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Short communication

# [(Dihydroindenyl)oxy]alkaonic acid inhibits the cystic fibrosis transmembrane conductance regulator

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**Abstract**

We investigated the effects of [(dihydroindenyl)oxy]alkaonic acid (DIOA) on the $Cl^-$ secretion in Calu-3 human airway epithelial cells that exclusively express the cystic fibrosis transmembrane conductance regulator (CFTR) as an apical $Cl^-$ channel. The 5′-nitro-2-(3-phenylpropylamino) benzoate (NPPB)-sensitive short-circuit current ($I_{sc}$) and apical conductance were markedly reduced by DIOA (100 μM) in the presence and absence of isoproterenol (10 nM). Replacement of the butyl group in DIOA with a methyl group attenuated the inhibitory effects. The $ED_{50}$ of DIOA ($17.0 \pm 1.0$ μM) was almost equivalent to that of NPPB ($15.6 \pm 2.1$ μM). In conclusion, DIOA inhibits CFTR as strongly as NPPB does. 

*Keywords:* Calu-3; CFTR (cystic fibrosis transmembrane conductance regulator); [(Dihydroindenyl)oxy]alkaonic acid; Short-circuit current

## 1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a major $Cl^-$ secretory pathway that has great responsibility for determining the amount of airway liquid on the human airway surface. In chronic airway inflammatory diseases like chronic bronchitis, bronchial asthma and bronchoectasis, a hypersecretory state limits airflow and causes mucociliary dysfunction (Tamaoki et al., 1992). It is, therefore, possible that regulation of CFTR would result in appropriate fluid volume in the respiratory tract.

There are several lines of evidence that blockers of $Cl^-$ channels and anion transporters have some similarities in their structural formulae (Wangemann et al., 1986). Indeed, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), which is a blocker of the outwardly rectifying $Cl^-$ channel, inhibits the $K^+$–$Cl^-$ cotransporter and the $Cl^-$–$HCO_3$-exchanger (Venglarik et al., 1994). Cragoe et al. (1982) screened a large number of indenyloxy alkaonic acids for the treatment of brain injury that produced swelling of astrocytes via $Cl^-$-cation transporters like the $K^+$–$Cl^-$ cotransporter and the $Cl^-$–$HCO_3$-exchanger. However, the effects of indenyloxy alkaonic acids on $Cl^-$ channels have been scarcely documented. In the present study, we investigated the effects of [(2-cyclopentyl-6,7-dichloro-2, 3-dihydro-2-*n*-butyl-1-oxo-1 H-inden-5-yl)oxy] acetic acid (DIOA), which is one of the indenyloxy alkaonic acids on $Cl^-$ secretion in Calu-3 human airway cells. These cells have been proven to express the CFTR exclusively as an apical $Cl^-$ channel, displaying electrophysiological properties consistent with human airway serous cells (Haws et al., 1994).

## 2. Materials and methods

Calu-3 cells were purchased frozen (−80 °C) from American Type Culture Collection (Rockville, MD, USA) and grown in $T_{75}$ tissue culture flasks at 37 °C in a humidified incubator with 5% $CO_2$ in air. We used a 1:1 mixture of Dulbecco's Modified Eagle's Medium and F12 (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (GIBCO), 100 μg/ml streptomycin and 100 U/ml penicillin (GIBCO) for the culture medium. For short-circuit current ($I_{sc}$) measurement, cells from the flasks were subcultured by the air interface method (Yamaya et al., 1992) at $10^6$ cells/$cm^2$ on human placen-

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tal collagen-coated Costar Snapwell inserts (0.4 μm pore size, 12 mm diameter, polyester; Costar, Cambridge, MA, USA) for 7–13 days.

The filter inserts with confluent monolayers were mounted in modified Ussing chambers (EasyMount Chamber; Physiologic Instruments, San Diego, CA, USA) with a solution containing NaCl, 140; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10, and HEPES, 10 with pH of 7.4 at 37 °C. The $I_{sc}$ was measured by a voltage clamp amplifier (VCC MC2; Physiologic Instruments). Every 20 s, we applied a 2-μA pulse for 0.5 s under an open-circuit to cause voltage deflections (Δ potential difference). This procedure enabled us to calculate transepithelial conductance ($G_t$) by Ohm's law ($G_t = 2\ \mu A/\Delta$ potential difference).

Replacing the butyl group in DIOA with a methyl group produces [(2-cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy]acetic acid (IAA-94). DIOA, IAA-94, 5′-nitro-2-(3-phenylpropylamino) benzoate (NPPB), bumetanide, phlorizin, nystatin, isoroterenol, forskolin, and 8-bromo-cAMP were obtained from Sigma (St. Louis, MO, USA). Isoroterenol and 8-bromo cAMP were dissolved in distilled water. All other drugs were dissolved in dimethylsulfoxide (DMSO). Data are expressed as means ± S.E.M. Statistical differences were determined by Student's *t* test or one-way analysis of variance. Values of $P < 0.05$ were considered to be significant.

## 3. Results

Transepithelial $Na^+$ transport in Calu-3 cells occurs in two steps: $Na^+$ entry across the apical membrane via the $Na^+$-glucose cotransporter and $Na^+$ extrusion across the basolateral membrane via the $Na^+$–$K^+$ pump (Ito et al., 1999). To observe only the transepithelial $Cl^-$ current in the present study, every experiment was carried out in the apical presence of phlorizin (200 μM), a $Na^+$-glucose cotransport blocker. As shown in Fig. 1(A), basolaterally applied isoproterenol (10 nM) biphasically increased $I_{sc}$: a transient component followed by a sustained one that was steadily maintained for more than 30 min. The isoproterenol-induced actions were markedly inhibited by pretreatment with 100 μM NPPB. The $Na^+$–$K^+$–2 $Cl^-$-cotransporter is largely responsible for the transepithelial $Cl^-$ transport as the $Cl^-$ entry steps across the basolateral membrane under bicarbonate-free conditions (Niisato et al., 1999). Basolateral bumetanide (100 μM) was also effective in preventing the $I_{sc}$ in response to isoproterenol (Fig. 1(A)). The NPPB-sensitive $I_{sc}$ measured 60 min after adding bumetanide or its vehicle (0.1% DMSO) were $3.2 \pm 0.3$ ($n = 5$) or $7.8 \pm 0.7$ ($n = 13$) μA $cm^{-2}$ in the presence of isoproterenol and $1.4 \pm 0.1$ ($n = 7$) or $2.5 \pm 0.2$ ($n = 12$) μA $cm^{-2}$ in its absence (Fig. 1(B)). The effects of DIOA and IAA-94 are shown in Fig. 1(A) and Fig. 1(B). NPPB-sensitive $I_{sc}$ measured 60 min after bilateral application of 100 μM DIOA were $0.1 \pm 0.4$ μA $cm^{-2}$ ($n = 4$) in the absence of 10 nM isoproterenol, and $0.2 \pm 0.1$ μA $cm^{-2}$ ($n = 4$) in its presence (Fig. 1(B)). However, IAA-94 at 100 μM caused a hardly detectable inhibition of the NPPB-sensitive component of isoproterenol-stimulated ($7.3 \pm 0.5$ μA $cm^{-2}$, $n = 4$) and unstimulated $I_{sc}$ ($2.5 \pm 0.1$ μA $cm^{-2}$, $n = 4$), and an even higher concentration (1 mM) was necessary for IAA-94 to inhibit the $Cl^-$ transport ($0.4 \pm 0.1$ in the absence of isoproterenol, $n = 8$; $0.5 \pm 0.4$ μA $cm^{-2}$ in its presence, $n = 8$) (Fig. 1(B)).

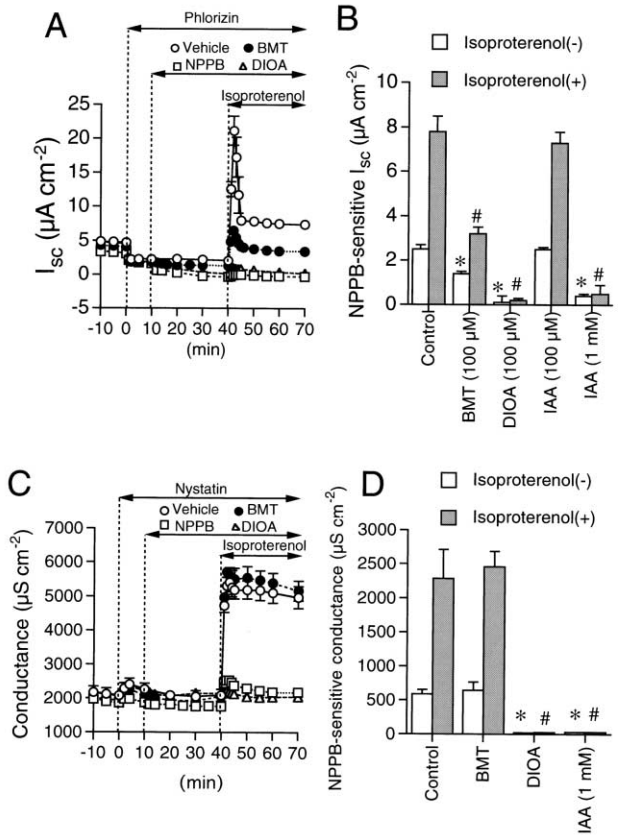


Fig. 1. Effects of DIOA on short-circuit current ($I_{sc}$) and apical conductance in Calu-3 cells. $I_{sc}$ was measured in the presence of 200 μM phlorizin. (A) Isoproterenol (10 nM) was applied from the basolateral side 30 min after application of 100 μM bumetanide (BMT, basolateral), 100 μM NPPB (bilateral), 100 μM DIOA (bilateral) or 0.1% DMSO (vehicle). (B) The NPPB-sensitive (100 μM) $I_{sc}$ was measured 30 min after application of isoproterenol (isoproterenol (+)) or its vehicle (distilled water, isoproterenol (−)). Application of IAA-94 (IAA, 100 μM) instead of DIOA caused a hardly detectable inhibition, but it was obtained at an even higher concentration (1 mM). (C) The apical conductance was measured after permeabilization of the basolateral membrane with 100 μM nystatin. After pretreatment with NPPB, BMT, DIOA or their vehicle for 30 min, isoproterenol was applied in the basolateral solution. (D) To evaluate the apical $Cl^-$ conductance, the NPPB-sensitive (100 μM) conductance was measured 30 min after application of isoproterenol (isoproterenol (+)) or its vehicle (distilled water, isoproterenol (−)) in the presence of BMT, DIOA, IAA (1 mM) or their solvent (control). $n = 4$–13, (#) and (*) indicate significant differences from the control in the presence and absence of isoproterenol, respectively ($P < 0.05$).

To observe the change of apical conductance, we permeabilized the basolateral membrane with 100 μM nystatin, as in previous reports (Ito et al., 2000). The conductance after adding nystatin was maintained for at least 100 min. As shown in Fig. 1(C), isoproterenol potentiated the apical conductance that was abolished by bilateral pretreatment with NPPB (100 μM). In contrast to the results shown in Fig. 1(A), however, pretreatment with bumetanide (100 μM) from the basolateral side had no effect on the isoproterenol-induced change of apical conductance (Fig. 1(C)). The NPPB-sensitive component of apical conductance measured 30 min after adding 10 nM isoproterenol ($2283 \pm 427$ μS cm$^{-2}$, $n = 7$) or its vehicle ($584 \pm 68$ μS cm$^{-2}$, $n = 7$, Fig. 1(D)) was unaffected by bumetanide ($2455 \pm 228$, $n = 4$; $642 \pm 123$ μS cm$^{-2}$, $n = 5$). On the other hand, pretreatment with DIOA (100 μM) and IAA-94 (1 mM) caused marked diminution of NPPB-sensitive apical conductance in both the presence of isoproterenol ($26 \pm 11$, $n = 7$; $33 \pm 4$ μS cm$^{-2}$, $n = 4$) and its absence ($22 \pm 7$, $n = 5$; $30 \pm 5$ μS cm$^{-2}$, $n = 4$) (Fig. 1(D)).

The effects of isoproterenol were mimicked by forskolin (10 μM, Fig. 2(A)) and 8-bromo-cAMP (1 mM, Fig. 2(B)). Increases in the peak values ($\Delta I_{sc}$) were $16.9 \pm 1.4$ ($n = 10$) and $15.8 \pm 2.2$ μA cm$^{-2}$ ($n = 10$), respectively, and were also inhibited by pretreatment with 100 μM DIOA ($1.5 \pm 0.2$, $n = 8$; $1.5 \pm 0.1$ μA cm$^{-2}$, $n = 6$). The sustained components produced by these cAMP-related agents assessed by the NPPB-sensitive $I_{sc}$ were $6.0 \pm 0.6$ ($n = 10$) in forskolin and $6.1 \pm 0.8$ μA cm$^{-2}$ ($n = 10$) in 8-bromo-cAMP, which were attenuated by DIOA ($0.4 \pm 0.4$, $n = 8$; $0.3 \pm 0.1$ μA cm$^{-2}$, $n = 6$, Fig. 2(C)).

DIOA, NPPB and IAA-94 caused concentration-dependent inhibition of basal $I_{sc}$. The $EC_{50}$ of DIOA ($17.0 \pm 1.0$ μM, $n = 4$) approximated that of NPPB ($15.6 \pm 2.1$ μM, $n = 4$), and it was significantly lower than that of IAA-94 ($209.3 \pm 7.0$ μM, $n = 8$). However, there were no significant differences in the Hill coefficients of the agents ($2.1 \pm 0.1$ in DIOA, $2.4 \pm 0.1$ in IAA-94 and $2.3 \pm 0.1$ in NPPB)

As shown in Fig. 2(D), the chemical features common to NPPB and DIOA are phenyl rings with an anionic group.

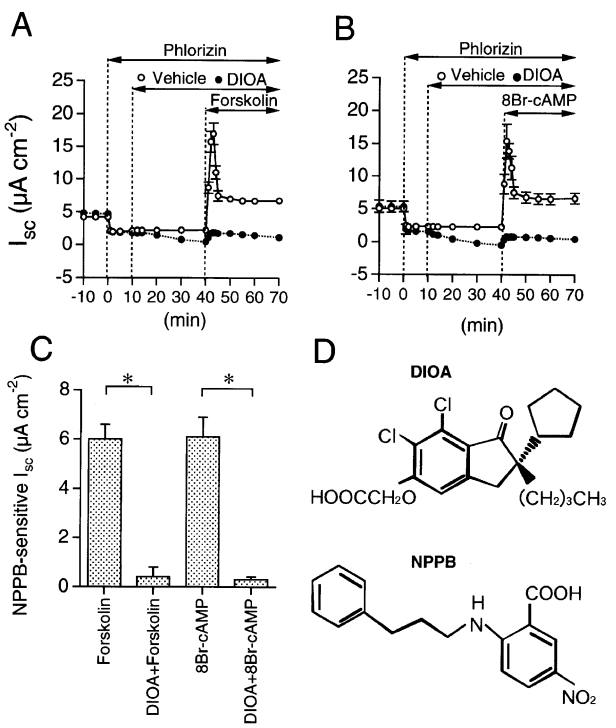


Fig. 2. Effects of DIOA on the forskolin- and 8-bromo cyclic AMP (8Br-cAMP)-induced short-circuit current ($I_{sc}$). Forskolin (10 μM, A) or 8Br-cAMP (1mM, B) was applied in the basolateral solution pretreated with 100 μM DIOA or 0.1% DMSO (vehicle) in the bilateral solutions. (C) In the presence of DIOA or its absence, the NPPB-sensitive $I_{sc}$ was measured 30 min after the application of each cAMP-related agent by adding NPPB (100 μM, bilateral). $n = 6$–10, (*) significantly different ($P < 0.05$). Structures of DIOA and NPPB are shown in (D).

## 4. Discussion

To date, it has been accepted that NPPB is the most potent blocker of $Cl^-$ channels. In several kinds of epithelial cells expressing CFTR $Cl^-$ channels, NPPB strongly inhibited transepithelial $Cl^-$ transport (Wangemann et al., 1986; Keeling et al., 1991), as shown in the present study. However, we here provide new evidence that DIOA, which has been believed to be a selective blocker of the $K^+$–$Cl^-$ cotransporter (Garay et al., 1988; Linton and O'Donnell, 1999), suppressed the transepithelial $Cl^-$ current in cultured Calu-3 human airway epithelial cells as potently as NPPB did. Under bicarbonate-free conditions, the transepithelial $Cl^-$ transport involves the apical $Cl^-$ channels and basolateral anion transporters like the bumetanide-sensitive $Na^+$–$K^+$–$2Cl^-$ cotransporter (Niisato et al., 1999). According to a previous report by Garay et al. (1988), however, DIOA does not inhibit the $Na^+$–$K^+$–$2Cl^-$ cotransporter. In addition, they showed that DIOA reduced the activity of the DIDS-sensitive $Cl^-$ $HCO_3^-$ exchanger. The mechanisms underlying the effects of DIOA and DIDS are thought to involve competition with $Cl^-$ for a common site on the anion transporters. The CFTR $Cl^-$ channel is classified as a member of the ATP-binding cassette superfamily of anion transporters. The targets of most CFTR inhibitors are acknowledged to be the $Cl^-$ binding sites of the pore regions (Zhang et al., 2000). Accordingly, the explanation may be that NPPB and DIOA share a common $Cl^-$ binding site in CFTR $Cl^-$ channels. To confirm this point, we observed the response of apical conductance. Since CFTR $Cl^-$ channels are exclusively detected in cell-attached patch clamp studies of the confluent Calu-3 cells (Haws et al., 1994; Moon et al., 1997), a change of apical conductance reflects $Cl^-$ conductance through the apical CFTR $Cl^-$ channels (Moon et al., 1997;

Ito et al., 2000). Actually, the pretreatment with NPPB (100 μM) prevented the isoproterenol-elicited potentiation of apical conductance, whereas bumetanide had no effect. Our data show that DIOA (100 μM) totally prevented the NPPB-sensitive (100 μM) component of apical conductance, suggesting that DIOA and NPPB are equally susceptible to CFTR-mediated $Cl^-$ conductance.

Competition studies produced several lines of evidence that NPPB and IAA-94 share a common binding site to outwardly rectifying $Cl^-$ channels and act as open-channel blockers accessible to the channel pore from the cytosolic side (Singh et al., 1991). In the present study, IAA-94 at 1 mM, but not at 100 μM, mimicked the actions of NPPB and DIOA on the apical conductance, indicating that IAA-94 inhibits CFTR $Cl^-$ channels in spite of a potency lower than DIOA and NPPB. Wangemann et al. (1986) showed that lipophilic compounds with an anionic $COO^-$ group could be more accessible to the $Cl^-$ channel protein, and that the lipophilicity was produced by the apolar residue (i.e., phenyl- or cycoalkyl group). Further, since CFTR $Cl^-$ channels are characterized by low conductance (7 pS) and a low degree of size selectivity, there may not be multiple anion binding sites within the pore (McCarty et al., 1993). Considering these results, NPPB, DIOA and IAA-94 may share a common binding site with the CFTR. This hypothesis is consistent with the lack of significant differences among the Hill coefficients of the concentration–inhibition curve, which ranged from 2.1 to 2.4.

In conclusion, the present study suggests that DIOA blocks CFTR $Cl^-$ channels as potently as NPPB does, and that the butyl-group at position 2 in DIOA is important for its susceptibility to the CFTR.

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## References

Cragoe Jr., E.J., Gould, N.P., Wortersdorf Jr., O.W., Ziegler, C., 1982. Agents for the treatment of brain injury: 1. (Aryloxy)alkaonic acids. J. Med. Chem. 25, 567–579.

Garay, R.P., Nazaret, C., Hannaert, P.A., Cragoe Jr., E.J., 1988. Demonstration of a [$K^+$, $Cl^-$ ]-cotransport system in human red cells by its sensitivity to [(dihydroindenyl)oxy]alkanoic acids: regulation of cell swelling and distinction from the bumetanide-sensitive [$Na^+$, $K^+$,$Cl^-$ ]-cotransport system. Mol. Pharmacol. 33, 696–701.

Haws, C., Finkbeiner, W.E., Widdicombe, J.H., Wine, J.J., 1994. CFTR in Calu-3 human airway cells: channel properties and role in cAMP-activated $Cl^-$ conductance. Am. J. Physiol. 266, L502–L512.

Ito, Y., Kume, H., Yamaki, K., Takagi, K., 1999. Tetracyclines reduce $Na^+/K^+$ pump capacity in Calu-3 human airway cells. Biochem. Biophys. Res. Commun. 260, 13–16.

Ito, Y., Mizuno, Y., Aoyama, M., Kume, H., Yamaki, K., 2000. CFTR-mediated anion conductance regulates $Na^+$–$K^+$-pump activity in Calu-3 human airway cells. Biochem. Biophys. Res. Commun. 274, 230–235.

Keeling, D.J., Taylor, A.G., Smith, P.L., 1991. Effects of NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) on chloride transport in intestinal tissues and the T84 cell line. Biochim. Biophys. Acta 1115, 42–48.

Linton, S.M., O'Donnell, M.J., 1999. Contributions of $K^+$–$Cl^-$ cotransport and $Na^+/K^+$-ATPase to basolateral ion transport in malpighian tubules of *Drosophila melanogaster*. J. Exp. Biol. 202, 1561–1570.

McCarty, N.A., McDonough, S., Cohen, B.N., Riordan, J.R., Davidson, N., Lester, H.A., 1993. Voltage-dependent block of the cystic fibrosis transmembrane conductance regulator $Cl^-$ channel by two closely related arylaminobenzoates. J. Gen. Physiol. 102, 1–23.

Moon, S., Singh, M., Krouse, M.E., Wine, J.J., 1997. Calcium-stimulated $Cl^-$ secretion in Calu-3 human airway cells requires CFTR. Am. J. Physiol. 273, L1208–L1219.

Niisato, N., Ito, Y., Marunaka, Y., 1999. Activation of $Cl^-$ channel and $Na^+/K^+/2Cl^-$ cotransporter in renal epithelial A6 cells by flavonoids: genistein, daizein, and apigenin. Biochem. Biophys. Res. Commun. 254, 368–371.

Singh, A.K., Afink, G.B., Venglarik, C.J., Wang, R., Bridges, R.J., 1991. Colonic $Cl^-$ channel blockade by three classes of compounds. Am. J. Physiol. 260, C51–C63.

Tamaoki, J., Chiyotani, A., Kobayashi, K., Sakai, N., Kanemura, T., Takizawa, T., 1992. Effect of indomethacin on bronchorrhea in patients with chronic bronchitis, diffusepanbronchiolitis, or bronchiectasis. Am. Rev. Respir. Dis. 145, 548–552.

Venglarik, C.J., Singh, A.K., Bridges, R.J., 1994. Comparison of -nitro versus -amino 4,4′-substituents of disulfonic stilbenes as chloride channel blockers. Mol. Cell. Biochem. 140, 137–146.

Wangemann, P., Wittner, M., Di Stefano, A., Englert, H.C., Lang, H.J., Schlatter, E., Greger, R., 1986. $Cl^-$ channel blockers in the thick ascending limb of the loop of Henle. Structure–activity relationship. Pfluegers Arch. 407 (S8), S128–S141.

Yamaya, M., Finkbeiner, W.E., Chun, S.Y., Widdicombe, J.H., 1992. Ion transport by cultures of human tracheobronchial submucosal glands. Am. J. Physiol. 261, L485–L490.

Zhang, Z.-R., Zeltwanger, S., McCarty, N.A., 2000. Direct comparison of NPPB and DPC as probes of CFTR expressed in *Xenopus* oocytes. J. Membr. Biol. 175, 35–52.