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Physiology

α_2-Adrenergic inhibition of Cl^- transport by opercular epithelium is mediated by intracellular Ca^{2+}

(*Fundulus heteroclitus*/chloride cell/ionomycin/thapsigargin/intracellular pH)

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ABSTRACT **We isolated the opercular epithelium of seawater killifish (*Fundulus heteroclitus*) to study the mediation of catecholamine inhibition of Cl^- secretion. The receptors are α_2-adrenergic, as they have a high affinity for the α_2-adrenergic agonist clonidine over phenylephrine and clonidine action is blocked by yohimbine. Pertussis toxin and indomethacin did not block the clonidine effect; hence inhibitory guanine nucleotide-binding proteins (G_i proteins) and prostaglandins (respectively) are not involved. Intracellular pH (pH_i) of single chloride cells was measured microspectrofluorometrically and resting pH_i was 7.22 ± 0.03. However, pH_i was unaffected by clonidine; hence pH_i and Na^+/H^+ exchange are not involved. The lipoxygenase inhibitors nordihydroguaiaretic acid and baicalein and the lipoxygenase products (12*S*)- and (12*R*)-12-hydroxyeicosatetraenoic acid stimulated Cl^- secretion. Protein kinase C is an unlikely site of action because the diacylglycerol kinase inhibitor R59022 had no effect alone and did not block the clonidine effect. Ionomycin (1 μM) in normal but not low-Ca^{2+} solutions mimicked the action of clonidine and both inhibitions were reversible by isoproterenol. Thapsigargin, a releaser of intracellular Ca^{2+}, inhibited Cl^- secretion and this effect was reduced in low-Ca^{2+} solutions. Low-Ca^{2+} solutions also blunted but did not block entirely the clonidine response, indicating that the primary Ca^{2+} release was from intracellular stores. Whereas α_1-adrenergic receptors commonly act via the Ca^{2+}/inositol trisphosphate pathway, to our knowledge this is the first report of a Ca^{2+}-mediated α_2-adrenergic response in a nonmammalian vertebrate.**

Marine teleosts drink seawater, absorb the water and ions in the intestine, and actively secrete the salt "load" across their gill and skin epithelia using mitochondria-rich "chloride" cells (reviewed in refs. 1–3). Whereas regulation of Cl^- transport in cystic fibrosis has received much attention (e.g., ref. 4), the regulation of anion transport—particularly the hormonal *inhibition* of Cl^- secretion—is less well understood. Marine teleost skin and gills are unusual among Cl^--transporting epithelia because even when unstimulated, the chloride cells in isolated epithelia secrete Cl^- at a high rate (50–150 $\mu A \cdot cm^{-2}$) when bathed on both sides with saline (1, 5). The resting transport rate can be inhibited by epinephrine, α-adrenergic agonists, urotensin II, and acetylcholine; it can be stimulated by β-adrenergic agonists, cAMP, phosphodiesterase inhibitors (e.g., 3-isobutyl-1-methylxanthine), and urotensin I (1–3). The high transport rate in unstimulated tissues indicates that *in vivo* an inhibitory stimulus must be applied to shut down Cl^- secretion. This response is physiologically relevant because euryhaline fish that enter freshwater stop secreting Na^+ and Cl^-. During the acute phase, circulating catecholamines and corticosteroids are increased *in vivo* (6) and the branchial α-adrenergic response is a reduction in NaCl loss. Physiological levels of epinephrine equivalent to that found in stressed fish (0.1 μM epinephrine; ref. 6) inhibit Cl^- secretion by chloride cells *in vitro* by about 50% (5). Importantly, the α_2-adrenergic receptor action is not blocked by cholinergic antagonists (7), so the receptors appear to be postsynaptic.

The intracellular mediator of the α-adrenergic receptor has not been identified for Cl^- secretion in teleosts. The mediation is not via inhibition of adenylate cyclase and reduction in intracellular cAMP, since α_2-receptor activation does not reduce intracellular resting levels of cAMP or cAMP levels that had been augmented by isoproterenol, 3-isobutyl-1-methylxanthine, or forskolin (8). There was no effect of nominally Ca^{2+}-free solutions or of the ionophore A23187 on the rate of Cl^- secretion by *Fundulus* opercular epithelium (8); hence Ca^{2+} did not seem to be involved. The α_2-receptors in many systems operate via an inhibitory GTP-binding protein (G_i protein) and inhibition of adenylate cyclase (9, 10) or (e.g., in platelets; ref. 11) an activation of Na^+/H^+ exchange. Van Praag *et al.* (12) suggested that eicosanoids could be important intracellular regulators in chloride cells. We set out to determine which of several possible intracellular mediators might transduce the α-adrenergic signal in chloride cells.

MATERIALS AND METHODS

Adult killifish (*Fundulus heteroclitus*; 4–7 g) from the Antigonish estuary were kept indoors for at least 10 days in full-strength seawater (salinity, 30 g/liter) under natural photoperiod at 20–25°C and were fed daily a marine fish blend (TetraMarine, TetraWerke, Germany) supplemented twice weekly with brine shrimp. The animals were killed by decapitation, and opercular epithelia were dissected and mounted in either standard Ussing membrane chambers with membrane area of 0.125 cm^2 (for pharmacological studies) or a microscope stage chamber (surface area, 0.125 cm^2) for intracellular pH (pH_i) measurements by microspectrofluorometry. In the Ussing chambers, transepithelial conductance (G_t), transepithelial potential (V_t, mucosal side grounded), and short-circuit current (I_{sc}, $\mu A/cm^2$) were monitored as described (13). For fluorescence microscopy, the subepithelial chromatophore layer and muscle fibers were microdissected from the epithelium to help identify individual chloride cells and to reduce background autofluorescence.

We know of no previous attempt to measure pH_i of chloride cells from fish. We employed the fluorophore 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM), a membrane-permeant derivative of carboxy-

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Abbreviations: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester; HETE, 12-hydroxy-5-*cis*-8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; pH_i, intracellular pH; G_t, transepithelial conductance; I_{sc}, short-circuit current.

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fluorescein with a pK_a of 6.98 (14). The dye enters cells in the esterified (BCECF-AM) form and is deesterified intracellularly by nonspecific esterases to form the highly fluorescent product BCECF. The technique has been applied successfully to epithelial cells in cell suspension and to single epithelial cells *in situ* (e.g., ref. 15).

The *in vitro* bathing solution was a killifish Ringer's solution containing (in mM) Na^+, 160; Cl^-, 137; K^+, 4.0; HCO_3^-, 5.0; Ca^{2+}, 2.0; Mg^{2+}, 0.5; HPO_4^{2-}, 1.0; SO_4^{2-}, 0.5; gluconate, 14.0; glucose, 4.0; and Hepes Na, 10 (Hepes was from United States Biochemical; other chemicals were from Sigma). The solution had a pH of 7.8 ± 0.1 when bubbled with 99% O_2/1% CO_2 and an osmotic activity of 335 milliosmole·kg^{-1}. Opercular epithelia, mounted in the microscope chamber, were superfused with killifish Ringer's solution at 25°C. The fluorescence microscope, epifluorescence measurement, and calibration procedures (using 0.02 mM digitonin to release BCECF) were the same as used previously (15). BCECF-AM was loaded at a final concentration of 67 μM, added to the serosal (upper) hemichamber for 40–60 min, after which time the excess was rinsed off and the serosal superfusion was continued so as to wash out any leaked BCECF. Individual chloride cells were identified by phase contrast as large (10–20 μm in diameter), spherical or ovoid, finely granular cells with centrally placed nucleus; identification was confirmed by 2-*p*-dimethylaminostyrylpyridylethyl iodide (DASPEI) fluorescence (16). A census pH_i measurement of 7–10 randomly selected cells was taken at the beginning and end of each experiment to obtain average pH_i values. Experimental manipulations were performed while pH_i of one cell was measured at 15- or 30-s intervals.

The pharmaceuticals, from Sigma, were used at the indicated final concentrations and were dissolved in saline or the described vehicle: clonidine (0.32 μM, except for dose–response tests), phenylephrine (5.0 μM, except in dose–response tests), pertussis toxin (1.0 μg/ml), yohimbine (3.2 μM, dissolved in ethanol), nordihydroguaiaretic acid (NDGA, 20.0 μM, dissolved in dimethyl sulfoxide, DMSO), indomethacin (1.0–25 μM), baicalein (1.5 μM, in ethanol), thapsigargin (1.0 μM, in DMSO), and diacylglycerol kinase inhibitor (R59022, Calbiochem; 10.0 μM in ethanol). The lipoxygenase products (12*S*)- and (12*R*)-12-hydroxy-5-*cis*-8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid [(12*S*)- and (12*R*)-HETE, 2.0 μM, in methanol] were gifts from Merck Frosst Labs (Pointe Claire, PQ, Canada). Ionophores A23187 (10.0 μM, in DMSO) and ionomycin (1.0 μM, in DMSO) were from Calbiochem. Final DMSO and ethanol concentrations were <0.1% and did not affect I_{sc}. Drug effects were monitored for 30 min of incubation or until a new steady-state I_{sc} was reached. Data are expressed as the mean ± SEM and were compared by appropriate paired or unpaired *t* tests.

RESULTS

α-Adrenergic Receptor Subtype. We compared the efficacies of the α_1-receptor agonist phenylephrine with that of clonidine, an α_2-receptor agonist, in serial dose–response additions to paired opercular epithelia and found that the ED_{50} for clonidine was 0.51 μM, whereas that for phenylephrine was about 70 times greater (36.0 μM; Fig. 1). Chloride cells had ED_{50} values of 0.05 μM and 0.5 μM for epinephrine and norepinephrine, respectively, and both catecholamines had a greater effect than did phenylephrine (5). The α_2-receptor antagonist yohimbine effectively blocks the action of the α_2-agonist clonidine (ref. 17; also see below). Also, the α_1-receptor antagonist prazosin has no effect on the action of clonidine (17). Together, these results confirm that the receptor in chloride cells that mediates the inhibition of Cl^- transport is of the α_2 subtype.

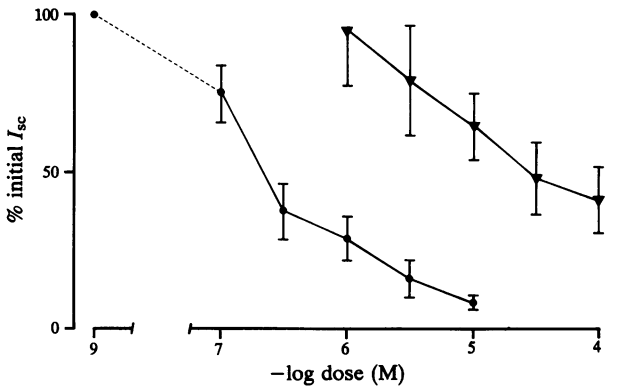

FIG. 1. Dose–response of inhibition of Cl^- secretion (as I_{sc}) by serial addition of α-adrenergic agonists phenylephrine (▼) and clonidine (●) (n = 6).

pH_i. We investigated the possible involvement of pH_i changes, specifically a reduction in pH_i associated with deactivation of the Na^+/H^+ antiporter. Chloride cells could be readily distinguished from other cell types in the epithelium by phase microscopy, and the photometer aperture had a 7-μm diameter at the plane of the tissue so that the signal from only one cell was measured. Individual chloride cells *in situ* in opercular epithelia had pH_i of 7.22 ± 0.03 (n = 24 membranes, 209 cells) in bathing solutions that were equilibrated with 1% CO_2 and had a pH of 7.7 ± 0.05 (n = 15). We found (by analysis of variance) that there was more cell-to-cell variation in pH_i within an opercular preparation than between fish. This suggests a rather broad range of pH_i among chloride cells. Addition of a maximally effective dose of clonidine (10 μM, serosal side) produced no immediate change in pH_i in cells that were monitored continuously for about 15 min after clonidine addition. The average pH_i of chloride cells after clonidine was likewise unchanged at 7.19 ± 0.03 (n = 19 membranes, 172 cells). Changes in pH_i could be detected in this preparation, inasmuch as the introduction of serosal bathing solutions that were equilibrated with 5% CO_2 produced a rapidly developing intracellular acidosis. In this case, pH_i started at 7.28 ± 0.07, n = 5 membranes, and dropped rapidly to 6.91 ± 0.03 ($P < 0.002$) in hypercapnia, and the effect was reversible, as pH_i rose again to 7.35 ± 0.09 during the wash period. Control exchanges of solutions produced no detectable change in the BCECF signal. Whereas chloride cells seem to regulate pH_i below the extracellular pH, changes in pH_i did not seem to be involved in the action of clonidine.

The effect of changes in pH_i on Cl^- transport was examined further in Ussing membrane chambers, where the transport rate as I_{sc} could be monitored during exposure to elevated (5%) and diminished (air) CO_2. Reduction of bath pH from 7.70 ± 0.05 to 7.02 ± 0.06 in 5% CO_2 produced marked cellular acidosis (above) but inhibited the Cl^- secretion rate as I_{sc} by only 24.9%, from 364 ± 47.6 to 273 ± 32.7 μA/cm^2 ($P < 0.025$; n = 6) (Fig. 2). Alkalosis in air produced a rise in bath pH to 7.95 ± 0.06 (n = 5) but no significant change in I_{sc}. The small reduction in Cl^- secretion in acidotic solutions could be accounted for by the narrow pH optimum for Na^+,K^+-ATPase and its inhibition by a drop in pH_i, such as has been observed in rabbit urinary bladder (18).

To test whether G_i proteins were involved in the reduction of Cl^- transport, we applied pertussis toxin, known to block norepinephrine-induced melanocyte aggregation in fish (19), to opercular epithelia with parallel running paired control membranes and observed no significant change in I_{sc} (Fig. 2). Pertussis toxin did not diminish the effect of 0.32 μM clonidine. Indomethacin, a blocker of prostaglandin production in *Fundulus* opercular epithelium (12), had no effect on

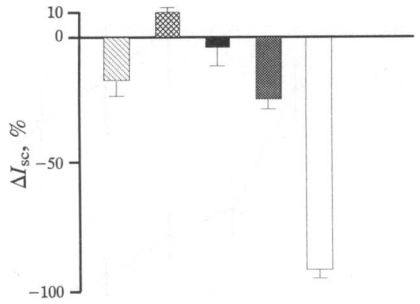

FIG. 2. Pertussis toxin (1 μg/ml; a G_i-protein blocker; n = 5; hatched bar), indomethacin (1–25 μM; a cyclooxygenase inhibitor; n = 6; cross-hatched bar), and calcium ionophore A23187 (10 μM; n = 5; filled bar) had little effect on Cl^- secretion rate (as I_{sc}). Reduction of bath pH in hypercapnic bathing solutions produced cellular acidosis (see text) and decreased I_{sc} by 25% (stippled bar). None of these pretreatments affected the α_2-adrenergic inhibition of I_{sc} (shown is the pooled clonidine effect, open bar).

resting I_{sc} and did not reduce the clonidine effect (Fig. 2). The Ca^{2+} ionophore A23187 at doses up to 10 μM, which are effective in many systems, had no effect alone and did not diminish the response to clonidine (Fig. 2).

Because Van Praag *et al.* (12) identified several lipoxygenase products as possible regulators of chloride cells, we attempted to block arachidonate 5-lipoxygenase and 12-lipoxygenase with the inhibitors NDGA and baicalein. These two drugs produced a mild stimulation of I_{sc} (Fig. 3) and so we looked for previously untested metabolites that might be inhibitory. Because the leukotrienes that had been tested previously (leukotrienes C_4, D_4, and E_4) were all stimulatory (12), we looked at the intermediates (12*S*)- and (12*R*)-HETE. Both these metabolites stimulated I_{sc} and did not block the effect of clonidine (Figs. 3 and 4); hence it appears that clonidine does not act via this pathway.

Ca^{2+} Mediation. On reexamination of the possibility of Ca^{2+} mediation of the clonidine response we used the Ca^{2+} ionophore ionomycin, which is more effective than A23187 in that the turnover number of ionomycin is 3–5 times greater than for A23187 (20). Ionomycin at 1.0 μM produced a sustained inhibition of I_{sc} of about 60% with a time course that was similar to that for clonidine (Table 1; Fig. 5). The effect of ionomycin and clonidine combined was the same as the effect of clonidine alone (Table 1), suggesting that the ionomycin effect was a portion of the total. To ensure that the ionomycin effect was specific to its action on Ca^{2+} influx, we designed a Ca^{2+}-depletion protocol that would reduce the Ca^{2+} in the medium but would not adversely affect the

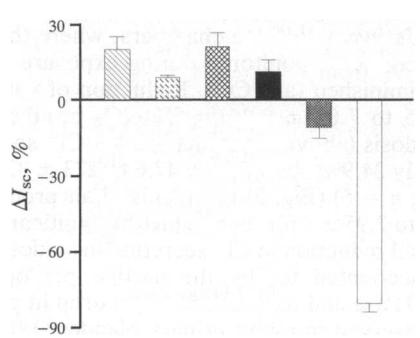

FIG. 3. Baicalein (1.5 μM; n = 9; hatched bar) and NDGA (20 μM; n = 8; dashed bar), inhibitors of 5- and 12-lipoxygenase, and the lipoxygenase products (12*S*)- and (12*R*)-HETE (2 μM; n = 7 and 5, respectively; crosshatched and filled bars) caused a modest stimulation of I_{sc}. The diacylglycerol kinase inhibitor R59022 (10 μM; n = 7; stippled bar) produced a small inhibition. None of the above pretreatments significantly affected the clonidine inhibition (open bar).

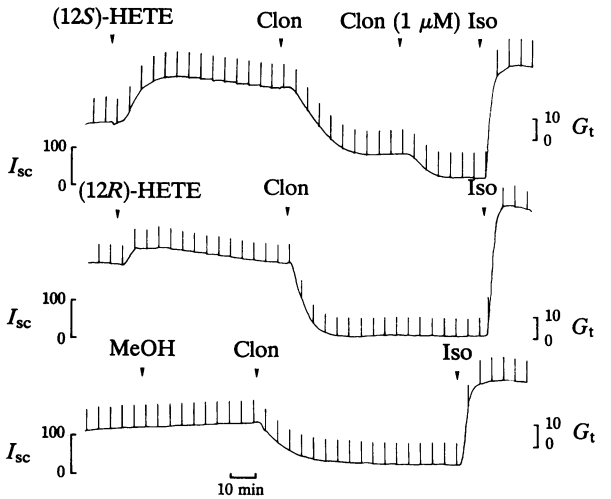

FIG. 4. Example recording of I_{sc} with a parallel-running membrane as a control. The solid line is I_{sc} (μA/cm²) and the transient deflections in the current record represent total epithelial conductance (G_t, mS/cm²). While the methanol vehicle had no effect on I_{sc}, both (12*S*)-HETE and (12*R*)-HETE stimulated I_{sc} by 10–30%. Neither pretreatment blocked the clonidine response or the subsequent stimulation with isoproterenol (Iso, 1.0 μM). Clon, clonidine (0.32 μM, unless indicated otherwise).

integrity of the epithelium. Ca^{2+}-free solutions on both sides cause rapid reductions in epithelial resistance that indicate an opening of the paracellular pathway and may have contributed to previous negative results with low-Ca^{2+} solutions (8). The low-Ca^{2+} medium (expected Ca^{2+} activity was <1.0 μM) was added to the serosal side 30–40 min before the test substances (clonidine or ionomycin); the mucosal side remained in the high-Ca^{2+} saline (1.6 mM). The values of G_t in three trials was 7.12 ± 0.84 (n = 7), 6.25 ± 0.57 (n = 10), and 6.78 ± 0.71 (n = 8) mS/cm² before low-Ca^{2+} solutions and was 9.56 ± 1.14, 8.15 ± 0.64, and 8.66 ± 1.11 mS/cm² after 30 min in low-Ca^{2+} solutions. Hence there was a consistent rise in G_t of about 2 mS/cm²; parallel-run time controls in

Table 1. Effect of low-Ca^{2+} (0.1 mM Ca^{2+} with 0.75 mM EGTA) solution on regulation of Cl^- transport (as I_{sc})

Treatment	I_{sc}, μA/cm²		P
	Normal Ca^{2+}	Low Ca^{2+}	
	(n = 13)	(n = 10)	
Control	124.5 ± 11.2	111.5 ± 10.9	0.422
Clonidine	10.1 ± 3.5	25.1 ± 6.3	0.038*
	(−91.9 ± 3.1%)	(−78.3 ± 4.5%)	(0.018*)
Isoproterenol	270.5 ± 21.1	176.2 ± 16.0	0.003*
	(n = 7)	(n = 8)	
Control	172.6 ± 29.6	113.9 ± 16.0	0.125
Ionomycin	83.0 ± 13.0	94.3 ± 13.6	0.607
	(−60.3 ± 4.3%)	(−12.4 ± 10.9%)	(0.002*)
Clonidine	4.0 ± 3.0	25.3 ± 5.6	0.008*
	(−96.1 ± 1.2%)	(−77.6 ± 6.9%)	(0.028*)
Isoproterenol	270.0 ± 26.1	132.0 ± 21.0	0.001*
	(n = 7)	(n = 7)	
Control	141.6 ± 18.8	123.7 ± 16.2	0.525
Thapsigargin	90.0 ± 21.1	100.0 ± 16.3	0.739
	(−38.6 ± 3.2%)	(−21.3 ± 5.7%)	(0.021*)
Clonidine	6.1 ± 1.9	22.9 ± 7.5	0.080
	(−95.1 ± 1.3%)	(−83.1 ± 5.7%)	(0.063)
Isoproterenol	150.0 ± 17.6	218.0 ± 16.5	0.009*

Preparations were treated sequentially with the indicated drugs. Statistical analysis was by unpaired two-tailed t test comparing normal and low Ca^{2+}. Significance (*), $P < 0.05$.

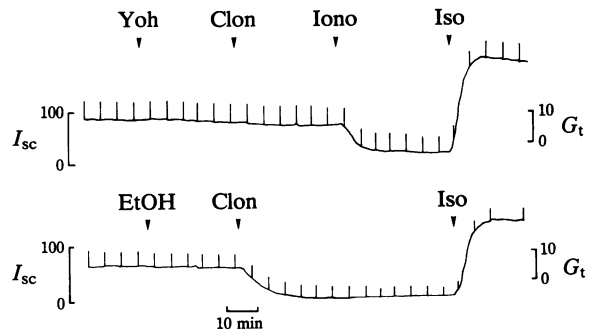

FIG. 5. Yohimbine (Yoh, 3.2 μM) and its ethanol vehicle had no effect on I_{sc} (μA/cm²) or G_t (mS/cm²) but yohimbine blocked the action of clonidine (Clon, 0.32 μM). The Ca^{2+} ionophore ionomycin (Iono, 1.0 μM) markedly reduced I_{sc} and with a time course similar to that of the clonidine response. The inhibitions of I_{sc} by clonidine and ionomycin were reversible with the addition of isoproterenol (Iso, 1.0 μM).

high-Ca^{2+} medium rose only slightly from 6.44 ± 0.50 (n = 7) to 6.66 ± 0.41 mS/cm².

Low-Ca^{2+} saline reduced the effect of ionomycin markedly from 60% inhibition to 12% (Table 1), as expected. Addition of clonidine to low-Ca^{2+}-treated epithelia decreased I_{sc} significantly less (77–83% vs. 92–96%) than the inhibition seen in the paired normal Ca^{2+} control (Table 1), and the final I_{sc} in low Ca^{2+} was significantly higher (23–25 vs. 4–10 μA/cm²) than in normal Ca^{2+}. This "blunting" of the clonidine response was also seen in the tissues that had been exposed to ionomycin (or thapsigargin) in low-Ca^{2+} saline first (Table 1). These relatively subtle changes suggest that Ca^{2+} is needed for the clonidine response. Because the clonidine effect was not entirely blocked in low-Ca^{2+} saline, it appeared that clonidine could release Ca^{2+} mainly from intracellular stores (because 30- to 40-min unilateral exposure to low-Ca^{2+} saline would most likely only partially deplete intracellular Ca^{2+} stores).

To help determine whether clonidine released intracellular Ca^{2+}, we attempted to increase intracellular Ca^{2+} by using thapsigargin, which releases Ca^{2+} from intracellular pools separate from those affected by protein kinase C and may inhibit Ca^{2+}-ATPase (21). Thapsigargin (1.0 μM, serosal side) reduced I_{sc} by about 40% in normal-Ca^{2+} saline and 20% in low-Ca^{2+} saline, indicating that intracellular release of Ca^{2+} inhibits I_{sc} and suggesting that thapsigargin's effect was at least partially dependent on high extracellular Ca^{2+} (Table 1). Clonidine inhibition was additive to that of thapsigargin.

DISCUSSION

Inhibition of Cl^- secretion by the chloride cells in opercular epithelium and gills of euryhaline teleost fish is an essential ionoregulatory mechanism connected with the early stages of acclimation by seawater euryhaline fish to dilute medium. Without this response, as with stenohaline marine teleosts, ion loss continues in the dilute medium and the animal dies of osmoregulatory failure. The action of catecholamines in the response is well recognized, and while both α- and β-adrenergic receptors are present, the response to nonselective agonists (epinephrine) is an α_2-receptor-mediated inhibition (refs. 5, 7, and 22; Figs. 1 and 5). Inhibition of adenylate cyclase was apparently not part of the intracellular mediation of the effect, because clonidine at maximal doses did not decrease resting cAMP levels (7). Previous studies were unable to determine the intracellular mediator of the α_2-receptor, although Ca^{2+} did not seem to be a likely candidate inasmuch as ionophore A23187 was ineffective and the Ca^{2+}-channel blocker verapamil did not block the effect of clonidine (7). The Ca^{2+}-channel blocker D600, however, partially inhibited I_{sc} (22). We found that α_2-adrenergic agonists inhibit Cl^- secretion with a high affinity (Fig. 1) and that the α_2-adrenergic antagonist yohimbine blocked the clonidine response (Fig. 5), confirming the receptor subtype.

The intracellular mediation of the α_2-receptor apparently involves a rise in intracellular Ca^{2+} because there is an inhibition of I_{sc} by the Ca^{2+} ionophore ionomycin (Table 1) and the ionomycin response mimics closely the time course of the clonidine inhibition (Fig. 5). The action of ionomycin is dependent on external Ca^{2+}, inasmuch as low-Ca^{2+} solution blocks the ionomycin response (Table 1). Both the ionomycin inhibition and the clonidine effects are reversible with increases in cAMP via stimulation of β-adrenergic receptors with isoproterenol (Fig. 5); hence ionomycin is not toxic at the doses used. Further evidence for Ca^{2+} involvement is that Ca^{2+}-depleted solutions on the serosal side "blunt" the clonidine inhibition (Table 1). The fact that low-Ca^{2+} solutions do not completely block the effect of clonidine is not surprising, given that intracellular sources of Ca^{2+} may be mobilized efficiently by clonidine but may not be depleted in our low-Ca^{2+} regime. We used thapsigargin at doses known to mobilize intracellular Ca^{2+} from a pool that is insensitive to protein kinase C (21), and the drug produced a mild inhibition of I_{sc} that was reversible and did not block the further effect of clonidine (Table 1). As with other systems (HeLa cells; ref. 21) a decrease in extracellular Ca^{2+} decreases the effectiveness of thapsigargin (Table 1), apparently because the thapsigargin-mobilized pool of Ca^{2+} is depleted by removal of external Ca^{2+}. Our results are also consistent with the fact that thapsigargin (less effective in our system) mobilizes a *portion* of the Ca^{2+} retained in cells and considerably more Ca^{2+} can be mobilized with ionomycin (which is more effective in our system) (23). Finally, the previously reported ineffectiveness of the calmodulin blocker trifluoperazine at 0.1 mM (22) was not repeated in a later work (7) wherein control addition of clonidine (1.0 μM) produced an inhibition of 94.3 ± 2.7% (n = 5), but after 1.0 mM trifluoperazine this inhibition was reduced significantly (P = 0.0012; unpaired t test), to 57.4 ± 6.4% (n = 8). Hence calmodulin blockade can reduce the effectiveness of clonidine, contrary to the conclusions of May and Degnan (7) but consistent with the intracellular Ca^{2+}-mediated response reported here.

There are two possible pathways in the phospholipase C cascade, one through diacylglycerol, diacylglycerol kinase, and protein kinase C and the other through inositol bisphosphate and trisphosphate in combination with Ca^{2+} (14). The diacylglycerol pathway is not involved because the diacylglycerol kinase inhibitor had no effect on the clonidine response (Fig. 3), and in preliminary experiments, phorbol 12-myristate 13-acetate at 10 μM (which activates protein kinase C) had little effect on I_{sc}. At present it is not possible to stimulate the inositol trisphosphate pathway directly because inositol trisphosphate, to be effective, must be microinjected and the extensive tubular system of chloride cells precludes the use of this procedure. On the basis of elimination of the diacylglycerol pathway and the strong evidence for a Ca^{2+} mediation of the effect, we conclude that inhibition involves release of intracellular stores of Ca^{2+} by inositol trisphosphate. Ca^{2+} mobilization has only recently been recognized as an intracellular response to α_2-adrenoceptor activation, and only in a few systems—notably astroglial cells (24) and vascular smooth muscle (25) from mammals.

Our results with pH_i of chloride cells demonstrate that pH_i is apparently not part of the clonidine response. The pH_i, at 7.22 ± 0.03, is consistently lower than the external pH, 7.7 ± 0.05 and is comparable to our previous values obtained for urinary bladder (15). In both tissues, it appears that protons are normally being pumped out of the cell, because the

expected ratio of H^+ activities (intracellular over extracellular) with an assumed transmembrane potential of −55 mV and passive H^+ distribution would be 9.2, whereas the observed ratio is 3.0. This is consistent with the normal operation of a Na^+/H^+ antiporter where there is a concentration gradient favoring Na^+ entry across the basolateral membrane. As with our previous observations with urinary bladder (15) a rise in the partial pressure of CO_2 produces cellular acidosis. It is interesting that *in vitro* acidosis did not greatly inhibit I_{sc}, implying that Cl^- secretion could continue while fish are recovering from hypercapnic acidosis, such as that imposed by exhaustive exercise.

The α_2-adrenergic response in chloride cells of the opercular epithelium is unusual because in most Cl^--transporting epithelia, increases in intracellular Ca^{2+} *activate* Cl^- transport. This is true for the parietal cells of the gastric mucosa (26), airway epithelia that secrete Cl^- (4), and colonic cells that take up Cl^- (27). The fundamental difference seems to reside in the "resting" or unstimulated rate of anion transport by these tissues. Only in the case of the opercular epithelium is the free-running anion transport rate very high, 2–6 microequivalents of Cl^- per cm^2 per hr (Table 1 and ref. 1) as opposed to 0.04–0.29 microequivalents of Cl^- per cm^2 per hr in dog tracheal epithelium (28); hence, fish have inhibitory control over the process. Chloride cells secrete Cl^- via a secondary active-transport mechanism in which, according to the "Silva" model of operation of these chloride cells (see refs. 1 and 2), the transmembrane electrochemical gradient favoring Na^+ entry across the basolateral membrane, maintained by Na^+,K^+-ATPase, drives Cl^- into the cytoplasm, via Na^+–K^+–$2Cl^-$ cotransport, where it accumulates above its electrochemical equilibrium so that Cl^- exits the cell passively via anion channels in the apical membrane and is secreted out of the animal into seawater. K^+ apparently recycles across the basolateral membrane via K^+ channels while Na^+ is secreted via a cation-selective paracellular pathway down its electrochemical gradient. This model is shared (with minor variations for uptake or secretion) among the airway epithelia (4), parietal cells (26), colon (27), elasmobranch rectal gland and marine teleost gill (1), corneal epithelium (29), and thick ascending limb of the loop of Henle (30). Because isoproterenol and other agents that increase cAMP stimulate ion transport after pretreatment with clonidine, thapsigargin, or ionomycin (Table 1), the point in the inositol trisphosphate pathway that is affected by component(s) of the cAMP pathway must be distal to the rise in intracellular Ca^{2+}. It is feasible that the final regulatory point could be the apical membrane anion channel itself, as in colon (27), or the basolateral Na^+–K^+–$2Cl^-$ cotransporter (1).

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