2001). A princípio, acreditava-se que a rota paracelular constituia uma estrutura impermeável e não regulável de selamento entre as células, que impedia o intercâmbio de substâncias entre dois compartimentos de composições químicas distintas delimitados pelo epitélio (Diamond 1977, Cereijido 1992). Porém, atualmente, sabe-se que esta via de transporte constitui uma importante rota modulável que permite o fluxo passivo de íons, pequenas e médias moléculas através do epitélio (Lutz and Siahaan, 1997; Fasano, 2000; Mitic et al., 2000; Anderson, 2001). Essas características do transporte paracelular devem-se principalmente às propriedades das junções intercelulares, em especial, à junção de oclusão.

1.2 As junções intercelulares

As junções intercelulares são definidas como uma série de especializações das membranas plasmáticas localizadas na região de contato entre duas células vizinhas (Diamond, 1977; Cereijido, 1992; Collares-Buzato, 2001). Morfologicamente, o complexo de junções intercelulares é composto por quatro unidades estruturais distintas e interdependentes (Figura 1), a saber: 1) a junção de

oclusão (*zonula occludens* ou *tight junctions*), que forma um cinturão ao redor da célula, onde ocorre uma fusão aparente de folhetos externos das membranas laterais de células adjacentes; 2) a junção aderente (*zonula* ou *fasciae adherens*), região especializada da membrana onde as duas membranas adjacentes ficam paralelas e separadas por um espaço de 15 a 20 nm, e onde, no lado citoplasmático, ocorre a ancoragem dos microfilamentos do citoesqueleto; 3) o desmossomo (ou *maculae adherens*), junção caracterizada por apresentar duas placas lineares, paralelas e elétron-densas às quais se associam os filamentos intermediários do citoesqueleto e 4) a junção comunicante (ou *gap junction*), coleção de canais intercelulares, localizados na membrana plasmática, que conectam os citoplasmas de duas células vizinhas (Collares-Buzato, 2001). No tecido epitelial de revestimento, a junção de oclusão, a junção aderente e o desmossomo formam o chamado Complexo Unitivo, que representa um dispositivo de adesão intercelular (Collares-Buzato, 2001).

O emprego de métodos tais como fracionamento subcelular, imunoprecipitação e imunocitoquímica permitiu um considerável avanço no conhecimento da composição e arranjo molecular de cada um dos componentes das junções intercelulares. Embora, bioquimicamente, as junções intercelulares sejam bem distintas entre si, elas apresentam uma estrutura molecular análoga. Cada unidade juncional é formada por proteínas transmembranas específicas que interagem com o citoesqueleto através de um complexo protéico associado à região citoplasmática da junção (Madara, 1998). Sabe-se, atualmente, que as proteínas integrais, denominadas ocludina e claudinas, são os principais constituintes da rede complexa de cordões de vedação da junção de oclusão, responsáveis pelo contato íntimo entre células adjacentes (Furuse et al., 1993 e 1998). A junção aderente e os desmossomos contém moléculas de adesão dependentes de cálcio, pertencentes à super-família das caderinas, como um de seus componentes (Geiger e Ayalon, 1992). Tais moléculas são glicoproteínas de superfície que promovem a coesão de células adjacentes e interagem com o citoesqueleto por intermédio das cateninas, no caso da junção aderente, e do complexo desmoplaquina/placoglobina, nos desmossomos (Knudsen et al., 1995; Ozawa et al., 1989; Reynolds et al., 1994). A junção comunicante, por sua vez, apresenta como seu único constituinte as proteínas pertencentes à super-família das conexinas, que são responsáveis em compor os canais intercelulares (Collares-Buzato, 2001).

Funções específicas foram atribuídas a cada um destes componentes do contato intercelular, tendo como base sua morfologia e composição bioquímica. A formação e a manutenção da arquitetura tecidual são conseqüências da firme adesão entre células vizinhas promovida pela junção aderente (Geiger e Ayalon, 1992). Adicionalmente, várias outras funções estão associadas à

junção aderente, como a formação e manutenção das outras junções intercelulares, conservação da polaridade celular e organogênese (Collares-Buzato et al., 1998; Eaton e Simons, 1995; Geiger e Ayalon, 1992; Gumbiner et al., 1988; Nose e Takeichi, 1986). Assim como a junção aderente, os desmossomos também promovem a adesão entre as células, porém, sem interferir na constituição e na função dos outros componentes juncionais (Cowin et al., 1984). Com relação à junção comunicante, sua atividade primordial é efetivar a comunicação intercelular (Collares-Buzato, 2001). A junção de oclusão, por sua vez, exerce um papel fundamental de barreira de difusão pela via paracelular, essencialmente em endotélios e epitélios de revestimento (Farqhuar e Palade, 1963; Gumbiner, 1987; Madara, 1990; Schneeberger e Lynch, 1992; Fasano, 2000; Mitic et al., 2000). Além disto, este tipo de junção realiza a manutenção da distribuição assimétrica (polaridade) de proteínas e lipídios entre os domínios apical e basolateral da membrana de células epiteliais (Diamond, 1977; Gumbiner, 1987; Cereijido et al., 1989; Handler, 1989; Schneeberger e Lynch, 1992).

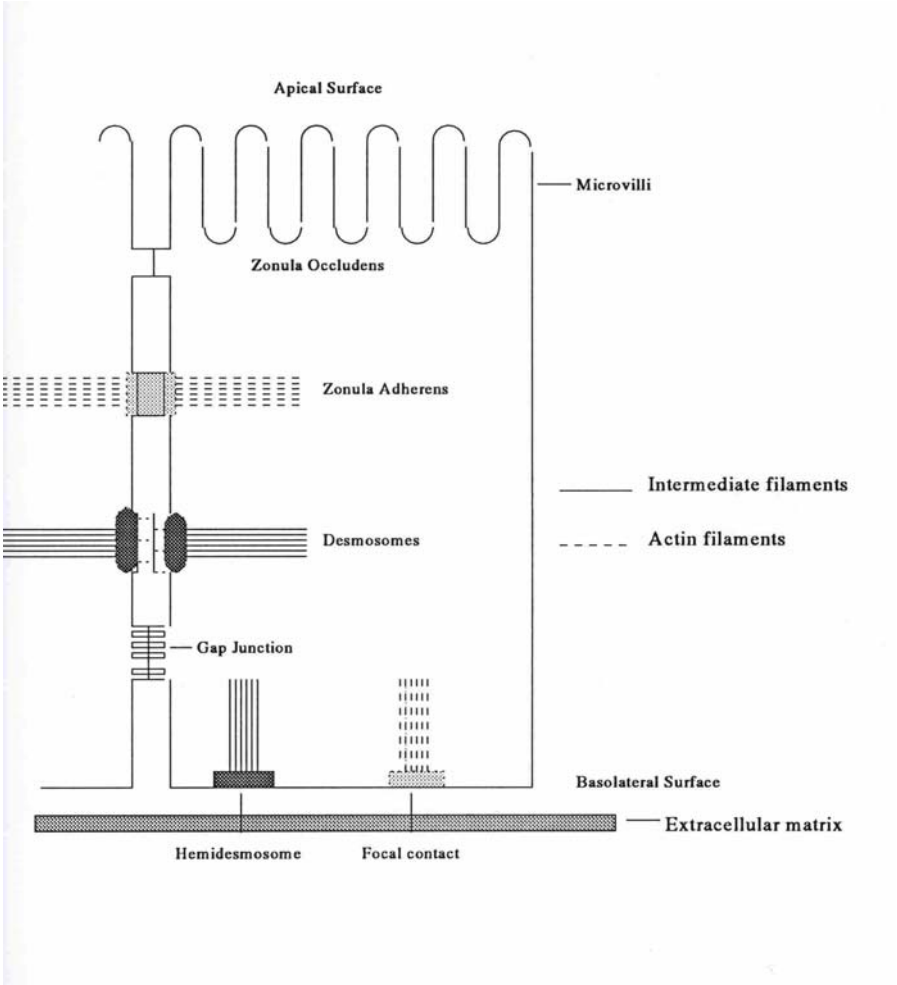

Figura 1: Representação esquemática de duas células mostrando as junções intercelulares. Reprodução com permissão do autor (Collares-Buzato, 1995).

1.3 A junção de oclusão e a permeabilidade paracelular

Dentre as junções intercelulares, a junção de oclusão (JO) é a que tem despertado recente interesse quanto ao estudo dos mecanismos envolvidos na regulação da sua estrutura bioquímica e função. Esse interesse advém de duas razões principais: 1) o importante papel da junção de oclusão em determinar a função de barreira paracelular nos epitélios, de maneira que o comprometimento da mesma está associado a várias doenças (Hollander, 1988; Jepson et al., 1995; Madara, 1990; Mitic et al., 2000) e 2) a possibilidade de regular a função de barreira paracelular visando a otimização da

administração terapêutica de drogas de difícil absorção transepitelial, como por exemplo nos epitélios intestinal, pulmonar e cerebral.

A JO é o componente mais apical do complexo juncional sendo a unidade que exerce a principal função de regulação da permeabilidade epitelial, influenciando o fluxo paracelular de fluidos e solutos (Lutz e Siahaan, 1997; Fasano, 2000; Nusrat et al., 2000). Pode-se afirmar que a JO é a principal estrutura anatômica que define as propriedades específicas da barreira paracelular e que tem sua permeabilidade fisiologicamente regulada por processos intracelulares (Anderson, 2001).

Ultra-estruturalmente, a JO constitui uma série de discretos sítios de "fusão" das membranas plasmáticas de células adjacentes. Em criofraturas, a JO apresenta-se como uma série de fibrilas intramembranosas contínuas e anastomosantes, as quais correspondem aos sítios de contato intercelular. Tal estrutura da JO pode ser explicada, em nível molecular, pela disposição lateral de partículas protéicas integrais da membrana de células epiteliais vizinhas, formando cordões de vedação que se entrelaçam e aderem firmemente entre si, semelhante ao que acontece no fechamento de um zíper (Farquhar e Palade, 1963; Staehelin 1973, 1974). As proteínas transmembranas, ocludina e claudinas, têm sido recentemente identificadas como as principais proteínas que formam a estrutura da JO (os cordões de vedação) e estão diretamente envolvidas com as características fisiológicas da junção. Portanto, estas proteínas determinam a barreira paracelular e definem as diversas propriedades do transporte paracelular (Furuse et al., 1998; Mitic et al., 2000; Anderson, 2001). A ocludina é uma fosfoproteína, com massa molecular de aproximadamente 65 kDa, que atravessa a membrana celular quatro vezes, formando quatro regiões hidrofóbicas inseridas na membrana e duas regiões citoplasmáticas terminais, NH_2 e COOH. Em conseqüência, formam-se dois "loops" extracelulares ricos em glicina e tirosina, responsáveis pela interação com outra molécula vizinha, e dois "loops" intracelulares, responsáveis pela interação com proteínas citoplasmáticas (Mitic et al., 2000; Anderson, 2001; Collares-Buzato, 2001). As claudinas, que constituem uma família composta de aproximadamente 20 diferentes subtipos, também apresentam quatro domínios moleculares. Entretanto, em comparação à ocludina, são proteínas significantemente menores, com massa molecular aproximada de 23 kDa, que apresentam dois domínios extracelulares e porções terminais NH_2 e COOH mais curtos (Mitic et al., 2000; Anderson, 2001; Collares-Buzato, 2001).

Atualmente, as claudinas são consideradas as principais moléculas de adesão intercelular, que podem se co-polimerizar com a ocludina para formar os cordões de vedação específicos da JO (Mitic et al., 2000; Anderson, 2001). Apesar da ocludina ter sido considerada, primeiramente, como

o principal elemento constituinte das JO, sabe-se hoje que as JO podem ser formadas na sua ausência, isto é, a ocludina parece ser um componente facultativo que pode interagir, direta ou indiretamente, com as claudinas (Anderson, 2001). A capacidade das junções de oclusão de restringir a passagem de íons e moléculas entre as células aumenta em progressão logarítmica com a complexidade dos cordões de vedação (Alberts et al., 1997). O número destes cordões parece se relacionar com o grau de expressão de ocludina e claudinas, enquanto que a permeabilidade e seletividade aos cátions de cada cordão dependem do número e tipo de claudinas que o compõe (Collares-Buzato, 2001).

A porção terminal COOH das proteínas ocludina e claudinas se liga a uma conhecida família de proteínas citoplasmáticas conhecida como *zonula occludens* - ZOs (ZO-1, ZO-2, ZO-3) (Figura 2). Tais proteínas citoplasmáticas formam um complexo justaposto à face interna da membrana que está envolvido na organização das proteínas transmembranas e nas suas ligações às outras proteínas citoplasmáticas, assim como aos microfilamentos de actina (Fanning et al., 1998; Inai et al., 1999; Anderson, 2001). A ZO-1 foi a primeira proteína integrante da JO a ser identificada; possui massa molecular de aproximadamente 225 kDa e um formato globular alongado com vários resíduos de fosfo-serinas em sua estrutura (Stevenson e Goodenough, 1984). A ZO-1 forma um complexo protéico com a ZO-2 e ZO-3, outros dois membros da família das ZOs que possuem massa molecular de 160 e 130 kDa, respectivamente, e com seqüências estruturais homólogas às de ZO-1 (Gumbiner et al., 1991; Jesaitis e Goodenough, 1994). Existem outras numerosas proteínas citoplasmáticas associadas à JO, como a cingulina, antígeno 7H6 e simplequinas, cuja estrutura molecular e função ainda não estão bem caracterizadas (Mitic e Anderson, 1998; Mitic et al., 2000; Anderson, 2001).

A interação das proteínas transmembranas com proteínas de sinalização e o citoesqueleto exerce um papel fundamental na regulação da permeabilidade paracelular (Madara, 1998). Em condições experimentais, tem sido demonstrado que a desorganização dos microfilamentos de actina com agentes como a faloidina e a citocalasina induz alterações na morfologia da JO e conseqüente quebra da barreira paracelular (Madara, 1986). Da mesma maneira, outros experimentos verificaram que o aumento do Ca^{+2} intracelular induz um aumento da contração da actina perijuncional, causando, portanto, aumento da permeabilidade paracelular (Tsuneo et al., 1991).

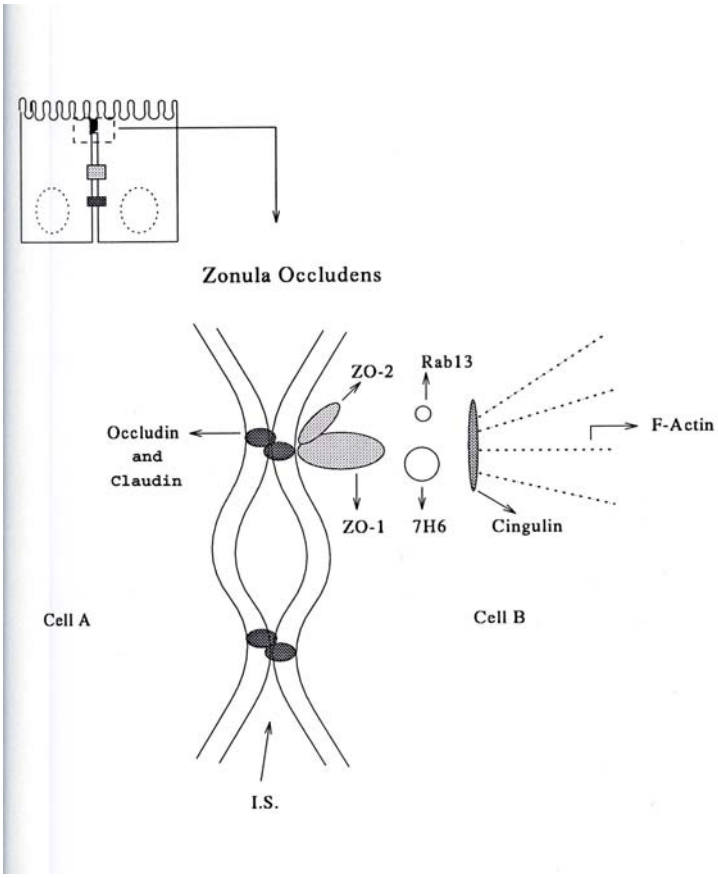

Figura 2: Diagrama mostrando um modelo hipotético da composição bioquímica e interação das proteínas que formam a JO. Legenda: I.S. = espaço intercelular. Reprodução com a permissão do autor (Collares-Buzato, 1995).

1.4 Métodos para a análise da função de barreira paracelular

A integridade funcional da barreira paracelular em epitélios pode ser analisada por métodos biofísicos e não-biofísicos (Madara e Hecht, 1989; Reuss, 1992; Lewis et al., 1995). Técnicas eletrofisiológicas têm sido amplamente utilizadas para avaliar a permeabilidade paracelular. Neste caso, a função de barreira paracelular é expressa em termos de resistência à passagem transepitelial da corrente elétrica. Uma vez que a corrente elétrica, em soluções eletrolíticas, é resultante do fluxo de íons, a resistência à passagem passiva do fluxo de íons através da rota paracelular é representada por uma medida direta do valor de resistência. Uma intensidade conhecida de corrente é transmitida através do epitélio por meio de dois eletrodos que permitem esta passagem (eletrodos Ag/AgCl), então, a diferença de potencial transepitelial resultante é detectada e medida por um voltímetro interno (Waltington et al., 1970). Esta diferença de potencial é convertida em medida de resistência à passagem de fluxo de íons e, finalmente, o cálculo da resistência elétrica transepitelial (R_t) é

expresso em Ohms (Ω).cm^2 por unidade de área. Embora a medida de R_t possa fornecer algumas informações sobre a integridade da barreira paracelular, alterações nesta variável não necessariamente refletem mudanças específicas na permeabilidade paracelular, pois a permeabilidade transcelular a íons também pode influenciar na medida deste parâmetro. Um epitélio pode ser visualizado como um circuito formado por duas principais resistências em paralelo: a resistência transcelular (Rc) e a resistência da via paracelular (Rp) (Figura 3). Além do que, a resistência transcelular é constituída pela resistência da membrana apical (Ra) em série com a resistência da membrana basolateral (Rb). Portanto, quando é feita a medida de R_t , ambos componentes (Rc e Rp) estão envolvidos.

Uma medida mais direta da integridade funcional da barreira paracelular é o fluxo transepitelial utilizando marcadores extracelulares. Esta técnica avalia a taxa de transferência de moléculas marcadas de um lado do epitélio para outro oposto. Tipicamente, alguns solutos hidrofílicos (por exemplo: tioureia, manitol, inulina, fenol vermelho, etc.) possuem uma insignificante permeabilidade através de biomembranas e, portanto, são usados para medir especificamente a taxa de fluxo através da via paracelular (McEwan et al., 1993; Thwaites et al., 1993; Lewis et al., 1995). Quando há uma diminuição na medida de R_t, ao mesmo tempo em que ocorre um aumento do fluxo de um marcador extracelular específico para o outro lado do epitélio, pode se afirmar que a permeabilidade através da junção está aumentada e, conseqüentemente, sua função de barreira está comprometida. Uma situação inversa, como aumento na R_t associado com diminuição do fluxo transepitelial de marcadores extracelulares, pode indicar um reforço na barreira epitelial (Claude, 1978).

Finalmente, estudos morfológicos usando microscopia eletrônica ou de fluorescência permitem a observação da morfologia geral e estrutura da JO, no caso de alteração da permeabilidade paracelular. Em particular, o uso de anticorpos monoclonais para localizar algumas conhecidas proteínas estruturais associadas à JO pode ser uma ferramenta poderosa para correlacionar alterações observadas em parâmetros biofísicos e de fluxo com modificações na organização destas moléculas específicas da JO.

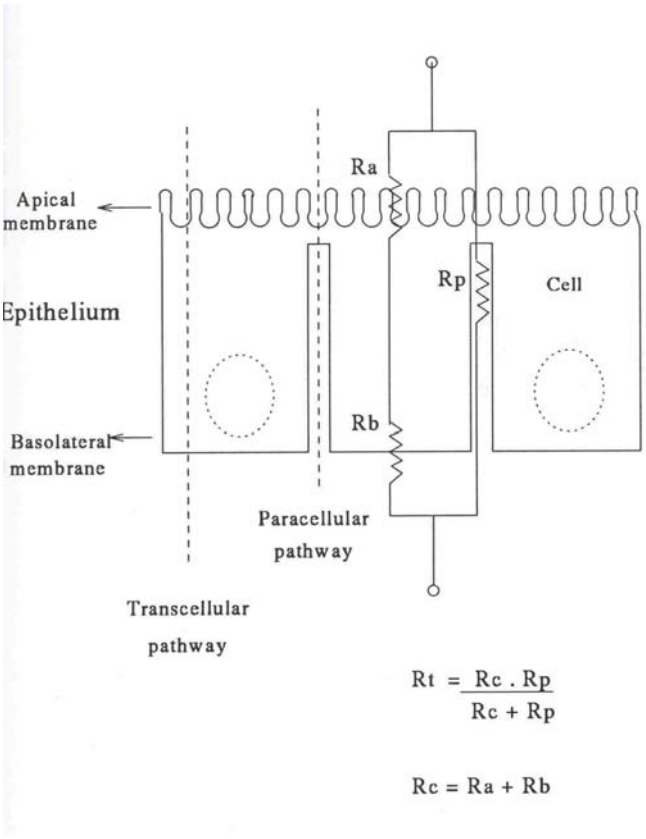

Figura 3: Circuito elétrico de um epitélio. A figura mostra a resistência da rota transcelular (Rc) e a resistência da rota paracelular (Rp) operando em paralelo. Rc contém duas resistências em série: as resistências da membrana apical (Ra) e da membrana basolateral (Rb). Reprodução com permissão do autor (Collares-Buzato, 1995).

1.5 Regulação da JO e da barreira paracelular

Há inúmeras evidências indicando que a junção de oclusão e a barreira epitelial podem ser moduladas em certas condições "in vivo" e em processos fisiopatológicos, como por exemplo: no processo de absorção intestinal de nutrientes (Madara, 1990); na espermiogênese (Byers e Pelletier, 1992); no trânsito de moléculas através dos capilares cerebrais (Rubbin e Staddon, 1999); na transmigração de patógenos e/ou células através de epitélios e endotélios durante infecção bacteriana intestinal (Jepson et al., 1995) e inflamação (Evans et al., 1983). Além disso, alterações da função e estrutura da junção de oclusão em epitélios podem ser induzidas experimentalmente. Tratamentos com determinadas substâncias aumentam a permeabilidade da junção de oclusão, por meio da ruptura das mesmas ou pela internalização/alteração conformacional das suas proteínas constitutivas. Como exemplo destas condições experimentais temos: retirada do cálcio extracelular, aumento de cálcio intracelular, diminuição do pH citoplasmático, ativação de tirosina quinases,

exposição a certos hormônios, citocininas e policátions (Collares-Buzato, 2001; Collares-Buzato et al., 1994a e 1994b). Por outro lado, outras situações podem acarretar em diminuição da permeabilidade da junção de oclusão, como o tratamento com vitamina A, proteases e glicocorticóides (Collares-Buzato, 2001; Polak-Charcon, 1992).

Apesar do considerável conhecimento relacionado às funções da junção de oclusão na fisiologia epitelial, os mecanismos intracelulares envolvidos na regulação desta junção (possíveis mensageiros intracelulares e seu sítio de ação, envolvimento e interrelação das proteínas juncionais no processo, dentre outros) ainda são pouco conhecidos. Um exemplo disto são os efeitos de macromoléculas policatiônicas e o controle hormonal mediado pelos glicocorticóides sobre a permeabilidade epitelial e a função da JO.

Certas proteínas policatiônicas liberadas pelas células polimorfonucleares durante o processo de inflamação contribuem para as doenças inflamatórias, injúrias teciduais, aumento da permeabilidade vascular e formação de edema, por meio de mecanismos ainda pouco conhecidos (Peterson e Gruenhaupt, 1990; Jones et al., 2001; Gautam et al., 2001). Exemplos de proteínas catiônicas liberadas por células do sistema imune são: a proteína básica principal (MBP), a proteína catiônica de eosinófilo (ECP), a neurotoxina derivada de eosinófilo (EDN), a peroxidase de eosinófilo (EPO) e a proteína ligante da heparina (HBP) (Jones et al., 2001; Gautam et al., 2001). Alguns estudos têm demonstrado a ação destes policátions sobre a função de barreira epitelial. A MBP induz alterações nas respostas da via aérea em animais experimentais (Coyle et al., 1993) e desestrutura moléculas de adesão intercelular (ICAM-1) em cultura de células epiteliais (Altman et al., 1993). Tem sido também demonstrado que a HBP (Proteína Ligante da Heparina), liberada pelos neutrófilos, induz um rearranjo Ca^{2+}-dependente do citoesqueleto e a formação de fendas intercelulares em monocamadas endoteliais "in vitro". "In vivo", a HBP aumenta o efluxo macromolecular em capilares (Gautam et al., 2001).

Os efeitos carga-dependentes de policátions sobre o transporte e/ou estrutura dos epitélios tem sido reproduzidos "in vitro" pela protamina. A protamina é uma proteína básica rica em arginina, com peso molecular de aproximadamente 5000 Da, obtida do esperma de salmão (Fromm et al., 1985).

A ação da protamina sobre a integridade da barreira epitelial parece ser diversa e dependente da espécie animal e do tipo de célula estudada (Fromm et al., 1985; Peterson e Gruenhaupt, 1990). Em epitélio da bexiga de *Necturus*, este policátion induz uma redução reversível na permeabilidade paracelular (Fromm et al., 1985; Bentzel et al., 1987) associada a um aumento no número de cordões de vedação da JO, como observado por "freeze-fracture" (Bentzel et

al., 1987). Em contrapartida, tem sido reportada, após tratamento com protamina, uma elevação na permeabilidade vascular em pulmões perfundidos e isolados de ratos, além de um aumento no fluxo de albumina através de células endoteliais em cultura (Peterson e Gruenhaupt, 1990). Peterson e Gruenhaupt (1990) demonstraram que o tratamento apical com protamina em cultura de células de rim de cão, MDCK (*Madin Darby canine kidney*), induziu um aumento dose-dependente na condutância transepitelial sugerindo uma ruptura da integridade de barreira da monocamada. O aumento na permeabilidade paracelular foi acompanhado por alterações nos filamentos de actina que compõem o citoesqueleto celular (Peterson e Gruenhaupt, 1990).

Quanto ao controle hormonal mediado pelos glicocorticóides sobre a permeabilidade paracelular de epitélios, é sabido que os glicocorticóides apresentam atividades anti-inflamatórias devido, em parte, ao seu papel de promover diminuição da permeabilidade capilar e, conseqüentemente, de edema (Guyton e Hall,1996). Embora não demonstrado experimentalmente, este efeito sobre os capilares, na região de inflamação, pode envolver regulação da junção de oclusão nas células endoteliais. Recentemente, tem sido demonstrado que o tratamento com dexametasona, um glicocorticóide sintético, induz formação da junção de oclusão em células epiteliais glandulares mamárias, "in vitro", como comprovado pelo aumento na R_t e diminuição no fluxo paracelular de inulina (Stelwagen et al., 1999; Zettl et al., 1992). Este efeito da dexametasona sobre a permeabilidade paracelular foi intensificado pelo tratamento combinado com prolactina e envolveu aumento na expressão de ocludina.

Não se sabe se os efeitos da protamina, dos glicocorticóides e da prolactina sobre a função de barreira epitelial constituem um fenômeno geral sobre a junção de oclusão em epitélios, assim como são desconhecidos os mecanismos intracelulares envolvidos na regulação dessa junção por tais substâncias.

2. OBJETIVO E JUSTIFICATIVAS

O presente projeto tem como objetivo investigar a atuação dos hormônios dexametasona (um glicocorticóide sintético) e prolactina e da substância policatiônica protamina sobre a função de barreira de permeabilidade seletiva da via paracelular, em células epiteliais renais de cão (MDCK), averiguando os possíveis mecanismos intracelulares envolvidos na regulação da junção de oclusão.

A linhagem de células MDCK foi escolhida para este estudo, uma vez que representa um tipo celular muito bem caracterizado em termos de propriedades funcionais e composição molecular de junções intercelulares. Além do que, as células MDCK constituem uma linhagem de rápido

crescimento “in vitro” e de fácil manutenção, e, portanto, compõe um modelo “in vitro” adequado para este tipo de estudo. Foram utilizadas duas cepas da linhagem MDCK que se distinguem pela natureza e composição diferente da JO: a cepa I, que apresenta a JO com características consistentes com um epitélio “tight” (R_t ~ 5000 Ωcm^2) e a cepa II, que forma um epitélio classificado como “leaky” com valores de R_t ~ 200 Ωcm^2 (Collares-Buzato et al., 1994a, 1998).

3. ORGANIZAÇÃO DA DISSERTAÇÃO

A presente dissertação de mestrado está estruturada em 5 capítulos, incluindo este.

Os Capítulos 2 e 3 apresentam os resultados de nossos estudos sob a forma de artigos a serem submetidos em periódicos internacionais. O Capítulo 2 refere-se ao artigo intitulado “Modulation of epithelial barrier by dexamethasone (DEX) and prolactin (PRL) in cultured MDCK cells” que apresenta os dados sobre o efeito do tratamento com dexametasona e prolactina sobre a função de barreira paracelular, em cultura de células MDCK. O Capítulo 3 refere-se ao artigo intitulado “Effect of protamine upon epithelial barrier function and involvement of cytoskeleton and TJ-associated proteins in cultured MDCK cell line” que mostra os resultados sobre as ações da protamina sobre a barreira paracelular e a estrutura da JO, utilizando duas cepas da linhagem de células MDCK. O Capítulo 4 apresenta as conclusões gerais do trabalho e o Capítulo 5 as referências bibliográficas referentes ao Capítulo 1 – Introdução Geral.

CAPÍTULO 2

MODULATION OF THE EPITHELIAL BARRIER BY DEXAMETHASONE (DEX) AND PROLACTIN (PRL) IN CULTURED MDCK CELLS

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Abstract:

Glucocorticoids and prolactin influence the barrier properties of mammary gland cells by stimulating the formation and maintenance of tight junction (TJ) structural components. In this work, we investigated the effect of dexamethasone (DEX) and prolactin (PRL) on the paracellular barrier function in MDCK epithelial cells. DEX (4 μM) + PRL (2 μg/ml) given simultaneously increased the R_t and tended to decrease the apical-to-basal phenol red flux when applied to confluent monolayers for up to 4 d, and after 24 h of cell seeding. An increase in the total number of viable cells per monolayer unit area, following treatment, was observed by cell counting of pre-stained with methyl-green dye and neutral red cell uptake. However, western blotting and immunocytochemistry revealed no changes in the expression and distribution of TJ-associated occludin, ZO-1 and claudin-1. Therefore, in contrast to mammary cells, the effect of glucocorticoids and prolactin on epithelial barrier function in MDCK monolayers is apparently not the result of an up-regulation of TJ proteins but may involve an increase in cell proliferation and/or viability.

Key words: dexamethasone, epithelial barrier, MDCK, prolactin, tight junction.

Running Title: Epithelial barrier function in MDCK cells.

Abbreviations: DEX: dexamethasone; **PRL:** prolactin; **MDCK:** Madin–Darby canine kidney; **TJ:** tight junction; **R_t:** transepithelial electrical resistance.

INTRODUCTION

Epithelia line the cavities and free surfaces of the body to form a border between the external and internal milieus. One of the most important attributes of epithelia is that they function as a barrier to prevent the penetration of noxious agents while at the same time providing vectorial transepithelial transport of nutrients, electrolytes and water between the external and internal compartments.

The main components of the epithelial barrier are the epithelial cells themselves, the cell membranes and the intercellular junctions. Epithelial cell proliferation maintains the epithelial integrity by preventing the formation of lacunas and consequent rupture of the barrier. The cell membrane has a relatively low passive permeability, especially to hydrophilic solutes, so that transcellular transport is governed mainly by specific cell membrane transporters and channels, including carrier-mediated and endocytotic mechanisms (Anderson, 2001; Diamond, 1977; Frömter and Diamond, 1972; Mitic et al., 2000). The intercellular junctions which connect adjacent cells limit transport across the epithelia through the paracellular spaces.

The TJ, the most apical element of the junctional complex (Farquhar and Palade, 1963), is considered the anatomical and functional site of the paracellular barrier (Diamond 1977; Gumbiner, 1987; Schneeberger and Lynch, 1992). Integral membrane proteins, such as occludin and claudins, have been identified as major adhesion molecules that constitute the TJ occluding strands. These proteins are directly involved in barrier function. Several other proteins have been localized in the cytoplasmic submembraneous plaque underlying the TJ region, including ZO-1, ZO-2, ZO-3, TH6 antigen, cingulin and symplekin, which organize the TJ transmembrane proteins, connecting them to other cytoplasmic proteins and to the cytoskeletal actin microfilaments (Anderson, 2001; Furuse et al., 1993; Gumbiner et al., 1991; Mitic and Anderson, 1998; Tsukita and Furuse, 1999).

Functional defects of the epithelial barrier are frequently associated with pathological states such as edema, mucosal inflammation, bacterial infection of the gut, and damage to the blood-brain barrier (Evans et al., 1983; Jepson et al., 1995; Rubbin and Staddon, 1999; Sonoda et al., 1999). In addition, dysfunction of the intestinal barrier associated with increased intestinal paracellular permeability have been considered an etiological factor that precedes the onset of Crohn's disease and celiac diseases (Hollander, 1988; Irvine and Marshall, 2000). There has been an increased interest in the study of the intracellular mechanisms that lead to disruption of the epithelial barrier in these disorders, as well as in determining how this barrier can be strengthened pharmacologically.

Appropriate experimental conditions can increase the barrier function by affecting primarily the TJ structure and consequently reducing the permeability of the paracellular pathway (Collares-Buzato, 2001; Polak-Charcon, 1992). One of these conditions is treatment with glucocorticoids, which can decrease capillary permeability and, consequently, reduce edema (Guyton and Hall, 1996). Dexamethasone (DEX), a synthetic glucocorticoid, has a direct effect on the formation and maintenance of TJs in cultured mammary gland epithelial cells (Stelwagen et al., 1999; Zettl et al., 1992). The increase in epithelial barrier function induced by DEX in mammary gland cells is markedly enhanced when this glucocorticoid is used in combination with the lactogenic hormone prolactin (PRL). PRL is well known for its stimulatory effect on the proliferation of mammary glands and milk production (Ben-Jonathan, 1996; Carretero et al., 2003; Ormandy and Sutherland, 1993; Steinmetz et al., 1993). This hormone also affects the growth and proliferation of other tissues, such as vascular muscle, human epithelial cells and immune system cells (Buckley, 2001; Sabharwal et al., 1992; Sauro and Zorn, 1991). An experimental investigation using neonatal rats has demonstrated that treatment *in vitro* with PRL induces the up-regulation of gap and adherens junctional proteins in pancreatic islets (Collares-Buzato et al., 2001).

The effects of glucocorticoids and PRL seen in mammary gland epithelial cells including the regulation of tight junctions have not been examined in other epithelia. In this work, we investigated the effect of these two hormones on the paracellular barrier function in cultured Madin–Darby canine kidney (MDCK) cells. MDCK cells constitute a very well established cell line that has been extensively used to investigate a variety of cellular processes, including epithelial transport, modulation of the epithelial barrier, and the function of intercellular junctions (Collares-Buzato et al., 1994a, b, 1998a, b).

MATERIALS AND METHODS

Reagents: Sterile plastic material for cell culture was supplied by Nunc (Roskilde, Denmark) or Corning (Corning, NY). Cell culture media and supplements were purchased from Cultilab or Nutricell (Campinas, SP, Brazil). Primary and secondary antibodies were supplied by Zymed (San Francisco, CA) or Sigma (St. Louis, MO). All others chemicals and reagents were supplied by Sigma or Merck. Ovine prolactin was obtained through NHPP, NIDDK and Dr. A. F. Parlow.

Cell culture: MDCK renal epithelial cells were obtained from the Adolfo Lutz Institute (São Paulo, SP, Brazil). The cells were grown in plastic flasks at 37°C in a humidified atmosphere of 5% CO_2/air, using Eagle´s minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 100 IU of penicillin/ml and 100 μg of streptomycin/ml. The cell culture medium was changed every 48 h, and the cells were passaged weekly at confluency with 2.5 mg of trypsin/ml and 100 μg EDTA/ml. For some experiments, the cells were seeded at a high density (4×10^5 – 6.4×10^5 cells/cm^2) onto specific tissue inserts incorporating a permeable inorganic membrane filter (Anocell, Nunc, with membrane area of 0.78 or 4.9 cm^2) or on coverslips (Thermanox, Nunc; 0.78 cm^2 of area).

Treatment with dexamethasone and prolactin: DEX (Decadron injected, Prodome) was diluted in sterile culture media (EMEM) and an aliquot was added to the culture media to give a final concentration of 4 μM. PRL was diluted in 1 mM Na_2HCO_3 solution and an aliquot was added to the culture media to give a final concentration of 2 μg/ml. MDCK were treated simultaneously with DEX and PRL at three time points, i.e. immediately after seeding, after monolayer confluency and following withdrawal of extracellular Ca^{2+}. *Immediately after cell seeding:* Treated MDCK cells were exposed to DEX and PRL immediately after seeding on permeable tissue culture supports and for four days thereafter. The control group was grown in culture media without DEX and PRL. The R_t of treated and control monolayers was measured every 24 h, at which time the culture medium with or without hormones was changed. *Confluent cell monolayers:* MDCK cells were grown for four days and DEX and PRL were then applied to the apical and basolateral surfaces of the monolayers. The control group received the same volume of culture media without hormone. Cell culture medium with or without hormones was changed every 48 h and transepithelial electrical resistance (R_t) measurements were taken aseptically every 24 h up to 96 h. *Ca^{+2} switch assay* (Collares-Buzato et al, 1994b): The culture medium of confluent MDCK monolayers was replaced by Hank's solution without Ca^{+2} (136 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$. $7H_2O$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 2 mM EGTA). After 30 min, the R_t was measured and the buffer solution was exchanged for Ca^{2+}-containing Hank's (same composition as described above except that EGTA was replaced by 1.25 mM $CaCl_2.2H_2O$). DEX and PRL were applied to the apical and basolateral surfaces of treated MDCK monolayers. The control group received the same volume of Ca^{+2}-containing Hank's without these hormones. R_t measurements were done at 1, 3 and 24 h after treatment. In all experimental conditions, control and treated MDCK monolayers

were cultured in EMEM supplemented with lower concentration of fetal calf serum (2%) since this serum may contain a detectable amount of prolactin (Sauro and Zorn, 1991). Cells grown in 2% FCS supplemented media had R_t values and a morphology that were indistinguishable from those of cells cultured in 10% FCS-containing medium.

Measurement of transepithelial electrical resistance (R_t): Transepithelial electrical resistance (R_t) was measured across MDCK monolayers using two Ag/AgCl "chopstick" electrodes coupled to a combined voltmeter and constant current source (EVOM, World Precision Instruments, UK).

Immunostaining of tight junction-associated proteins: Following exposure to DEX and PRL, some monolayers were used to assess the expression and distribution of the tight junction-associated proteins occludin, ZO-1 and claudin-1. MDCK cells were fixed and immunostained for these proteins using a standard indirect immunofluorescence protocol (Collares-Buzato et al, 1998a). Briefly, monolayers were fixed and permeabilized with –20 °C methanol and incubated overnight at 4°C with primary antibodies against occludin (diluted 1:100, polyclonal rabbit anti-occludin antibody, Zymed, USA), ZO-1 (diluted 1:50, polyclonal rabbit anti-ZO-1 antibody, Zymed, USA) or claudin-1 (diluted 1:50, polyclonal rabbit anti-claudin-1 antibody, Zymed, USA), followed by a 2 h incubation with a FITC conjugated specific second antibody (Sigma) at 37°C, in the dark. Subsequently, the monolayers were washed several times with phosphate-buffered saline solution (10 mM PBS, pH 7.4, 136 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4), then detached from the Anocell inserts and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA 94010). Cell staining was detected by confocal laser scanning microscopy (CLSM; Bio-Rad MRC 1024, Bio-Rad, Richmond, CA). Both the treated and control groups, were examined in the same experimental session to allow direct comparison.

Cytochemistry for F-actin: Following treatment with DEX and PRL, MDCK cells were fixed in 3.7% formalin (in PBS) for 30 min then permeabilized with 0.1% Triton X-100 for 10 min (in 10 mM PBS, pH 7.4) and incubated for 2 h with TRITC-labelled phalloidin (diluted 1:50, in 10 mM PBS, pH 7.4) at 37°C, in the dark. The monolayers were then washed several times with PBS buffer (pH 7.4), detached from Anocell inserts and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA 94010). Cell staining was detected by CLSM. Both the treated and control groups were examined in the same experimental session to allow direct comparison.

Western blotting: MDCK cells grown on Anocell membranes (4.9 cm^2) were scraped off and added to an anti-protease cocktail formula (100 mM Tris, 10 mM EDTA, 2 mM phenyl methyl sulfonyl fluoride, PMSF, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 0.03 mg of aprotinin/ml) and sonicated for 15 s. The total protein content of the homogenate was determined using a DC protein assay kit (Bio Rad Laboratories). Aliquots of cell homogenates (20 μg of total protein) from each experimental group (control and treated) were incubated for 60 min at 37^{o}C with one-third of the total homogenate volume of 5X concentrated Laemmli sample buffer (1 M sodium phosphate, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% sodium dodecyl sulfate-SDS, 2% mercaptoethanol). These aliquots were separated by electrophoresis in 6.5% (for detection of ZO-1) or 10% (for detection of occludin and claudin-1) polyacrylamide gels. The electrotransfer of proteins nitrocellulose membranes (Bio-Rad) was done for 1 h at 120 V using a transfer solution containing 25 mM Trizma base, 192 mM glycine, 20% methanol and 0.02% SDS. After checking the efficiency of transfer with Ponceau S dye, the membranes were blocked with 5% dry skimmed milk in TTBS (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween 20) overnight at 4^{o}C. The junctional proteins were detected in the membrane after a 1 h incubation at room temperature (RT) with primary antibodies (polyclonal rabbit anti-occludin, diluted 1:7000; polyclonal rabbit anti-ZO-1, diluted 1:500; polyclonal rabbit anti-claudin-1, diluted 1:500; all from Zymed and diluted in TTBS plus 3% dry skimmed milk) followed by a 1 h incubation at RT with HRP-conjugated specific second antibody (goat or guinea pig anti-rabbit IgG, Sigma, diluted 1:1000 in TTBS plus 1% dry skimmed milk). Immunoreactive bands were detected by chemiluminescence (Super Signal West Pico chemiluminescent substrate - Pierce).

Phenol red flux: The paracellular permeability of MDCK monolayers following treatment with DEX+PRL was assessed using the phenol red flux assay (Jovov et al., 1991). After 4 days of treatment, MDCK cells grown on Anocell membranes (4.9 cm^2) were transferred to a new 6-well plate containing 2% FCS-supplemented EMEM without phenol red. The apical medium was replaced by culture medium containing 20 mg of phenol red/ml. After a 4 h incubation at 37 ^{o}C, an aliquot of apical and basal media was collected and read at 492 nm in a microplate reader (Multiskan MS, Labsystems). The phenol red flux was expressed as a percentage of the mean absorbance of the control group (taken as 100% dye flux). DEX and PRL were present in the treated group throughout this incubation.

Analysis of cell proliferation and viability: Cell proliferation and viability were analyzed using the neutral red viability assay (Borenfreud and Puerner, 1984) and by counting cells pre-stained with methyl-green dye. Confluent cell monolayers were exposure to DEX (4 μM) and to PRL (2 μg/ml) for four days. For the neutral red viability assay, MDCK cells were seeded in 96 well-plates at a cell density of 1.96×10^5 cells per well. Following exposure to DEX and PRL, the medium were removed and 200 μl of MEM supplemented with 2% FCS and containing neutral red (5 μg/ml) were added to each well. The plate was incubated for 3 h at 37ºC, after which the medium containing the dye was removed and each well was washed twice with formol-calcium solution (40% formaldehyde, 1% anhydrous calcium chloride). Finally, 200 μl of an acetic acid-ethanol solution (containing 1 ml of glacial acetic acid in 100 ml of ethanol) were added to each well followed by incubation for 15 min at RT. The plate was read at 540 nm in a microplate reader (Multiskan MS). The cell viability was determined by comparing the absorbances of treated cells with the mean absorbances obtained with control wells (without treatment), which corresponded to 100% cell viability. For cell counting, MDCK cells were seeded on coverslips and, following treatment with DEX and PRL, were fixed with 4% formaldehyde for 10 min and then stained with methyl-green dye which binds to DNA in the nucleus. Total cell counts and the number of cell nuclei with condensed chromatin were determined from 6 monolayers per group, with 10 areas of 0.02 mm^2 being determined in each of monolayers using light microscopy.

Statistical Analysis: All numerical results were expressed as the mean ± standard error (SE). The statistical significance between two groups was determined by Student's t-test. For multiple comparisons, the statistical significance was assessed by ANOVA followed by the Bonferroni test. The significance level was set at $P < 0.05$.

RESULTS

As shown in Figure 1a, the treatment of confluent monolayers of MDCK with DEX (4 μM) and PRL (2 μg/ml) increased the transepithelial electrical resistance (R_t) by 53% after 24-96 h. When the treatment was initiated just after cell seeding, a significant increase in R_t of approximately 27% was only observed after 24 h compared with the control group (Fig. 1b). Nevertheless, the treatment with these hormones did not significantly alter the R_t after disruption of the TJ using the Ca^{2+} switch assay (Fig. 1c).

The phenol red flux assay was used to assess whether treatment with DEX + PRL affected the paracellular barrier function in confluent MDCK monolayers (Fig. 2). This method has been extensively used to assess the increase or decrease in paracellular permeability, although for the latter, the phenol red flux assay is less sensitive. A 96 h-exposure of the cells to these substances did not significantly after the apical-to-basal phenol red flux, compared to the controls, although there was a tendency towards a decrease.

As assessed by western blotting, treatment with DEX + PRL for 4 days did not change the expression of the tight junction-associated proteins occludin (Fig. 3a), ZO-1 (Fig. 3b) and claudin-1 (Fig. 3c), in confluent MDCK monolayers. In addition, MDCK monolayers labelled with FITC-conjugated phalloidin (Fig. 4) or immunostained for occludin (Fig. 5a, b), claudin-1 (Fig. 5c, d) and ZO-1 (Fig. 5e, f) showed no alteration in their cellular distribution or expression of these cytoskeletal and junctional proteins after treatment with DEX + PRL when compared with control monolayers. The expression and localization of occludin and ZO-1 proteins in treated MDCK just after cell seeding were also analyzed by western blotting and immunofluorescence. No changes were observed in the expression and distribution of the tight junction-associated proteins compared to the control cells (data not shown).

The effects of treatment with DEX + PRL for 4 days on cell viability and the proliferation of confluent MDCK monolayers were assessed by the neutral red assay and by counting cells pre-stained with methyl-green dye. As shown in Figure 6, the treatment significantly increased the neutral red absorbance by approximately 50% as compared to the controls. The counting of methyl-green-stained cells revealed that exposure to DEX + PRL significantly increased the number of cells per area by approximately 13% (Fig. 7). In addition, the treatment decreased the percentage of nuclei with condensed chromatin compared to the controls (data not shown).

DISCUSSION

Studies *in vivo* and *in vitro* have established that glucocorticoids and PRL are essential for the induction and maintenance of lactation in mammary glands (Dembinski and Shiu, 1987; Forsyth, 1983; Neville et al., 2001). The onset of lactation is correlated with an increase in the structural development of tight junctions (Pitelka, 1978), and glucocorticoids, in combination with PRL, have a direct effect on the formation and maintenance of mammary TJ (Stelwagen et al., 1999; Zettl et al., 1992). During lactogenesis, TJs form when the mammary gland switches from a

growth state to a differentiated state (when milk synthesis and secretion are initiated), and this switch coincides with decreasing levels of progesterone (which prevents mammary cell differentiation and inhibits TJ formation) and, concomitantly, with an increase in the levels of PRL and glucocorticoids (Convey, 1974; Linzell and Peaker, 1974; Nguyen and Neville, 1998). Mammary TJs are leaky during pregnancy and close around parturition to form a tight barrier that prevents the paracellular movement of molecules across the mammary epithelium (Nguyen et al, 2001). The importance of TJs in maintaining milk synthesis is further demonstrated by the fact that experimental disruption of TJs in the lactating mammary gland results in a significant decrease in milk synthesis (Stelwagen et al., 1995, 1997).

In this work, we investigated the ability of glucocorticoids and PRL in enhancing the epithelial barrier function by affecting the structure of TJs in MDCK cells. These cells constitute a very well-established epithelial cell line obtained from a total kidney suspension of a normal adult Cocker Spaniel and show many morphological and functional characteristics of distal and/or collecting tubule cells (Barker and Simmons, 1981; Valentich, 1981). In addition, these cells respond to glucocorticoids, indicating that they express membrane receptors to this class of hormones (Shu et al., 2001; Taub et al., 1979). A significant increase in the transepithelial electrical resistance (R_t) and a tendency towards a decrease in the apical-to-basal flux of extracellular marker were observed after chronic treatment of confluent MDCK monolayers with DEX (4 μM) and PRL (2 μg/ml). However, the increase in R_t was not associated with alterations in the expression and distribution of the tight junction-associated proteins occludin, ZO-1 and claudin-1, or in cytoskeletal F-actin.

Recent studies have documented a similar effect of DEX (1 μM) and PRL (1 μg/ml) on R_t in monolayers of a nontransformed mouse mammary epithelial cell line. However, in contrast to MDCK cells, both hormones at these doses increased the synthesis of the transmembrane TJ protein occludin, indicating the formation of tight junctions (Nguyen et al., 2001; Stelwagen et al., 1999; Zettl et al., 1992). Stelwagen et al. (1999) also showed that DEX and PRL modulated the expression of the TJ-associated protein ZO-1, since there was a small increase in the expression of the α^--isoform of ZO-1 relative to the α^+-isoform.

These results clearly show that DEX and PRL influence the barrier function properties of mammary cells by stimulating the synthesis of structural components of the tight junction or modulating the expression of regulatory factors that influence the localization, stability or assembly

of TJ protein in these cells. However, our results suggest that this effect may not be a general phenomenon in epithelia. In contrast to MDCK cells, mammary gland cells express the α^--isoform of ZO-1, which has been associated with cells displaying structurally dynamic TJs, including podocytes and Sertoli cells (Balda and Anderson, 1993). In these cells, TJs can rapidly and actively open and reseal. This ability may explain the differences in the TJ responses of MDCK and mammary gland cells to DEX and PRL.

Although no changes were observed in the TJ structure and function, MDCK cells treated with DEX and PRL showed a significant higher number of viable cells compared to the control group, as indicated by the neutral red assay and the number of cells stained with methyl-green dye. The epithelial barrier consists of epithelial cells, cell membranes and intercellular junctions. Thus, the main effect of DEX and PRL on barrier function in MDCK renal cells may be associated with the induction of epithelial cell proliferation or the inhibition of cell death, and not the stimulation of TJ formation, as observed in mammary gland cells. Since epithelial cell proliferation supports the epithelial barrier integrity, treatment with DEX+PRL may prevent the rupture of the barrier by avoiding the formation of lacunas in the epithelia that results from the detachment of dead cells from the MDCK monolayer. DEX and PRL may also influence the lactogenesis of mammary glands by preventing apoptosis. In late pregnancy and after weaning, the level of glucocorticoids drops and these is a concomitant increase in cell death by apoptosis in mammary gland cells. In culture, insulin, PRL and glucocorticoids maintain mammary explants in a state of lactation by inhibiting involution and programmed cell death (Feng et al., 1995; Nguyen et al., 2001).

Nguyen et al. (2001) have suggested that part of the effect of PRL on the barrier function of mammary epithelium is through its action on the maintenance of epithelium integrity by preventing apoptosis, with secondary effects on epithelial permeability. Interestingly, PRL and glucocorticoids induce cell proliferation in other tissues, including kidney cells (Holley and Kieman, 1974; Horster, 1980; Sauro and Zorn, 1991; Wilson and Horster, 1983). Both of these hormones induce proliferation in primary cultures of rabbit distal nephron epithelia, with dexamethasone causing maximal stimulation of [^{3}H] thymidine uptake by cortical collecting tubule cells in unsupplemented culture media (Horster, 1980; Wilson and Horster, 1983). Sakai et al. (1999) have shown that parietal epithelial cells of Bowman's capsule synthesize PRL *de novo* and suggested that PRL expressed in this tissue functioned in an autocrine/paracrine fashion to maintain parietal and tubular cell proliferation. Thus, these hormones may be important maintaining the barrier function of renal epithelium *in vivo*.

In conclusion, DEX and PRL increased the epithelial barrier function in confluent MDCK monolayers. An increase in R_t was also detected in treated MDCK cells after 24 h of cell seeding, a time point that probably corresponded to the exponential phase of MDCK cell growth in culture. However, no difference in this biophysical parameter was observed between DEX+PRL treated and control groups after the Ca^{2+} switch assay. This finding may indicate that these hormones do not affect the formation and/or assembly of epithelial TJ. Immunocytochemical analysis showed no alteration in the distribution of the junctional proteins occludin, ZO-1 and claudin-1. In addition, no significant changes were observed in F-actin distribution, as detected by cytochemistry with phallodin. Western blotting confirmed that there were no changes in the expression of TJ-associated proteins. However, the neutral red viability assay and cell counting of methyl green-stained MDCK cells showed that there was an increase in the number of viable cells per area following treatment. Thus, in contrast to mammary gland cells, the effects of DEX and PRL on renal epithelial barrier function appear to involve an increase in cell proliferation and/or viability rather than an increase in the expression of TJ-associated proteins.

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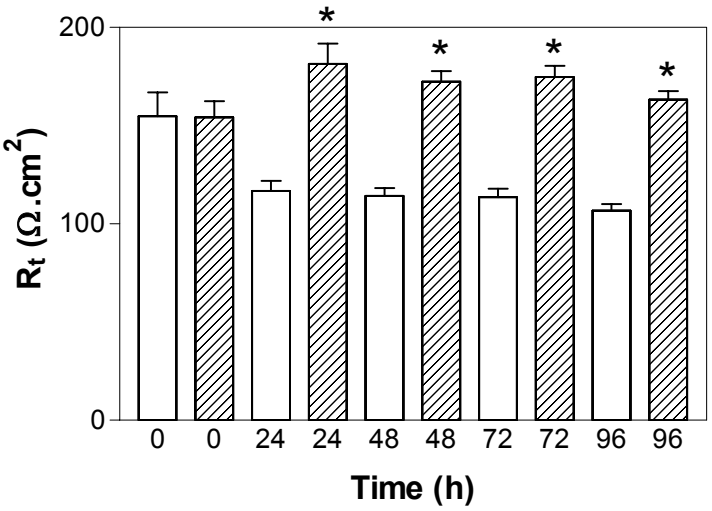
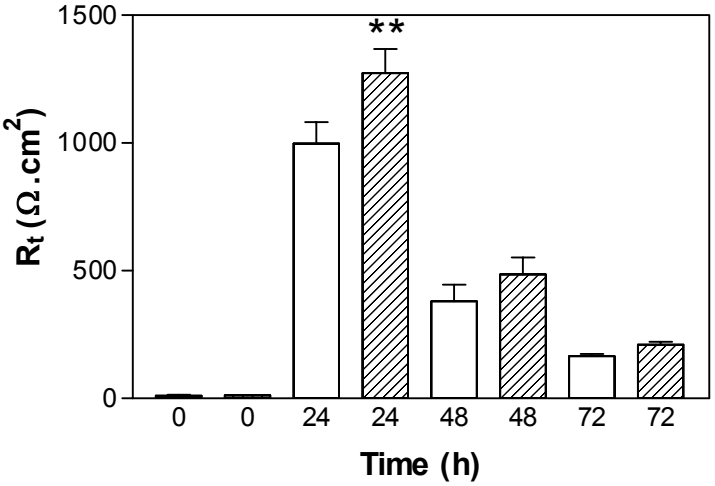
Figure 1: Transepithelial electrical resistance (R_t) across MDCK monolayers exposed to dexamethasone (DEX, 4μM) and prolactin (PRL, 2μg/ml) in culture medium for 4 days. Both substances were applied to the apical and basolateral surfaces of the monolayers. The control group was cultured for the same time but without treatment. (**a**) In confluent monolayers (three days of culture), DEX and PRL significantly increased the R_t compared to the control group. (**b**) During monolayer formation after cell seeding, a significant increase in R_t was observed only at 24 h of treatment. (**c**) DEX and PRL caused no significant alterations in R_t following TJ disruption using the Ca^{2+} switch assay compared to the control group. The columns are the mean ± SE of 5 to 13 monolayers per group from three independent experiments. *P<0.001 and **P<0.01, compared to the corresponding control (ANOVA and Bonferroni's test).

Figure 1

a) Confluent Monolayers



b) After cell seeding



c) Ca^{2+} switch assay

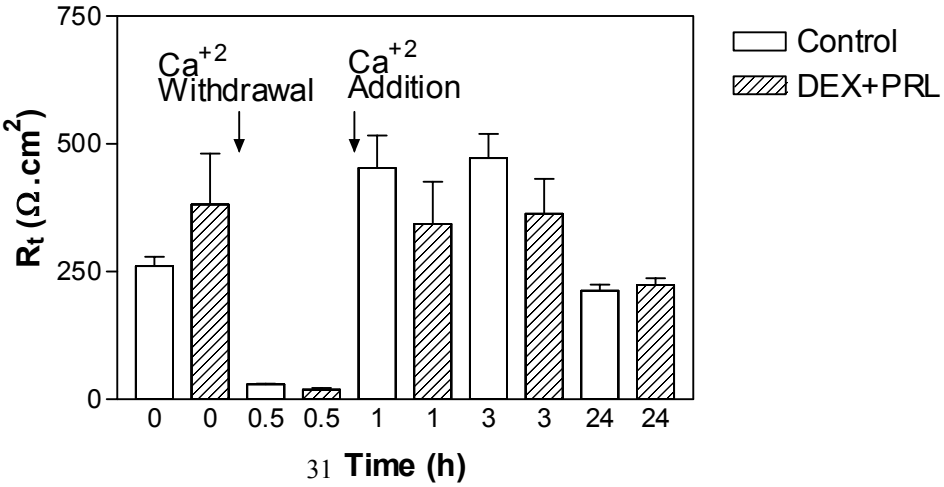


Figure 2: The apical-to-basal phenol red flux in confluent MDCK monolayers exposed to DEX (4μM) and PRL (2μg/ml) in culture medium for 4 days. Phenol red at concentration of 20 mg/ml was applied at the apical bathing media. An aliquot of the basal phenol red-free bathing media and of the apical bathing media were collected after 4 h incubation. Phenol red flux assay showed a tendency of decrease in the apical-to-basal phenol red flux compared to the control. The control group was taken as 100% dye flux value.

Figure 2

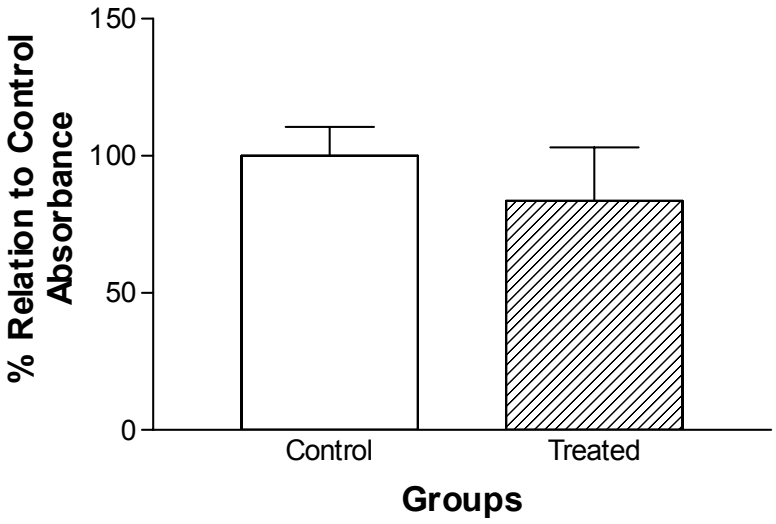

150
100
50
0
% Relation to Control Absorbance
Control
Treated
Groups

Figure 3: Expression of the tight junction-associated proteins occludin (**a**, **a'**), ZO-1 (**b**, **b'**) and claudin-1 (**c**, **c'**), in confluent MDCK monolayers exposed to DEX (4μM) and PRL (2μg/ml) in culture medium for 4 days. Western blotting showed no change in the expression of these three junctional proteins compared to the control group. Panels **a**, **b** and **c** are representative immunoblots, while panels **a', b'** and **c'** are the means± SEM of the arbitrary densitometric values for occludin (**a'**), ZO-1 (**b'**) and claudin-1 (**c'**) from three independent experiments.

Figure 3

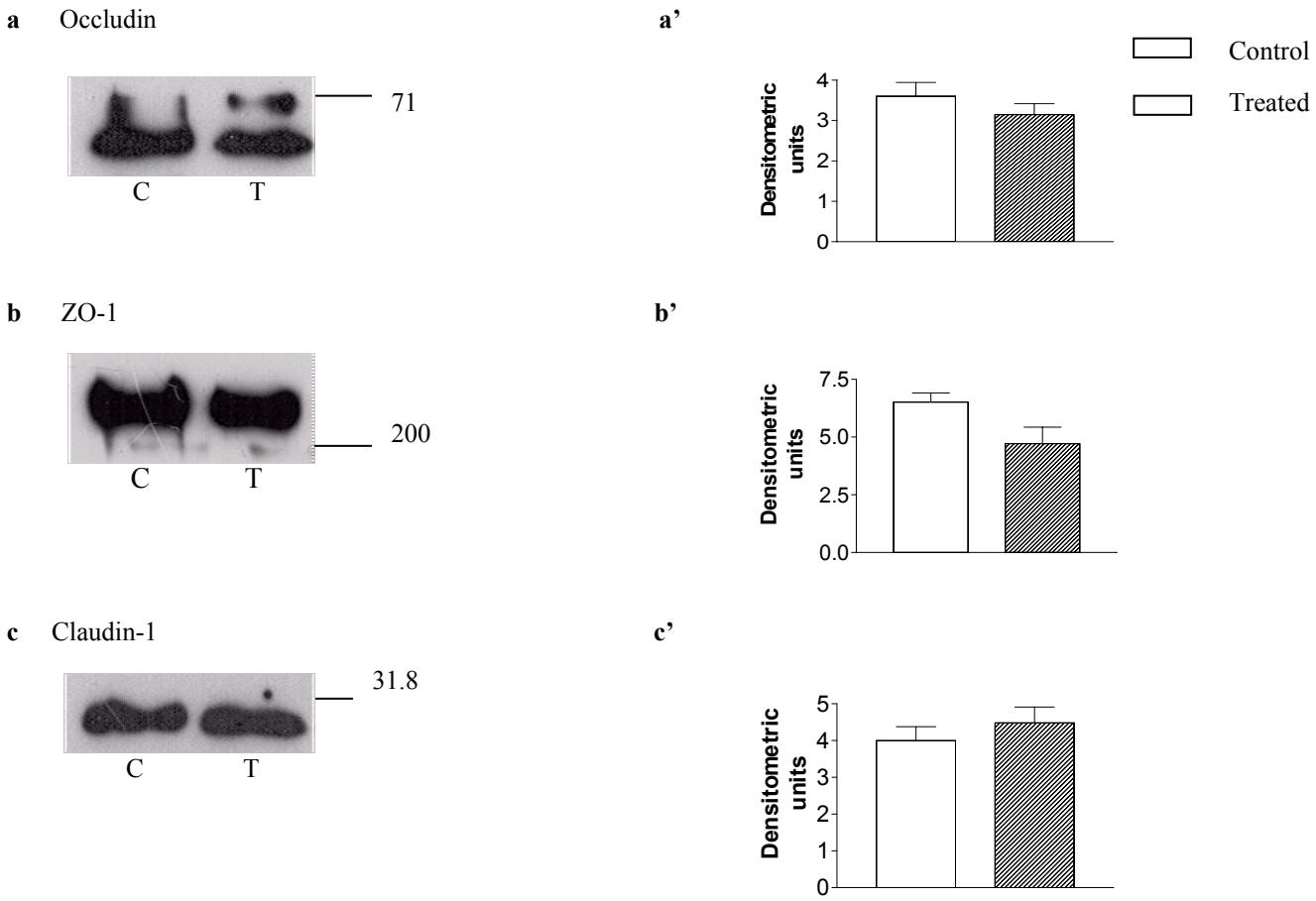

a Occludin
71
C
T
a'
Control
Treated
Densitometric units
4
3
2
1
0
b ZO-1
200
C
T
b'
Densitometric units
7.5
5.0
2.5
0.0
c Claudin-1
31.8
C
T
c'
Densitometric units
5
4
3
2
1
0

Figure 4: F-actin distribution in confluent MDCK cells following treatment with dexamethasone (DEX) and prolactin (PRL). MDCK monolayers were stained with TRITC-labelled phalloidin to reveal F-actin microfilaments. Confluent MDCK monolayers from the treated group were exposed to DEX (4μM) and PRL (2μg/ml) in culture medium for 4 days (**b**, **d** and **f**). The substances were applied to the apical and basolateral surfaces of the monolayers. The control group was cultured for the same time but without stimulation (**a**, **c** and **e**). The panels display representative confocal "en face" (X-Y) images obtained at the apical (**a** and **b**), middle (**c** and **d**), and basal (**e** and **f**) levels of the monolayer, using the same level of CLSM sensitivity to obtain the images of both experimental groups. Note that there was no change in the F-actin distribution after this treatment in MDCK. Bar, 30 μm.

Figure 4

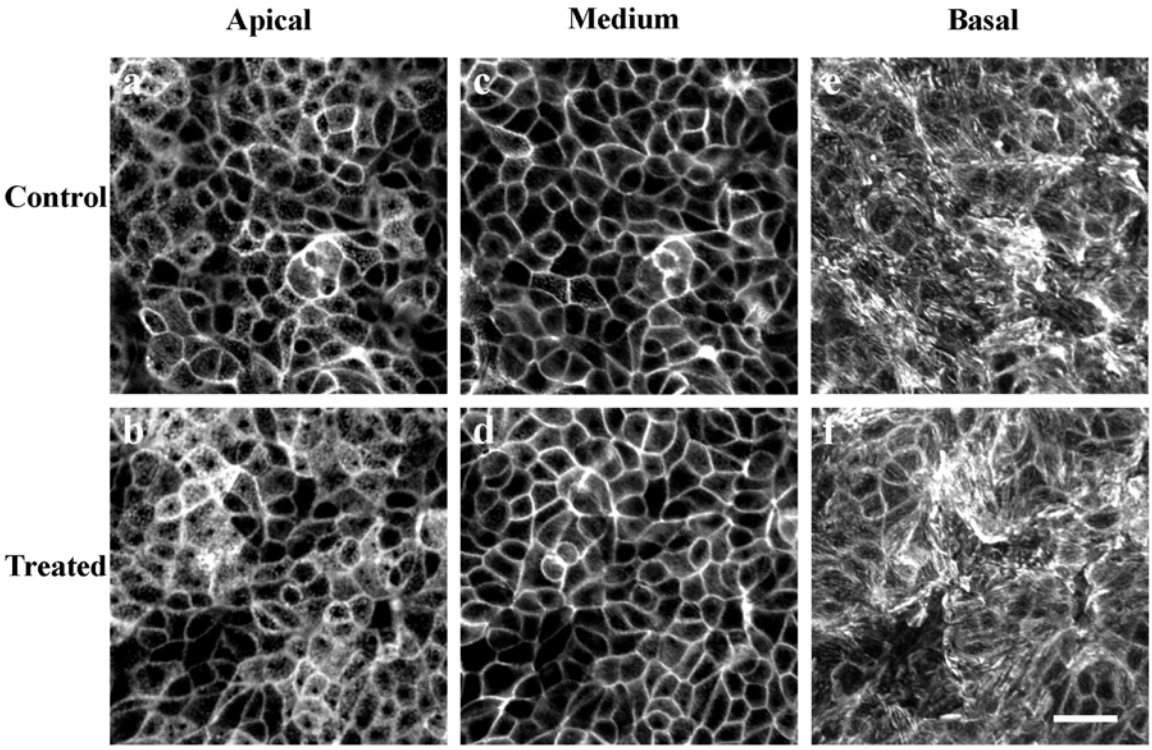


Figure 5: Cellular expression and distribution of the tight junction-associated proteins occludin (**a** and **b**), claudin-1 (**c** and **d**) and ZO-1 (**e** and **f**) in confluent MDCK monolayers following treatment with dexamethasone (DEX) and prolactin (PRL). The monolayers were fixed and immunostained for these proteins using a standard indirect immunofluorescence protocol. Confluent MDCK monolayers of the treated group were exposed to DEX (4μM) and PRL (2μg/ml) in culture medium for 4 days (**b**, **d** and **f**). The control group was cultured for the same time but without stimulation (**a**, **c** and **e**). These photomicrographs are representative confocal "en face" (X-Y) images obtained in the middle level of the monolayer using the same level of CLSM sensitivity to obtain the images of both experimental groups. Note again that the treatment produced no significant changes in the expression and distribution of occludin, claudin-1 and ZO-1 compared to the control cells. Bar, 30 μm.

Figure 5

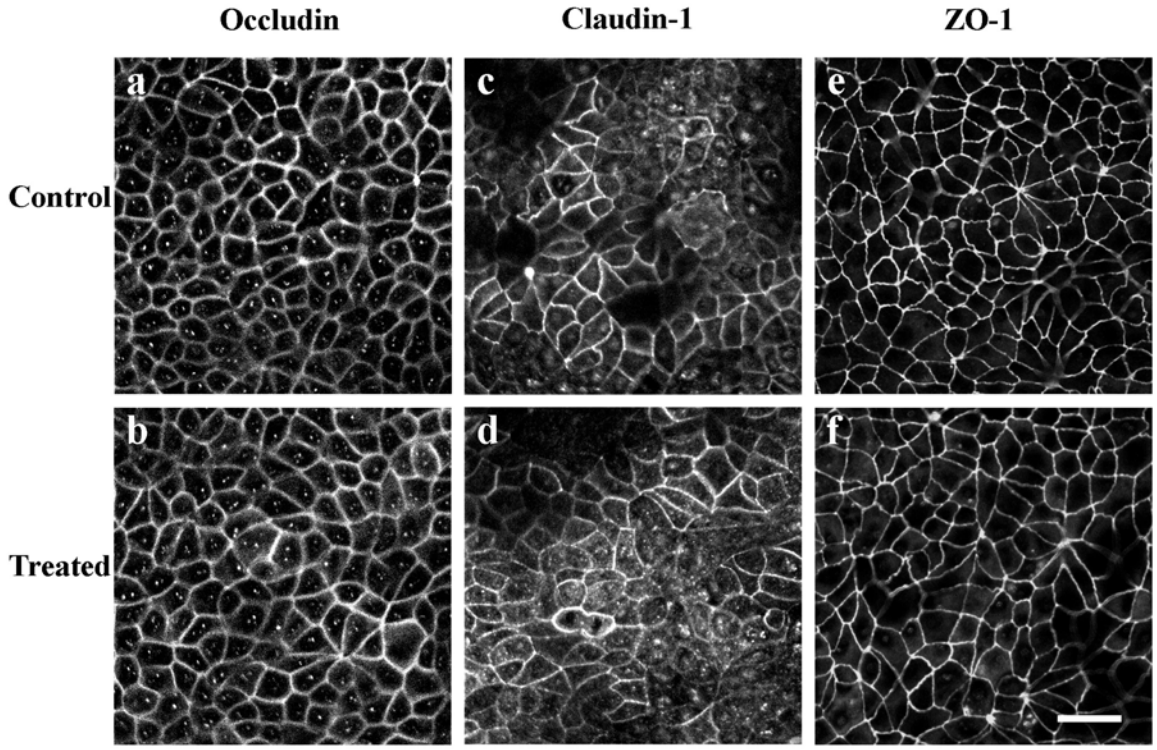


Figure 6: Viability of confluent MDCK cells following exposure to dexamethasone (DEX) and prolactin (PRL), as assessed by neutral red cell uptake. MDCK cells were exposed to DEX (4μM) and PRL (2μg/ml) in culture medium for 4 days. Treatment with DEX+PRL significantly increased the neutral red uptake compared to the control. *P<0,05 (Student's t-test).

Figure 6

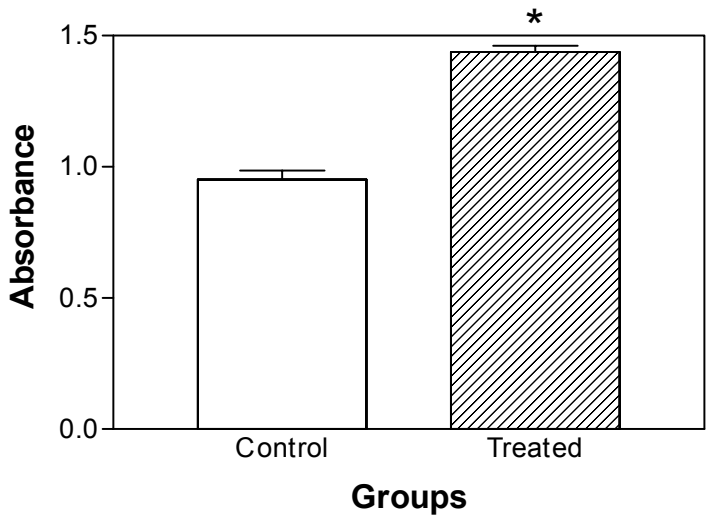
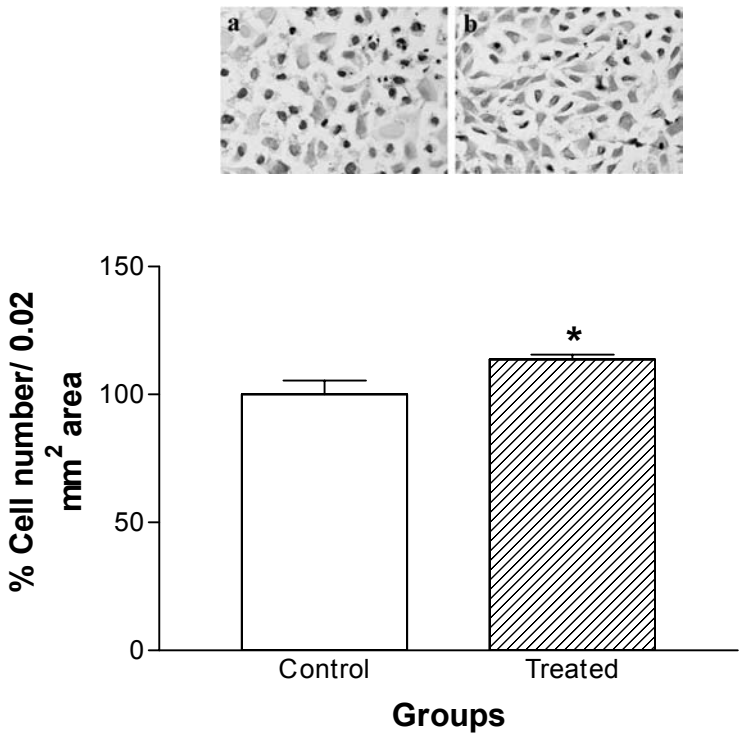


Figure 7: Cell proliferation following treatment with dexamethasone (DEX) and prolactin (PRL), as assessed by counting methyl green-stained cells in treated and control MDCK monolayers. MDCK cells were exposed to DEX (4μM) and PRL (2μg/ml) in culture medium for 4 days. The control group was cultured for the same time but without treatment. A significant increase in the number of cells per area unit was observed compared to the control group (*P<0,05 Student's t-test). The mean total number of control cells (without treatment) was taken as 100%. The values were obtained from 6 monolayers per group, with 10 areas of 0.02 mm^2 being determined in each of monolayers. Inserts **a** and **b** show control and DEX+PRL treated monolayers, respectively.

Figure 7


a
b
150
100
50
0
% Cell number/ 0.02 mm2 area
*
Control
Treated
Groups

CAPÍTULO 3

EFFECT OF PROTAMINE UPON EPITHELIAL BARRIER FUNCTION AND INVOLVEMENT OF THE CYTOSKELETON AND TIGHT JUNCTION-ASSOCIATED PROTEINS IN CULTURED MDCK CELLS

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Abstract

Natural and synthetic polycationic proteins, such as protamine, have been used to reproduce the tissue injury and epithelial permeability caused by positively charged substances released by polymorphonuclear cells during inflammation. Protamine has diverse, and often conflicting effects on epithelial permeability. The effects of this polycation on the distribution and expression of tight junction (TJ)-associated proteins have not yet been investigated. In this work, we examined the influence of protamine on paracellular barrier function and TJ structure using two strains of the epithelial Madin-Darby canine kidney (MDCK) cell line that differed in their TJ properties ("tight" TJ-strain I and "leaky" TJ-strain II). Protamine induced concentration-time- and strain-dependent alterations in transepithelial electrical resistance (R_t) only when applied to apical or apical+basolateral monolayer surfaces, indicating a polarity of action. In MDCK II cells, protamine 50 μg/ml caused a significant increase in R_t that returned to control values after 2 h. However, the treatment of this MDCK strain with a higher concentration of protamine (250 μg/ml) significantly decreased the R_t after 30 min. In contrast, treated MDCK I monolayers showed a significant decrease in R_t after apical treatment with protamine at both concentrations. The protamine-induced decrease in R_t was paralleled by an increase in the phenol red basal-to-apical flux in both MDCK strains, suggesting disruption of the paracellular barrier. Marked changes in cytoskeletal F-actin distribution and a significant reduction in the junctional expression of the tight junctional proteins

occludin and claudin-1 but subtle alterations in ZO-1 were observed following protamine-elicited paracellular barrier disruption. In conclusion, protamine induces alterations in the epithelial barrier function of MDCK monolayers that may involve the cytoskeleton and TJ-associated proteins. The various actions of protamine on epithelial function may reflect different degrees of interaction of protamine with the plasma membrane and different intracellular processes triggered by this polycation.

INTRODUCTION

One of the most important attributes of epithelia is their ability function as a barrier that prevents the permeation of noxious agents to the internal milieu and, at the same time, contribute to the vectorial transepithelial transport of nutrients, electrolytes and water (Madara, 1990). These roles depend on membrane specializations in the cell-cell contact region which forms the junctional complex, i.e. the tight junction (TJ), the zonula adherens and desmosomes. The TJ has been considered the specific anatomical site corresponding to the route-limiting permeability barrier of epithelia since cellular membranes of adjacent cells are at their closest apposition in this region (Stevenson et al., 1988; Schneeberger and Lynch, 1992; Nusrat et al., 2000; Anderson et al., 2001).

A number of experimental interventions and pathophysiological states can alter the paracellular permeability and modify the expression, cellular distribution and/or phosphorylation of several TJ-associated proteins, and also change the functional interactions between these proteins and the cytoskeleton (Madara et al., 1986; Polak-Charcon, 1992; Collares-Buzato et al., 1994a, 1994b, 1998; Jepson et al., 1995; Collares-Buzato, 2001).

The effect of natural and synthetic polycationic proteins, such as protamine and poly-L-lysine, upon epithelial permeability has been investigated in several tissues *in vivo* and *in vitro*. These polycations have been used to reproduce the charge-mediated effects, such as damage to the cell membrane and increased capillary permeability, of some positively-charged substances released by polymorphonuclear cells during inflammation (Fromm et al., 1985; Bentzel et al., 1987; Chang et al., 1987; Peterson and Gruenhaupt, 1990; McEwan et al., 1993). In the case of protamine, understanding its mechanisms of action is of interest because of its clinical use to reverse the anticoagulant effect of heparin, and also its possible involvement in the development of nephrotoxicity and noncardiogenic pulmonary edema (Olinger, 1980; Fromm et al., 1985).

Protamine has diverse, sometimes conflicting, effects on epithelial permeability since it may increase or decrease the transepithelial electrical resistance, depending on the tissue type, and this may or may not be accompanied by changes in apical membrane resistance. These results are difficult to compare not only because of differences in the animal species and cell types used, but also because of the rather wide range of concentrations of protamine tested. Alterations in the structure and function of TJ have been indicated as a possible cause of the changes in epithelial permeability. However, the effect of protamine upon the distribution and expression of TJ-associated proteins has not yet been investigated.

In this work, we examined the action of protamine on paracellular barrier function and on the structure and function of the TJ using cultured Madin–Darby canine kidney (MDCK) cells as an *in vitro* model system of epithelia. To determine whether the protamine-induced changes in epithelial barrier function were dependent upon the nature of the junctional complex, we used two strains of MDCK cells: strain I, which displays characteristics consistent with those of "tight" epithelia ($R_t \sim 5000\ \Omega cm^2$) and strain II, which has "leaky", cation-selective tight junctions with R_t values of $\sim 200\ \Omega cm^2$ (Collares-Buzato et al., 1994a, 1998).

MATERIALS AND METHODS

Reagents: Sterile plastic material for cell culture was supplied by Nunc (Roskilde, Denmark) or Corning (Corning, NY). Cell culture media and supplements were purchased from Cultilab or Nutricell (Campinas, SP, Brazil). Primary and secondary antibodies were supplied by Zymed (San Francisco, CA) or Sigma (St. Louis, MO). All other chemicals and reagents were supplied by Sigma and Merck.

Cell culture: Two strains of MDCK renal epithelial cells were studied in this work: the low resistant MDCK II was obtained from the Adolfo Lutz Institute (São Paulo, SP, Brazil) and the high resistant MDCK I was donated by Dr. Barry H. Hirst (University of Newcastle upon Tyne, UK). The cells were grown in plastic flasks at 37°C, in an humidified atmosphere of 5% CO_2/air, with Eagle´s minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The culture medium was changed every 48 h and, at confluence, the cells were passaged weekly with trypsin (2,5 mg/ml) and EDTA (100 μg/ml). For

the experiments, cells were seeded at a high density (0.5×10^6 cells/cm^2) on to specific tissue inserts incorporating a permeable inorganic membrane filter (Anocell, membrane area of 0.78 cm^2) and grown in supplemented EMEM for 4 days.

Treatment with protamine: Protamine base (Sigma) was diluted in buffered Krebs-Ringer (27.4 mM NaCl, 1 mM KCl, 0.2 mM $MgSO_4.7H_2O$, 0.06 mM KH_2PO_4, 0.06 mM $NaH_2PO_4.H_2O$, 2.8 mM Trizma base, 0.56 mM $CaCl_2.2H_2O$, and 1.8 mM glucose) to a final concentration of 50 μg/ml or 250 μg/ml. The protamine-containing Krebs-Ringer (pH 7.4, adjusted with HCl after protamine addition) was applied to the apical and/or basolateral surfaces of the MDCK II monolayers. MDCK I monolayers were treated with protamine only on their apical surface. The control cells received the same volume of Krebs-Ringer without the polycation. The cells were maintained at of 37°C throughout the experiment.

Measurement of the transepithelial electrical resistance (R_t): Transepithelial electrical resistance (R_t) was measured across MDCK monolayers using two Ag/AgCl "chopstick" electrodes coupled to a combined voltmeter and constant current source (EVOM, World Precision Instruments, UK). R_t measurements were obtained at 10, 30, 60 and 120 min after treatment.

Immunostaining of tight junction-associated proteins: Following exposure to protamine, some monolayers were prepared for analysis of the expression and distribution of the tight junction-associated proteins occludin, ZO-1 and claudin-1. MDCK cells were fixed and immunostained for these proteins using a standard indirect immunofluorescence protocol (Collares-Buzato et al., 1998). Briefly, monolayers were fixed and permeabilized with methanol at –20 °C and then incubated overnight at 4°C with primary antibodies against occludin (dilution 1:100, polyclonal rabbit anti-occludin antibody, Zymed, USA), ZO-1 (dilution 1:50, polyclonal rabbit anti-ZO-1 antibody, Zymed, USA) or claudin-1 (dilution 1:50, polyclonal rabbit anti-claudin-1 antibody, Zymed, USA), followed by a 2 h incubation with FITC-conjugated specific secondans antibody (Sigma) at room temperature (RT), in the dark. Subsequently, monolayers were washed several times with phosphate-buffered saline solution (10 mM PBS, pH 7.4, 136 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4), and then detached from the Anocell inserts and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Cell staining was detected by confocal laser scanning microscopy (CLSM, Bio-Rad MRC 1024, Bio-Rad, Rechmond, CA). To allow

comparison between the treated and control groups, the microscopic examination of both groups was done in the same experimental session. Staining was absent from negative control inserts in which the primary antibodies were omitted.

Cytochemistry for F-actin: Following treatment with protamine, MDCK cells were fixed with 3.7% formalin solution (in 10 mM PBS, pH 7.4) for 30 min, permeabilized with 0.1% Triton X-100 for 10 min (in 10 mM PBS, pH 7.4) and incubated for 2 h with TRITC-labelled phalloidin (dilution 1:50, in 10 mM PBS, pH 7.4) at 37°C, in the dark. The monolayers were then washed several times with PBS buffer (pH 7.4), detached from the Anocell inserts, and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Cell staining was detected by CLSM. To allow comparison between the treated and control groups, the microscopic examination was done in the same experimental session.

Phenol red flux: The phenol red flux was assayed as described before, with some modifications (Jovov et al., 1991). After 4 days in culture, MDCK cells seeded on permeable tissue culture supports were transferred to a new plate containing buffered Krebs-Ringer and phenol red (20 mg/ml) (basolateral surface). The apical medium was replaced by a Krebs-Ringer solution without phenol red. The apical surface of the treated group received Krebs buffer containing protamine, while the control group received the same volume of Krebs without polycation. After 30 min, 1 h and 2 h of incubation, 0.4 ml samples were taken from the apical surface and, at the end of the 2 h incubation period, the whole basal bathing solution was collected. All of the samples were read at 492 nm in a microplate reader (Labsystems Multiskan MS). The basal-to-apical phenol red flux ($F_{b\rightarrow a}$) was calculated as:

$$F_{b\rightarrow a} = Abs_{ap} \times 100\% / Abs_{ap} + Abs_{bas}$$

where $F_{b\rightarrow a}$ is the basal-to-apical phenol red flux, Abs_{ap} is the absorbance of the apical sample and Abs_{bas} is the absorbance of the basal sample. The flux results were expressed as a percentage of the mean $F_{b\rightarrow a}$ value of the control group (taken as 100% dye flux).

Analysis of cell viability: The potential cytotoxic effect of protamine in MDCK cells was analyzed using the neutral red dye assay (Borenfreud and Puerner, 1984). For this procedure, MDCK cells

were seeded in 96-well plates at a density of 1.96×10^5 cells per well. After 4 days in culture, the EMEM was replaced by buffered Krebs-Ringer containing different concentrations of protamine in the treated group, and by Krebs-Ringer without protamine in the control group. Following a 2 h treatment at 37ºC, the buffer was removed and 200 µl of Krebs containing neutral red (5 µg/ml) were added to each well. The plate was incubated for 3 h at 37ºC after which the buffer containing the dye was removed and the wells were washed twice with formol-calcium (40% formaldehyde, 1% anhydrous calcium chloride, w/w). Finally, 200 µl of an acetic acid-ethanol solution (1 ml of glacial acetic acid in 100 ml of ethanol) were added to each well followed by incubation for 15 min at RT. The plate was read at 540 nm in a microplate reader (Labsystems). As a positive control for cytotoxicity, some monolayers were treated with 0.5% Triton X-100 (diluted in Krebs-Ringer, pH 7.4) for 2 h at 37ºC. The cell viability was expressed as a percentage of the mean absorbance values obtained for the control wells (taken as 100%).

Statistical analysis: All numerical results were expressed as the mean ± standard error (SE). Statistical comparisons between two groups were done using Student's t-test. For multiple comparisons, the significance was assessed by ANOVA followed by the Bonferroni test. The level of significance was set at $P \leq 0.05$.

RESULTS

As shown in Figure 1, protamine (50 and 250 µg/ml) caused significant alterations in the transepithelial electrical resistance (R_t) only when applied to the apical and apical+basolateral surfaces of MDCK II monolayers, indicating polarity for the effect of protamine in these cells. Figure 2 shows the time-course and dose-dependence for the apical treatment with protamine in MDCK II and I monolayers. In MDCK II cells, protamine (50 µg/ml) increased the R_t by approximately 50% after 10 min of exposure when compared to the control (Figure 2a). However, treatment with a higher concentration of protamine (250 µg/ml) significantly decreased the R_t by approximately 82% compared to the control, after a 30 min exposure (Figure 2b). In contrast to MDCK II cells, protamine-treated MDCK I monolayers showed a significant decrease in R_t after exposure to protamine at both concentrations: the lower concentration (50 µg/ml) decreased the R_t by approximately 74% after a 30 min of exposure (Figure 2c), while the higher concentration (250

μg/ml) reduced the R_t by approximately 82% after 10 min when compared to the control cells (Figure 2d).

The phenol red flux assay was used to assess whether protamine specifically affected the paracellular barrier function in MDCK II and I monolayers (Figure 3). MDCK II cells exposed to protamine (50 μg/ml) for 2 h showed a 160% increase in the phenol red $F_{b\rightarrow a}$ when compared to the controls (Figure 3a) and treatment with a higher concentration (250 μg/ml) resulted in an even greater increase (approximately 260%) in the phenol red $F_{b\rightarrow a}$ (Figure 3b). In MDCK I cells, a 30 min exposure to the highest protamine concentration also significantly increased the basal-to-apical phenol red flux in this strain, but to a much lesser extent than in MDCK II cells (approximately 47% increase compared to the controls) (Figure 3c).

To ascertain that the observed changes in epithelial permeability were not due to cell death induced by protamine, the viability of MDCK II monolayers was assessed using the neutral red assay following treatment with protamine (Figure 4). Protamine did not alter the neutral red absorbance compared to the control cells, indicating that neither of the protamine concentrations used (50 and 250 μg/ml) had any cytotoxic effect on cultured MDCK cells.

To assess whether protamine altered the cytoskeleton in the two MDCK strains, monolayers were stained with FITC-conjugated phalloidin to reveal F-actin (Figures 5 and 6). Following a 2 h exposure to 50 μg of protamine/ml, MDCK II cells showed a marked disorganization of the cytoskeletal microfilaments seen as F-actin condensation in some areas of intercellular contact (Figure 5). This cytoskeletal alteration was only observed when protamine was applied to the apical and apical+basolateral surfaces (Figures 5c and 5d). After a 1 h exposure to this same concentration of protamine, the cells already showed disorganization of the F-actin microfilaments, but a lower number of cells were affected (Figure 5, insert). Figure 6 shows that treatment with protamine at a concentration of 250 μg/ml caused a greater alteration in the cytoskeleton compared to that seen with 50 μg/ml. At this higher concentration, MDCK II cells showed disorganized stress fibers in the basal region and intense staining of the apical surface compared to the controls (data not shown). In MDCK I cells, protamine (250 μg/ml) produced similar effects to those seen in MDCK II cells, although a lower number of cells was affected in the former strain.

The expression and localization of the TJ-associated proteins occludin, claudin-1 and ZO-1 in MDCK II and MDCK I strains after treatment with protamine were analyzed by immunofluorescence (Figures 7 - 11). Following treatment with protamine (50 and 250 μg/ml), MDCK II cells showed a significant decrease in the expression of occludin compared to the controls

(Figures 7a, 7b and 8). Some cells of the monolayers were more affected than others and showed a complete lack of occludin in the junctional area (Figure 8). The effect of 250 μg of protamine/ml on the cellular distribution and expression of occludin was more intense than that observed with 50 μg/ml. Double-staining for occludin and F-actin microfilaments showed that some treated cells had marked condensation of the cytoskeletal microfilaments in regions of intercellular contact associated with either a complete lack of occludin or an intense punctual reaction for this protein in the junctional area (Figure 9).

As shown in Figure 7, a 2 h exposure to 50 μg of protamine/ml did not alter the immunostaining for claudin-1 (Figures 7c and 7d) and ZO-1 (Figures 7e and 7f). As indicated in Figure 10, immunostaining for claudin-1 showed that protamine (250 μg/ml)-treated MDCK II cells also had a marked decrease in the junctional reaction for this protein. These protamine-treated strain II cells also had a greater amount of punctuate labelling within the cytoplasm (sometimes resembling vesicles) in comparison to the control cells (Figure 10). These data suggest the internalization of claudin-1 after the exposure to protamine.

Protamine produced no significant alterations in the junctional expression and distribution of ZO-1 in either strain of MDCK cells (Figure 11), although MDCK I monolayers were generally less affected since they showed only marked alterations in immunolabelling for occludin but not for claudin-1 or ZO-1 after treatment with protamine (250 μg/ml) (Figures 8, 10 and 11). In treated MDCK II cells, the ZO-1 immunoreaction was less evenly distributed at cell periphery in comparison to the control cells.

DISCUSSION

A number of agents, including cationic proteins, are released from polymorphonuclear leukocytes (PMN) in inflammatory disease, and it is now widely accepted that such cationic proteins may contribute to the pathogenesis of this state by inducing tissue injury, an increase in vascular permeability, and edema at sites of inflammation (Henson and Johnston, 1987; Gautam et al., 2001; Jones et al., 2001). The main examples of cationic proteins released by cells of the immune system are the major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), eosinophil peroxidase (EPO), and heparin-binding protein (HBP). Some studies have shown the influence of these polycations on epithelial barrier function. Thus, MBP

alters airway responses in experimental animals by damaging the airway epithelium (Frigas et al., 1980; Motojima et al., 1989; Coyle et al., 1993). Gautam et al. (2001) showed that HBP released by neutrophils induces Ca^{2+}-dependent cytoskeletal rearrangement and intercellular gap formation in cultured endothelial cells, and increases macromolecular efflux in microvessels *in vivo*.

Some polycations have been used to reproduce the charge-dependent effects of cationic proteins on epithelia functions *in vitro*. One of these cationic polypeptides is protamine, a naturally occurring arginine-rich protein (pI 9.7-12) obtained commercially from salmon sperm. The molecular weight of protamine is relatively low (approximately 5,000 Da), although the chain length can vary depending on the species of fish used to obtain the polycation (Fromm et al., 1985). Several studies have shown protamine can disturb of the epithelial barrier and increase the vascular permeability in rat lung (Chang et al., 1987) and brain (Olesen and Crone, 1986), as well as enhance the transport properties of alveolar and mesothelial epithelia (Alavi et al., 1982; Saumon et al., 1995). Experiments *in vitro* have revealed more diverse effects of protamine on epithelial functions. Thus, this polycation increases the R_t across *Necturus* gallbladder "leaky" epithelium (Fromm et al., 1985; Bentzel et al., 1987) and cultured human and bovine cilliary epithelial cells (Straub and Wiederholt, 1991), but decreases this biophysical parameter in "tight" epithelial MDCK cells (Peterson and Gruenhaupt, 1990) and in type II pneumocytes in culture (Rochat et al., 1988). All of these studies have claimed that the protamine-induced changes in R_t predominantly or exclusively from alterations in the paracellular permeability and in the TJ. However, the actions of this polycation on membrane permeability are controversial, and vary from claims of no effect to reports of increases or decreases in the membrane conductance to K^+, Cl^- and Na^+ (Poler and Reuss, 1987; Bentzel et al., 1987; Fromm et al., 1990; Smith et al., 1997). Such divergent effects may partly reflect the different animal species, tissues and concentrations of protamine used in these studies.

MDCK cells constitute a very well-established epithelial cell line originally obtained from a total kidney suspension of a normal adult Cocker Spaniel and shows many morphological and functional features of distal and/or collecting tubule cells (Barker and Simmons, 1981; Valentich, 1981). In this study, we have demonstrated a concentration–cell strain- and time-dependent effect of protamine on R_t. The exposure of MDCK II monolayers to protamine (50 μg/ml) significantly increased the R_t after 10 min, with a return to control values after 2 h. A higher concentration of protamine (250 μg/ml) caused an irreversible decrease in this parameter after a 30 min exposure. In contrast, both concentrations of protamine markedly decreased the R_t across "tight" MDCK I

monolayers. Therefore, our *in vitro* system reproduced these various epithelial actions of protamine described in the literature.

The barrier function of epithelia is achieved by limiting transport through two putative pathways: the transcellular route, governed by specific transporters and channels in the cell membrane (Mitic et al., 2000; Anderson, 2001) and the paracellular route, consisting of the lateral intercellular space and intercellular junctions (Collares-Buzato et al., 1994a; Anderson, 2001). Thus, although the measurement of R_t can provide some information about the integrity of the paracellular barrier, changes in this variable do not necessarily reflect specific changes in paracellular permeability since transcellular ion permeability may also influence the measurement of R_t. An epithelium can be viewed as an electrical circuit formed by two major resistances in parallel: the transcellular resistance and the resistance of the paracellular pathway itself. For this reason, methods such as the phenol red flux assay have been used to determine whether change in resistance involves the cellular or paracellular route (Jovov et al., 1991). As shown here, there was a significant increase in the basal-to-apical flux of this extracellular marker after a 2 h treatment with both concentrations of protamine in MDCK II cells and following a 30 min exposure to this polycation in MDCK I monolayers. These results indicate that protamine increases the paracellular permeability in both strains of MDCK cells.

Previous studies have demonstrated that the effect of protamine on barrier integrity was seen only after the addition of polycation to the apical surface of the cells, with no effect being observed when protamine was applied to the basolateral cell membrane (Alavi et al., 1982; Bentzel et al., 1987; Fromm et al., 1985; Peterson and Gruenhaupt, 1990). These findings were confirmed by our results showing that alterations in the transepithelial electrical resistance were only observed after apical and apical+basolateral-treatment with protamine (50 and 250 μg/ml) in MDCK II monolayers. Peterson and Gruenhaupt (1990) suggested that there may be sensitive binding sites for protamine on the apical membrane that are not present on the basolateral surface of epithelial cells. In addition, Fromm et al. (1985) suggested that in *Necturus* gallbladder epithelia protamine binds to the apical cell membrane and disrupts the microfilament connections with the inner surface of the membrane. This loss of microfilament attachment would release a sequence of intracellular events that would lead to additional tight junction strand formation or reallocation of TJ strands, thereby decreasing the permeability of *Necturus* gallbladder epithelium.

Some of the effects of polycations on epithelial barrier integrity (an increase or decrease in epithelial permeability) may be mediated by changes in the actin cytoskeleton (Alavi et al., 1982; Bentzel et al., 1987; Fromm et al., 1985; Peterson and Gruenhaupt, 1990, McEwan et al., 1993). In

agreement with this idea, we observed that changes in R_t measurements were associated with strain -and concentration-dependent alterations in the distribution of the cytoskeletal F-actin induced by protamine, including microfilament condensation in the intercellular contact region, contraction of the perijunctional actin ring, disarray of the stress fibers at the basal level, and intense staining of the apical surface region compared to non-treated cells. Manipulation of the cytoskeletal architecture modulates paracellular permeability (Madara et al., 1986). Various stimuli such as nutrients, hormones, soluble and cellular inflammatory mediators, and bacterial adherence may all regulate TJ permeability via actomyosin tension/contraction in the epithelial lining of the gastrointestinal tract. Thus, cytokines and leukocytes of the intestinal epithelium regulate TJ structure and paracellular permeability by influencing the TJ protein complex and/or its association with the underlying actin cytoskeleton (Nusrat et al., 2000). Bentzel et al. (1980) showed that in *Necturus* gallbladder microfilament-active agents, such as cytochalasin B and phalloidin, increased the transepithelial resistance and concluded that structural changes in the TJ were mediated via the cytoskeleton. Similarly, Meza et al. (1980) demonstrated that the microtubules and microfilaments were involved in the structural alterations in TJ following cytochalasin B treatment of MDCK monolayers.

The alterations in R_t measurements and cytoskeleton redistribution observed here were also associated with marked changes in the expression and distribution of the tight junction-associated proteins occludin and claudin-1. In contrast, there were no significant alterations in the immunolabelling for ZO-1 following treatment with protamine. To our knowledge, this is the first study to demonstrate that protamine-induced epithelial barrier disruption is associated with alterations in TJ structure and biochemistry. Occludin and claudins are transmembrane proteins that form the TJ strands and are directly involved in TJ barrier function. These proteins are the most likely candidates for creating the paracellular barrier (Anderson, 2001; Mitic et al., 2000; Tsukita and Furuse, 2000). Mutation or overexpression of these proteins in cultured cells has been shown to affect the epithelial permeability to charged and noncharged solutes. Inai et al. (1999) reported that when claudin-1 was overexpressed by transfection in cultured MDCK monolayers, the electrical resistance increased several fold compared to that of the control cells and was associated with a decrease in the paracellular flux of FITC-labelled dextrans. In experimental conditions, impairment of the paracellular barrier function has often been related to alterations in the junctional expression and localization of occludin and claudins.

Both occludin and claudins bind to a complex of cytoplasmic proteins, e.g. ZOs (ZO-1, ZO-2, ZO-3, cingulin, 7H6 antigen and symplekin) that have been identified in the cytoplasmic

submembraneous plaque underlying membrane contacts (Fanning et al., 1998; Itoh et al., 1999; Fasano, 2000). These proteins appear to organize the occludin and claudins within the TJ region and couple them to actin microfilaments (Anderson, 2001). Interactions between TJ-associated proteins and the cytoskeleton play a pivotal role in the regulation of paracellular permeability (Madara, 1998). Thus, after interacting with anionic sites of the apical membrane and triggering a still unknown intracellular signalling system, protamine elicits a disorganization of cytoskeletal microfilaments. Because of this change in cytoskeletal structure, the interaction with the TJ protein complex may be impaired, thereby resulting in disruption of the paracellular barrier. As a result, the whole TJ complex is disassembled and the proteins are internalized.

To assess whether the effect of protamine on barrier function was dependent on the nature of the TJ complex, we compared the protamine-induced responses in two strains of MDCK cells (strain II - low resistance and strain I - high resistance). Different responses to protamine were observed in these strains, suggesting that the effects of protamine on barrier function in MDCK cells may be dependent on the properties of the TJ and the paracellular barrier. In strain I "tight" monolayers, protamine (50 and 250 μg/ml) decreased the R_t values in association with an enhanced basal-to-apical phenol red flux. Using morphological methods, MDCK I cells showed similar changes in the localization and expression of the junctional proteins and F-actin when compared with strain II "leaky" MDCK cells. However, the protamine-induced alterations in the cytoskeleton and TJ were less intense in MDCK I cells compared to MDCK II cells. In contrast to our findings, McEwan et al. (1993) showed that the mechanisms involved in the changes in paracellular permeability after exposure to poly-L-lysine were independent of TJ characteristics of the various cell lines tested.

In conclusion, treatment with protamine *in vitro* induced diverse effects on epithelial barrier function in MDCK cells. The changes in R_t and in the transepithelial flux of an extracellular marker were dependent on the length of exposure to protamine, on the polycation concentration used and on the MDCK strain. Our results agreed with data reported in the literature on the effect of protamine on epithelial function. Thus, our *in vitro* system using two strains of MDCK cells provides a good model for investigating the epithelial action of polycations. The protamine-induced disruption of the epithelial paracellular barrier was associated with the rearrangement of F-actin and with a marked decrease in the junctional immunoreaction for TJ-associated proteins. These results strongly suggest that both the cytoskeleton and TJ are possible cellular targets for protamine.

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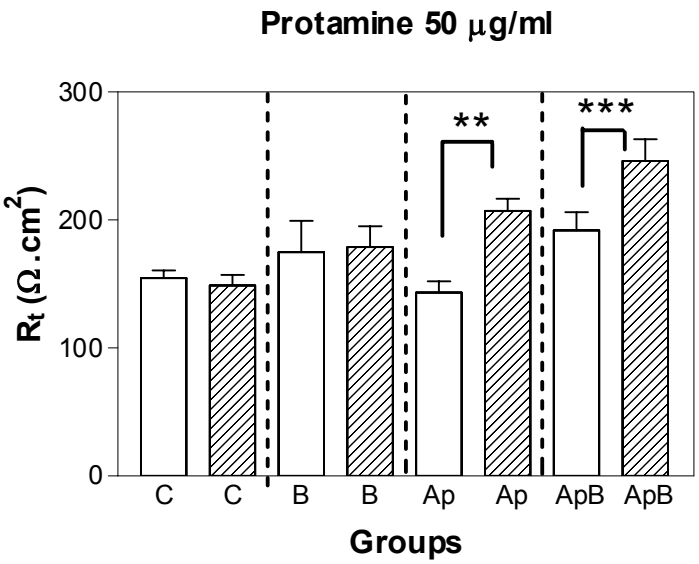
Figure 1: Effect of protamine treatment on transepithelial electrical resistance (R_t) across MDCK II monolayers: dependence of the dose and the local of exposure. MDCK II monolayers were treated with protamine, diluted in Krebs Ringer buffer given a final concentrations of 50 μg/ml (**a**) or 250 μg/ml (**b**), up to 1h. This substance was applied to the apical (Ap), the basal (B) or the apical+basolateral (ApB) surfaces of the treated monolayers. The control group (C) remained in Krebs for the same time period. Note that the exposure to 50 μg/ml of protamine induced a significant increase in R_t (**a**), while the treatment with protamine 250 μg/ml resulted in a significant decrease in R_t (**b**), as compared to the control group. These significant alterations in R_t were observed only when the treatment with protamine, at both concentrations, was applied to apical and apical+basolateral surfaces. All data are expressed as the mean ± SE of 5 to 20 monolayers per group from four to five independent experiments * P<0,001, ** P<0,01, *** P<0,05 (ANOVA and Bonferroni's test).

Figure 1

MDCK II

a



b

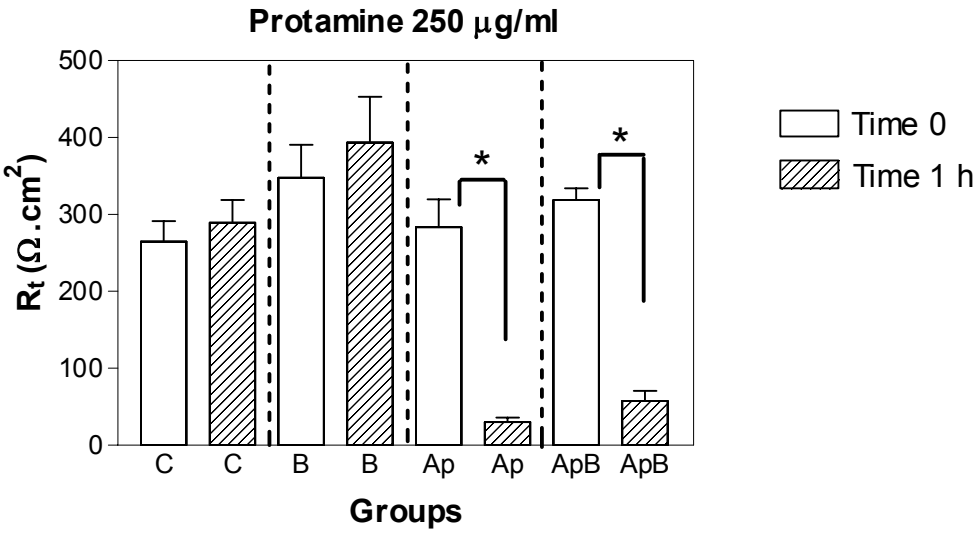
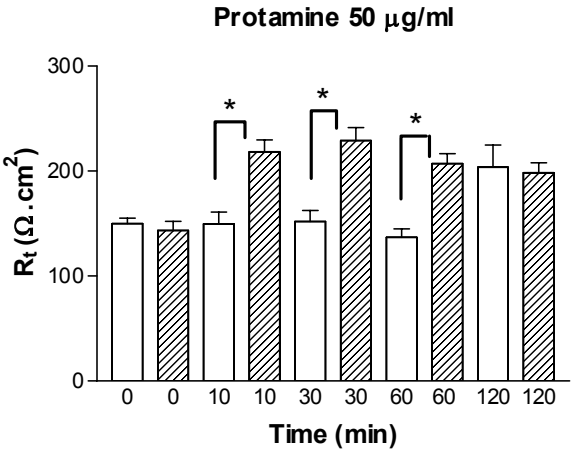


Figure 2: Effect of protamine treatment on transepithelial electrical resistance (R_t) across MDCK II and MDCK I monolayers: dependence of the dose and time of exposure. MDCK II and I were exposed to 50 (**a** and **c**) or 250 μg/ml (**b** and **d**) of protamine, diluted in Krebs Ringer buffer, up to 2h. This substance was applied to the apical surface of treated monolayers; control monolayers remained in buffer for the same time period. It was observed that the protamine at concentration of 50 μg/ml induced a significant increase in R_t of MDCK II cells, after 10 min of exposure; R_t restored back to control values after 2 h-incubation. However, protamine at concentration of 250 μg/ml induced a significant and irreversible decrease in this parameter of MDCK II after 30 min of treatment, as compared to the control group. In MDCK I cells, the treatment with protamine at both concentrations (**c** and **d**) induced a significant decrease in R_t as compared to the control group. This data suggests that the effect of protamine is dose and time-dependent. The MDCK II data are expressed as the mean ± SE of 12 to 16 monolayers per group from two to four independent experiments. The MDCK I data are expressed as the mean ± SE of 5 monolayers per group from five independent experiments.* P<0,001, ** P<0,01 (ANOVA and Bonferroni's test).

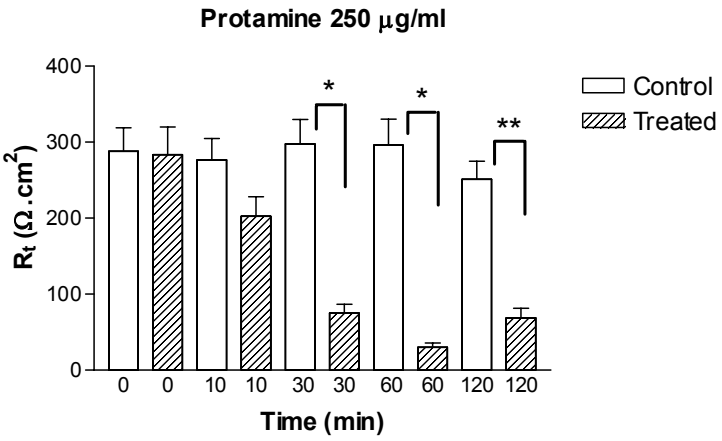
Figure 2

MDCK II

a

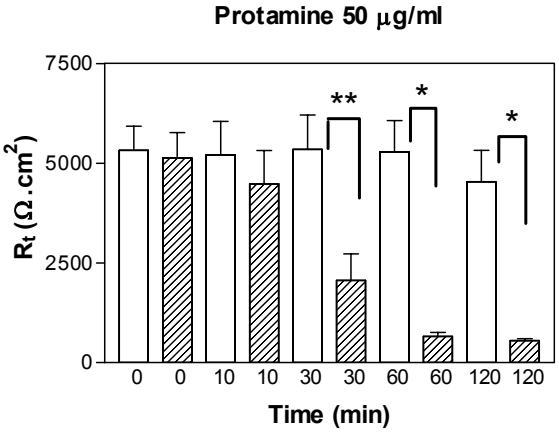


b



MDCK I

c



d

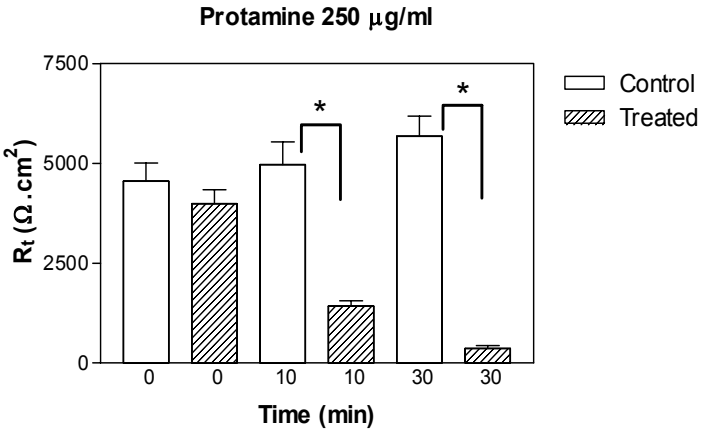
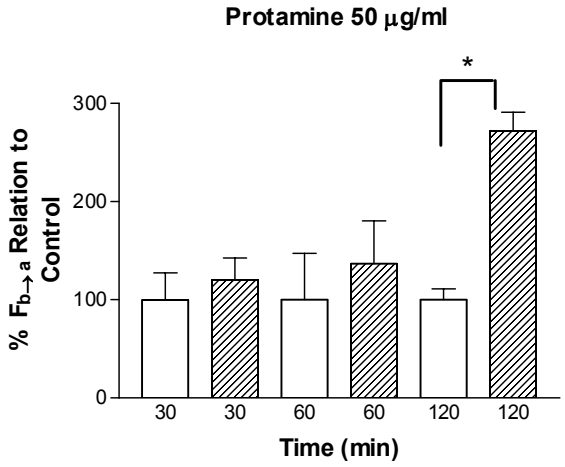


Figure 3: Effect of protamine on the basal-to-apical phenol red flux ($F_{b\rightarrow a}$) in MDCK II and MDCK I monolayers. MDCK II cells from the treated group were exposed to 50 (**a**) or 250 μg/ml (**b**) of protamine, diluted in Krebs Ringer buffer, at the apical surface, up to 2 h. MDCK I cells were treated only with protamine at concentration of 250 μg/ml (**c**). The apical surface of the control monolayers received the same volume of the Krebs without protamine. Phenol red at concentration of 20 mg/ml was applied at the basal bathing buffer of control and treated groups. A sample of the apical phenol red-free bathing buffer was collected at 30 min, 1 h and 2 h, and a sample of the basal bathing buffer were collected after 2 h incubation. As assessed by phenol red flux assay, MDCK II and I monolayers treated with protamine show a significant increase in the basal-to-apical phenol red flux as compared to the control only after 2 h of exposure (**a** and **b**) in MDCK II and at 30 min of treatment in MDCK I (**c**). The MDCK II data are expressed as the mean ± SE of 2 to 5 monolayers per group from one to two independent experiments. The MDCK I data are expressed as the mean ± SE of 4 monolayers per group from two independent experiments. The flux results were expressed as percentage of $F_{b\rightarrow a}$ value mean of the control group (taken as 100% dye flux) * $P<0,05$ (ANOVA and Bonferroni's test; Student's test).

Figure 3

MDCK II

a



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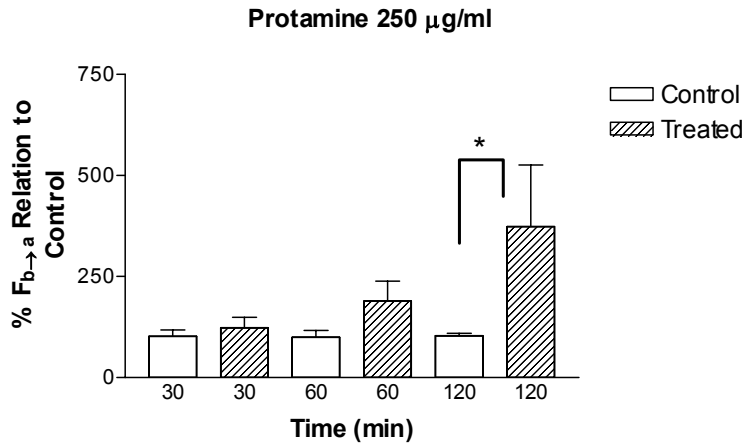


MDCK I

c

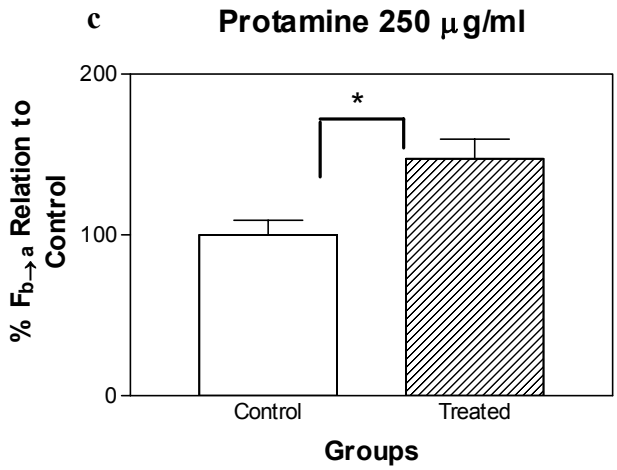


Figure 4: Cell viability of MDCK cells after treatment with protamine. Cell viability was assessed by neutral red cell uptake as described in Materials and Methods section. Treated strain II MDCK monolayers were apically exposed to protamine-containing Krebs buffer at concentrations of 50, 100 and 250 μg/ml. The control group was exposed to a Krebs buffer without protamine. Note that this treatment induced no alteration in the neutral red uptake as compared to the control. The treatment with Triton 0.5% (diluted in Krebs Ringer, pH 7.4) represents a positive control of cytotoxicity.

Figure 4

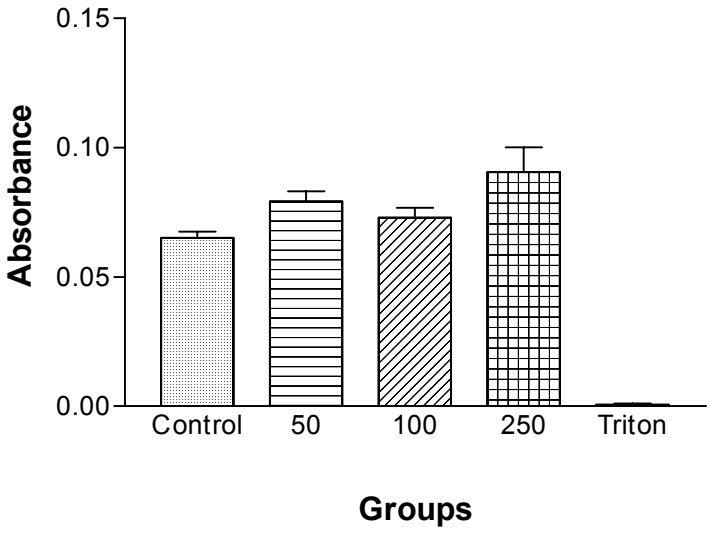


Figure 5: Polarized effect of protamine treatment upon F-actin distribution in MDCK II cells. Strain II MDCK monolayers were stained with TRITC-labelled phalloidin to reveal F-actin microfilaments. Monolayers from the treated group were exposed to 50 μg/ml of protamine, diluted in Krebs Ringer buffer, up to 2 h. This polycation was applied to the basal (**b**), the apical (**c**) or the apical+ basolateral (**d**) surfaces of the treated monolayers. The figure **e** shows 1h treatment with 50 μg/ml protamine. The control group remained in Krebs buffer for the same time period (**a**). Each panel is a representative confocal "en face" (X-Y) image obtained at the same level of the monolayer with an identical CLSM sensitivity. Note that a F-actin condensation at the intercellular contact region is observed only when this treatment was applied at the apical or the apical+basolateral surfaces (**c**, **d** and **insert**) as compared to the control. The disarrangement of cytoskeletal microfilaments after 1h of treatment was less dramatic in comparison to that observed after 2h-protamine exposure (insert). The arrowheads show a F-actin condensation at the intercellular contact region. Bar represents 30 μm.

Figure 5

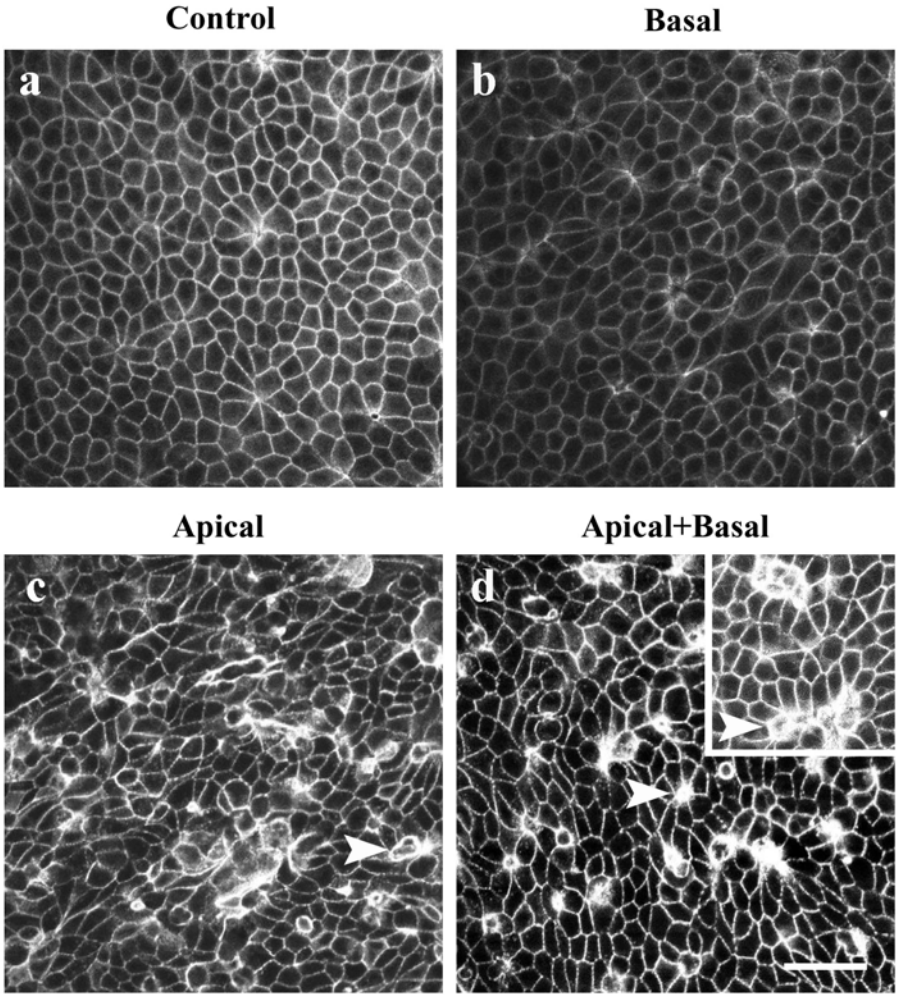


Figure 6: F-actin distribution in both strains of MDCK monolayers following protamine treatment at concentration of 250 μg/ml. MDCK II and MDCK I strains were stained with TRITC-labelled phalloidin to reveal F-actin microfilaments. Treated monolayers of the strain II (**b**) and strain I (**d**) were exposed to 250 μg/ml of protamine, diluted in Krebs Ringer buffer, up to 30 min. This polycation was applied to the apical surface of the treated monolayers. The control group (**a** and **c**) remained in Krebs buffer for the same time period. These photomicrographs are representative confocal "en face" (X-Y) images obtained at the medium level of the monolayer using an identical CLSM sensitivity. Note condensation of F-actin microfilament at some areas of the intercellular contact region in both strains of MDCK after treatment (arrowheads). It was observed that in MDCK I this alteration was less expressive in comparison to that observed in MDCK II. Bar represents 30 μm.

Figure 6

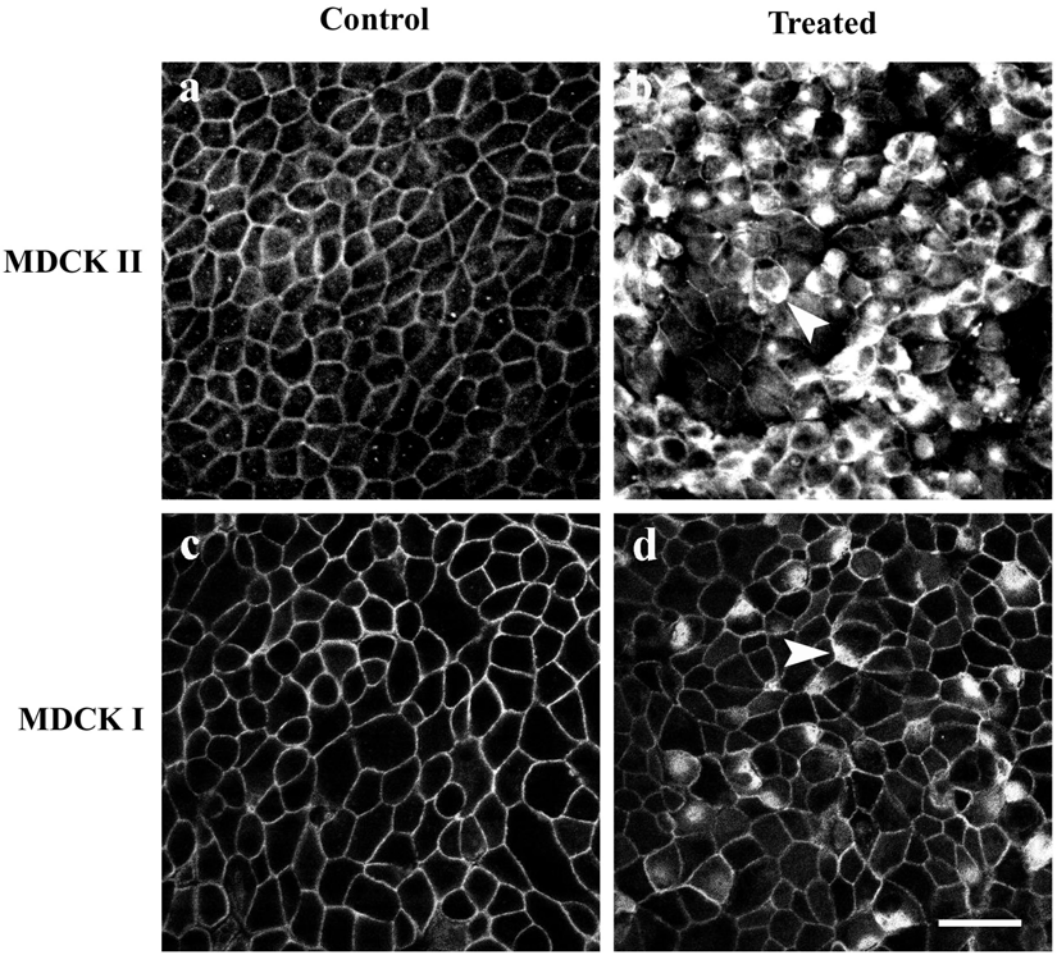


Figure 7: Cellular expression and distribution of junctional proteins in MDCK II monolayers following 50 μg/ml protamine treatment. The strain II of MDCK monolayers were immunostaining for the tight junction-associated proteins occludin (**b**), claudin-1 (**d**) and ZO-1 (**f**) using an indirect immunofluorescence methodology. The monolayers from the treated groups were exposed to 50 μg/ml of protamine, diluted in Krebs Ringer buffer, at apical surfaces, up to 2 h. The control group remained in Krebs buffer for the same time period (**a**, **c** and **e**). Each panel is a representative confocal "en face" (X-Y) image obtained at the medium level of the monolayer using an identical CLSM sensitivity. Note that the treatment with 50 μg/ml protamine induced a significant decrease in immunolabelling level for occludin but not for claudin-1 and ZO-1. Bar represents 30 μm.

Figure 7

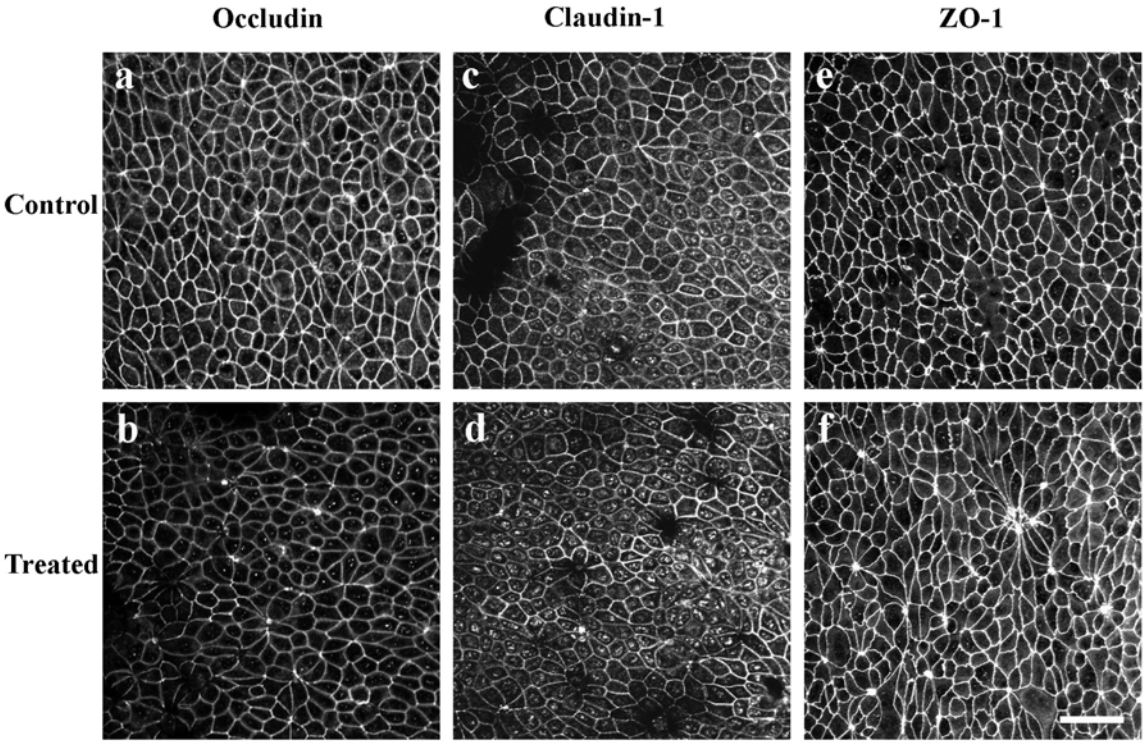


Figure 8: Cellular expression and distribution of the tight junction-associated protein occludin in both strains of MDCK monolayers following 250 μg/ml protamine treatment. The monolayers were fixed and immunostained for occludin using a standard indirect immunofluorescence protocol. **b** and **d; "**En face" (X-Y) confocal images, of MDCK II and MDCK I, respectively, showing monolayers treated with 250 μg/ml of protamine at apical surfaces, for 30 min. **a** and **c;** "En face" (X-Y) confocal images showing control monolayers that remained in Krebs buffer for the same time period. Note that this treatment induced a marked decrease in the junctional expression of occludin, as compared to the control, in both strains. In strain I, this alteration was less expressive in comparison to that observed in strain II. The grafics show the measurement of the fluorescence intensity of occludin immunoreaction in control and treated groups in both MDCK strains. The columns are the mean ± SE of 7 to 8 monolayers per group from one experiment. All confocal images were taken using the same sensitivity of the CLSM. Bar represents 30 μm. *P<0,05 (Student's t-test).

Figure 8

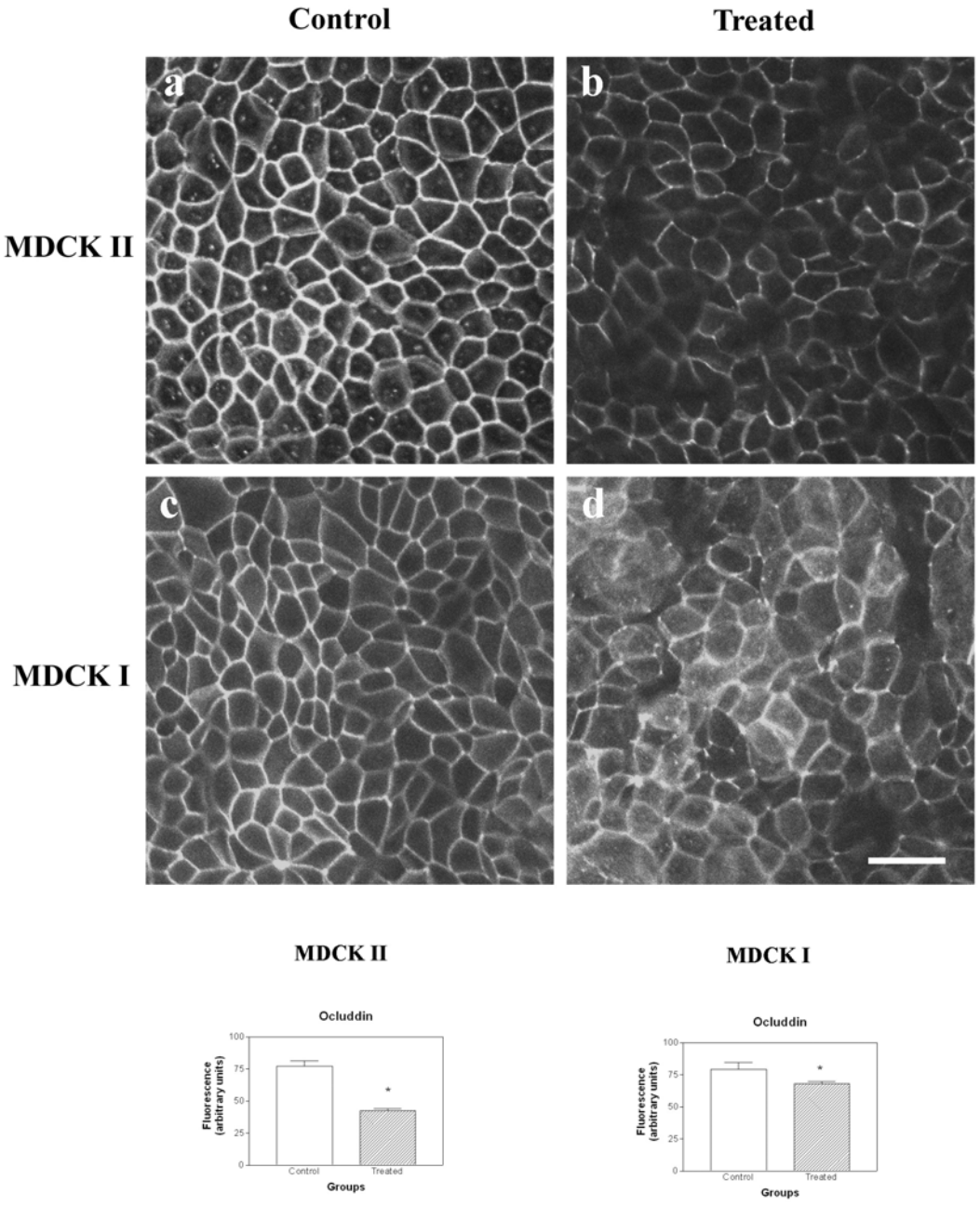

Control
Treated
MDCK II
MDCK I
a
b
c
d
MDCK II
Ocluddin
Fluorescence (arbitrary units)
100
75
50
25
0
Control
Treated
Groups
*
MDCK I
Ocluddin
Fluorescence (arbitrary units)
100
75
50
25
0
Control
Treated
Groups
*

Figure 9: Double immunolabelling for occludin and F-actin in MDCK II cells following protamine treatment. The MDCK II monolayers were stained with TRITC-labelled phalloidin to reveal F-actin microfilaments (**a** and **b**) and immunostaining for occludin protein (**c** and **d**). The **e** and **f** confocal images are superimposing F-actin and occludin images. The monolayers from the treated group were exposed to 250 μg/ml of protamine for 30 min (**b**, **d** and **f**). The control group remained in Krebs buffer for the same time period (**a**, **c** and **e**). These photomicrographs are representative confocal "en face" (X-Y) images obtained at the medium level of the monolayer using an identical sensitivity level of the CLSM. Note that treated cells showed marked disarrangement of F-actin associated with changes in the distribution of occludin, such as a complete lack of occludin reaction or intense punctual reaction at the junctional area. Bar represents 30 μm.

Figure 9

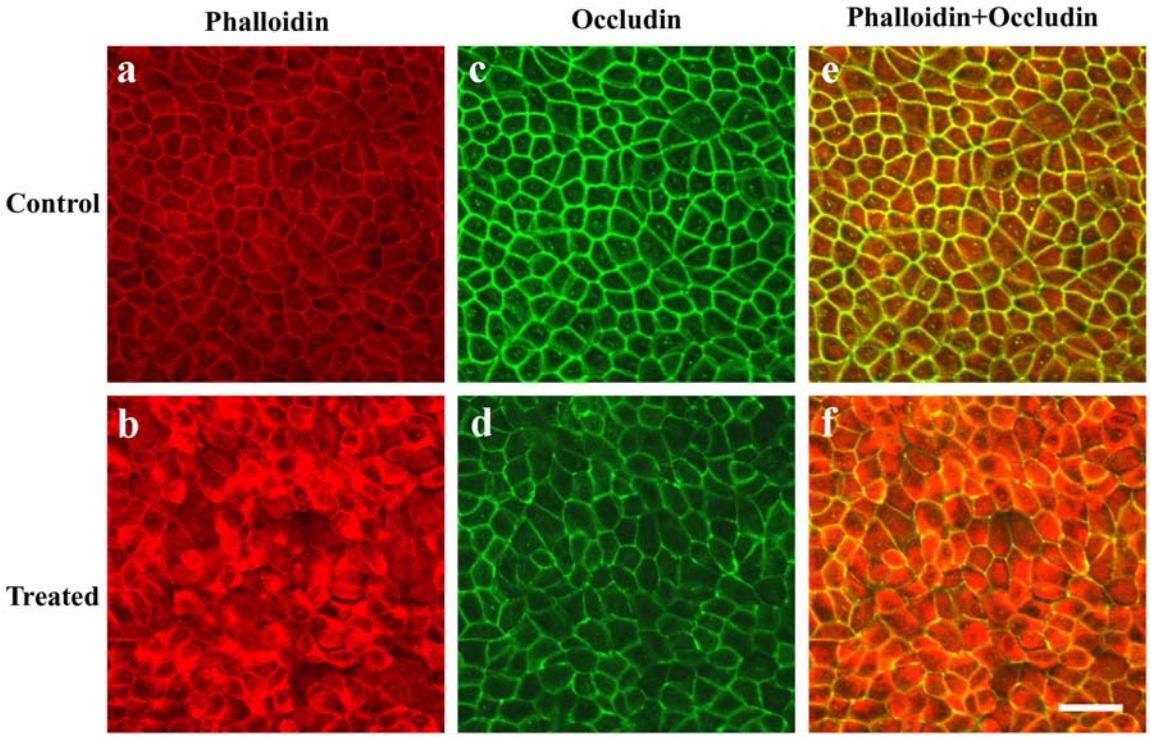

Phalloidin
Occludin
Phalloidin+Occludin
Control
Treated
a
b
c
d
e
f

Figure 10: Cellular expression and distribution of the protein claudin-1 in MDCK II and MDCK I monolayers following 250 μg/ml protamine treatment. The monolayers were fixed and immunostained for this tight junction-associated using a standard indirect immunofluorescence methodology. **b** and **d ;**"En face" (X-Y) confocal images, of strains II and II, respectively, showing monolayers treated with 250 μg/ml of protamine at apical surfaces, for 30 min. **a** and **c;** "En face" (X-Y) confocal images showing control monolayers that remained in Krebs buffer for the same time period. Note a marked decrease in junctional expression of claudin-1 only in treated strain II MDCK monolayers, as compared to the control. In addition, note a much more punctative labelling within the cytoplasm of treated cells. The grafics show the measurement of the fluorescence intensity of claudin-1 immunoreaction in control and treated groups in both MDCK strains. The columns are the mean ± SE of 4 to 7 monolayers per group from one experiment. All confocal images were taken using the same sensitivity of the CLSM. Bar represents 30 μm. *P<0,05 (Student's t-test).

Figure 10

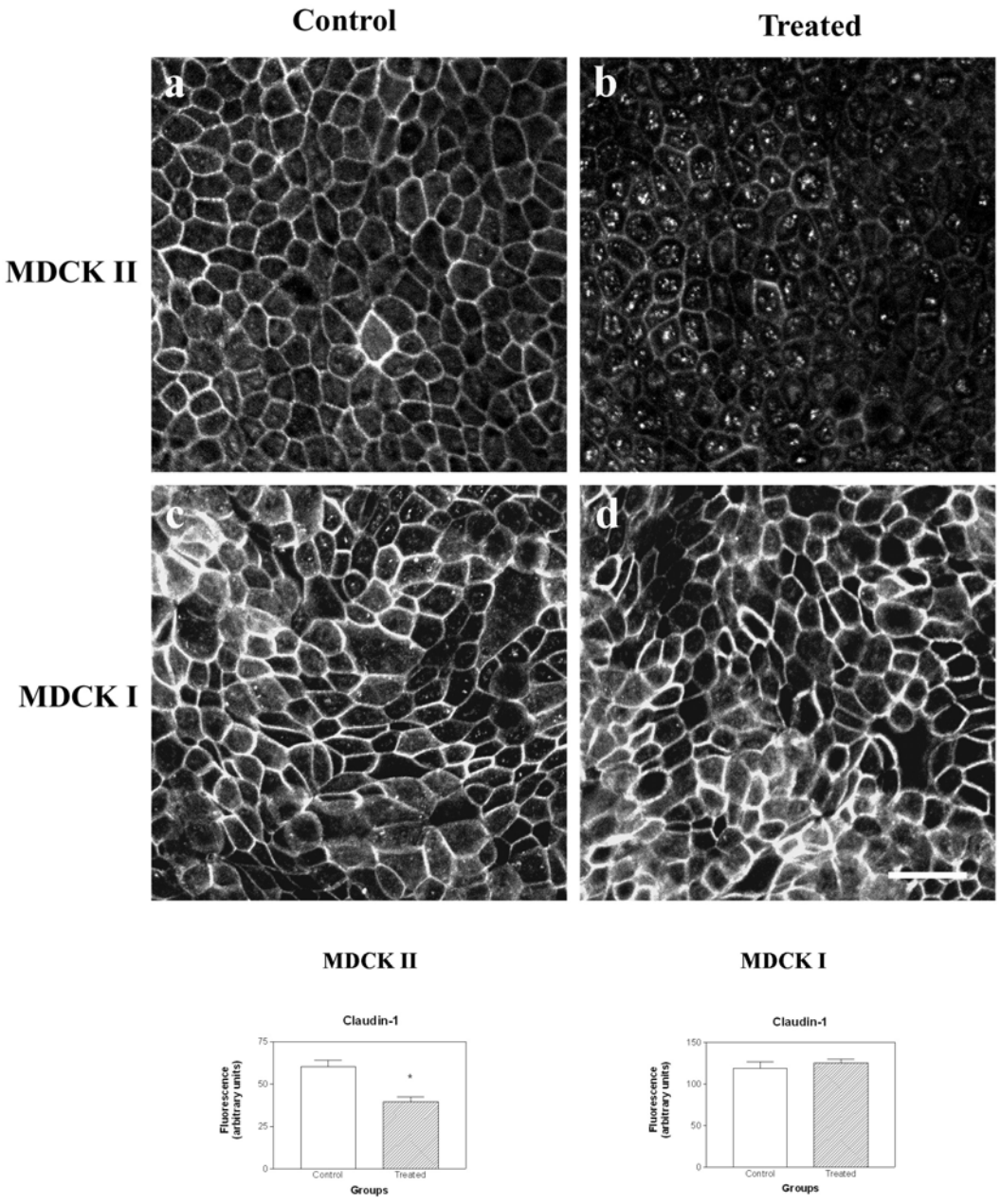
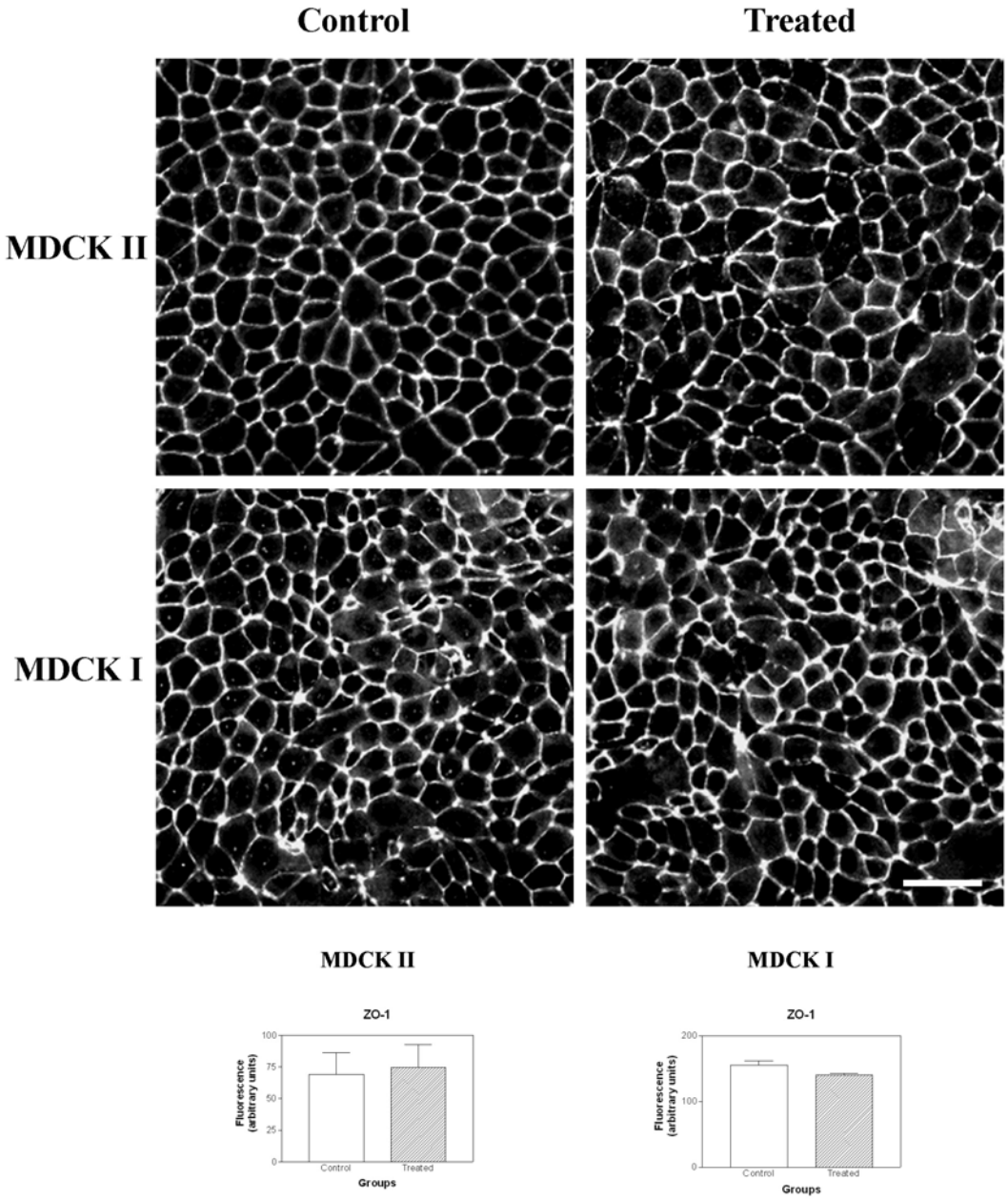

Control
Treated
a
b
MDCK II
c
d
MDCK I
MDCK II
MDCK I
Claudin-1
Fluorescence (arbitrary units)
75
50
25
0
*
Control
Treated
Groups
Claudin-1
Fluorescence (arbitrary units)
150
100
50
0
Control
Treated
Groups

Figure 11: Cellular expression and distribution of the tight junction-associated protein ZO-1 in the two strains of MDCK monolayers after 250 μg/ml protamine-exposure. The monolayers were fixed and immunostained for ZO-1 using a standard indirect immunofluorescence protocol. **b** and **d;** "En face" (X-Y) confocal images, of strains II and I, respectively, showing monolayers treated with 250 μg/ml of protamine at apical surfaces, for 30 min. **a** and **c;** "En face" (X-Y) confocal images showing control monolayers that remained in Krebs buffer for the same time period. Note protamine treatment had no visible effect on the junctional expression of ZO-1 in both strains, as compared to the control. The grafics show the measurement of the fluorescence intensity of ZO-1 immunoreaction in control and treated groups in both MDCK strains. The columns are the mean ± SE of 4 monolayers per group from one experiment. All confocal images were taken using the same sensitivity of the CLSM. Bar represents 30 μm.

Figure 11


Control
Treated
MDCK II
MDCK I
MDCK II
MDCK I
ZO-1
Fluorescence (arbitrary units)
100
75
50
25
0
Control
Treated
Groups
ZO-1
Fluorescence (arbitrary units)
200
100
0
Control
Treated
Groups

CAPÍTULO 4

CONCLUSÕES GERAIS

Com relação ao efeito do tratamento com Dexametasona (DEX) e Prolactina (PRL) sobre a barreira epitelial, verificamos que:

- O tratamento "in vitro" com DEX e PRL induz aumento da função de barreira epitelial em monocamadas confluentes de células MDCK;

- O aumento da função de barreira foi demonstrado por um aumento da resistência elétrica transepitelial (R_t) medida através de monocamadas confluentes tratadas com tais substâncias. Concomitantemente, observou-se uma tendência à diminuição do fluxo apical-basal de fenol vermelho ($F_{a \rightarrow b}$) das células tratadas quando comparado ao grupo controle, sugerindo uma diminuição da permeabilidade paracelular.

- O aumento da função de barreira paracelular induzido pelo tratamento com DEX e PRL não foi associado a modificações na localização e/ou na expressão das proteínas associadas à JO, ocludina, ZO-1 e claudina-1, nem mesmo na distribuição dos microfilamentos de F-actina.

- Por meio da técnica de coloração com metil-verde e pela análise da viabilidade pela técnica de coloração com *neutral red*, foi observado um aumento do número de células viáveis por área, após tratamento. Portanto, podemos afirmar que o efeito do tratamento com DEX e PRL envolveu aumento na proliferação e/ou viabilidade celular.

Com relação ao efeito do tratamento com protamina sobre a barreira epitelial, verificamos que:

- A protamina induziu alterações variáveis sobre a função de barreira epitelial como avaliado pela medida da R_t, em monocamadas de MDCK. O efeito da protamina sobre R_t foi dependente da aplicação na superfície apical da monocamada, do tempo de exposição, da dose empregada e da cepa utilizada da linhagem MDCK;

- Em tempos prolongados de tratamento (>2 h) e/ou doses altas de protamina, esta proteína policatiônica induziu invariavelmente uma diminuição da função de barreira epitelial em cultura de células MDCK, como avaliado pela medida de R_t e $F_{b\rightarrow a}$ de fenol vermelho.

- A diminuição da função de barreira foi associada a uma desorganização do citoesqueleto, bem como a uma diminuição significativa da expressão das proteínas associadas à JO, ocludina e claudina-1.

CAPÍTULO 5

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