Extracellular cyclic ADP-ribose potentiates ACh-induced contraction in bovine tracheal smooth muscle

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Franco, Luisa, Santina Bruzzone, Pinfang Song, Lucrezia Guida, Elena Zocchi, Timothy F. Walseth, Emanuele Crimi, Cesare Usai, Antonio De Flora, and Vito Brusasco. Extracellular cyclic ADP-ribose potentiates ACh-induced contraction in bovine tracheal smooth muscle. Am J Physiol Lung Cell Mol Physiol 280: L98–L106, 2001.—Cyclic ADP-ribose (cADPR), a universal calcium releaser, is generated from NAD⁺ by an ADP-ribosyl cyclase and is degraded to ADP-ribose by a cADPR hydrolase. In mammals, both activities are expressed as ectoenzymes by the transmembrane glycoprotein CD38. CD38 was identified in both epithelial cells and smooth myocytes isolated from bovine trachea. Intact tracheal smooth myocytes (TSMs) responded to extracellular cADPR (100 μM) with an increase in intracellular calcium concentration ([Ca²⁺]) both at baseline and after acetylcholine (ACh) stimulation. The nonhydrolyzable analog 3-deaza-cADPR (10 μM) elicited the same effects as cADPR, whereas the cADPR antagonist 8-NH₂-cADPR (10 μM) inhibited both basal and ACh-stimulated [Ca²⁺]i levels. Extracellular cADPR or 3-deaza-cADPR caused a significant increase in ACh-induced contraction in tracheal smooth muscle strips, whereas 8-NH₂-cADPR decreased it. Tracheal mucosa strips, by releasing NAD⁺, enhanced [Ca²⁺]i in isolated TSMs, and this increase was abrogated by either NAD⁺-ase or 8-NH₂-cADPR. These data suggest the existence of a paracrine mechanism whereby mucosa-released extracellular NAD⁺ plays a hormonelike function and cADPR behaves as second messenger regulating calcium-related contractility in TSMs.

acetylcholine; CD38; nicotinamide adenine dinucleotide/cyclic adenosine 5'-diphosphate-ribose-mediated paracrine mechanisms; calcium-related contraction of tracheal strips; adenosine 5'-diphosphate-ribose cyclase; cyclic adenosine 5'-diphosphate-ribose hydrolase.

CYCLIC ADP-ribose (cADPR) is a recently discovered second messenger involved in the mobilization of calcium from intracellular stores. First identified in sea urchin eggs (4, 19), where it was demonstrated to regulate the process of fertilization (18), cADPR has since been shown to be a naturally occurring nucleotide in a variety of mammalian cell types (28). cADPR is generated from NAD⁺ by an ADP-ribosyl cyclase and is degraded by a cADPR hydrolase (1, 11, 12, 17, 20, 26, 31). In mammals, both activities are expressed at the outer surface of the cell by CD38 (20), a long-known lymphocyte activation antigen and a type II transmembrane glycoprotein of 46 kDa (22).

The localization of CD38 raises the question of how ectocellularly generated cADPR can reach its receptor-operated intracellular target stores (27) and release calcium therefrom. This topologic paradox has recently been addressed and explained on the basis of two transport systems that mediate NAD⁺ and cADPR trafficking across cell membranes (5). Transport of cADPR can occur because of a unique property of CD38, which behaves as a catalytically active transporter of its product cADPR. Thus cADPR, once produced at the active site, can cross the plasma membrane directly through channel-generating structures (dimers and tetramers) of transmembrane CD38 (3, 7). Access of cytoplasmic NAD⁺ to the catalytic site of CD38, either ectocellular or intravesicular (i.e., within exocytotic or endocytotic membrane vesicles), is enabled by an equilibratory NAD⁺ transport system, which is present in several CD38-positive and CD38-negative cell types (5, 33).

A different mechanism allowing extracellularly or ectocellularly generated cADPR to cross the plasma membrane is direct permeation of cADPR to its intracellular target stores. Such an influx of cADPR, which occurs via a CD38-unrelated transport system, has been described in murine B lymphocytes (11), rat cerebellar granule neurons (6), and human hematopoietic progenitors (23). In these cells, externally added cADPR induces intracellular calcium mobilization that then activates cellular responses ranging from depolarization to proliferation (5).
Tracheal smooth muscle cells have recently been shown to respond to acetylcholine (ACh)-induced muscarinic receptor stimulation by releasing calcium from ryanodine-sensitive receptors expressed in the sarcoplasmic reticulum (24). Moreover, permeabilized tracheal smooth myocytes (TSMs) are susceptible to extracellularly added cADPR, which triggers intracellular calcium movements. These observations would suggest an involvement of this cyclic nucleotide in the physiological regulation of airway smooth muscle tone, provided its effects are also exerted on intact cells.

Based on these findings, the aims of the present study were to elucidate whether 1) bovine tracheal tissue cells express cADPR-metabolizing enzyme activities at their outer surface, 2) extracellularly added or produced cADPR affects intracellular calcium levels in isolated intact smooth muscle cells derived from tracheal strips, and 3) extracellular cADPR affects ACh-induced contraction of these tracheal strips via intracellular calcium mobilization. In investigating these problems on intact smooth myocytes and tracheal strips, we discovered a hitherto unknown paracrine cross talk between the NAD$^+$-releasing and cADPR-generating epithelium and the cADPR-responsive muscle layer. This paracrine interplay between two different cell types may prove to be a common mechanism of cADPR function in several mammalian tissues.

EXPERIMENTAL PROCEDURES

Materials. Anti-human CD38 monoclonal antibody (MAb) (21) was kindly provided by Prof. F. Malavasi (Department of Genetics, Biology, and Biochemistry, University of Turin, Turin, Italy). cADPR was prepared enzymatically from NAD$^+$ with ADP-ribosyl cyclase from Aplysia californica (courtesy of Prof. H. C. Lee, Department of Pharmacology, University of Minnesota, Minneapolis, MN) and purified by high-performance liquid chromatography (HPLC) as previously described (10). 3-Deaza-cADPR was synthesized as previously described (29). Fura 2-AM was purchased from Calbiochem (Darmstadt, Germany). Unless otherwise specified, all chemicals were of the highest purity grade available from Sigma (St. Louis, MO).

Tissue preparation. Bovine trachea were freshly obtained from the local abattoir and processed. The lower middle portion of the trachea was removed and immersed in aerated KH$_2$PO$_4$, 3.4 mM KCl, 2.4 mM CaCl$_2$, 110.5 mM NaCl, 25.7 mM NAD, 2.5 mM cADPR, respectively, by incubating dissociated myocytes with 50 mM DMEM containing 20% FCS, briefly homogenized as described above, and centrifuged at 100 g for 5 min to remove undigested tissue pieces. The supernatant containing the dissociated myocytes was then centrifuged at 600 g for 5 min, and the cell pellet was washed twice and resuspended (1 mg/ml) in DMEM-20% FCS. Routine fluorescence-activated cell-sorting analysis (see below) of the cell suspension with an anti-α-actin MAb (Sigma) revealed > 99% staining of the recovered myocytes. Integrity of the epithelial and muscle cells was routinely checked by the trypan blue exclusion test. Cytosol volumetric analyses of fresh myocyte suspensions were performed by incubating intact cells in 50 μM/ml of the IB4 MAb (21) in DMEM-20% FCS for 20 min at 4°C. The cells were then washed 3 times with 0.5 ml cold PBS-glucose. FITC-conjugated anti-mouse IgG in complete medium for 30 min at 4°C and then washed again, and fluorescence intensity was quantitated by flow cytometry with a FACScan (Becton Dickinson, Milan, Italy). No measurable fluorescence was observed on myocytes incubated only with the second antibody.

Assay of enzyme activities. NAD$^+$-ase, GDP-ribosyl cyclase, and cADPR hydrolase activities were assayed on 1 mM NAD$^+$, 1 mM nicotineamide guanine dinucleotide, and 0.5 mM cADPR, respectively, by incubating dissociated myocytes, epithelial cells (~300 μg total protein/assay), or whole tracheal tissue pieces (~5 mg total protein/assay) in 0.4 ml of PBS-glucose at 37°C. The use of intact cells for the assay of ectoenzyme activities on the corresponding substrates is a common procedure for activities related to cADPR metabolism in mammals and has been validated in many cell types (16, 20, 26, 32, 33). At different times, 60-μl aliquots of the incubation mixture were withdrawn and centrifuged for 30 s at 5,000 g. The corresponding supernatants were then deproteinized with TCA (10% final concentration) and centrifuged, and the excess TCA was removed with diethyl ether (10). HPLC analysis of the samples was performed to estimate the corresponding products generated by the three enzyme activities [ADP-ribose (ADPR) for NAD$^+$-ase, cyclic GDP-ribosyl (cGDPR) for GDP-ribosyl cyclase, and ADPR from cADPR for cADPR-hydrolase] and released into the extracellular medium. Briefly, HPLC analysis was performed on a Hewlett-Packard HP1090 instrument. A 5-cm, 4.6-μm ODS-Hypersil C18 reverse-phase (Hewlett-Packard) column was used. Solvent A was 0.1 M containing 5 mM tetrabutylammonium, pH 5.0; solvent B was solvent A containing 30% (v/v) methanol. The solvent program was a linear gradient (at a flow rate of 0.4 ml/min) starting at 100% solvent A and increasing to 100% solvent B in 30 min. The nucleotides were detected with a HP1040 A diode-array spectrophotometric detector set at 260 nm. Elution times of the standard nucleotides were 4.5 min for cGDPR, 5.6 min for cADPR, 8.0 min for nicotineamide guanine dinucleotide, 12.0 min for NAD$^+$, 16.5 min for GDP-ribosyl (GDPR), and 23 min for ADPR. Protein determination was performed on aliquots of the sonicated incubation mixture as previously described (2). Immunochemical detection of CD38. Isolated myocytes (1 mg), epithelial mucosal cells (1 mg), and murine 3T3 Swiss fibroblasts (0.5 mg, CD38-negative control; Ref. 30) were lysed by sonication (30 s at 3 W on ice) in 0.8 ml of PBS at 4°C. Human red blood cell membranes, used as a CD38-positive control, were prepared as previously described (31).
L100 EFFECTS OF CYCLIC ADP-RIBOSE ON TRACHEAL SMOOTH MUSCLE

Modified Laemmli (14) sample buffer (8% SDS without β-mercaptoethanol and EDTA) was added to each sample (50 μg of protein), heated for 5 min at 100°C, and subjected to 10% SDS-PAGE (14). Proteins were blotted on nitrocellulose membranes as previously described (32), and saturation of the membranes, incubation with the first anti-CD38 Mab (IB4), and addition of the second antibody (anti-mouse IgG; Amersham, Milan, Italy) were performed following instructions of the Amersham ECL Plus immunodetection kit.

Fluorometric calcium measurements. Isolated TSMs (100-μl suspension in DMEM-20% FCS) were incubated either without additions (control) or in the presence of 1, 10, or 100 μM cADPR (HPLC purified; Ref. 10); 10 nM 3-deaza-cADPR, or 10 μM 8-NH2-cADPR at 37°C for 2 h. Fura 2-AM (10 μM) was added during the last 45 min of incubation. Myocytes were then centrifuged at 200 g for 5 min, washed in 2 ml of a zero-calcium solution (135 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 10 mM glucose, and 5 mM HEPES, pH 7.4), resuspended in 0.5 ml of zero-calcium buffer, and laid on a confluent layer of adherent murine 3T3 Swiss fibroblasts (ATCC, VA) (cyclic AMP-sensitive) on a 20-mm-diameter glass coverslip that formed the bottom of a 200-μl chamber. The presence of a feeder of murine fibroblasts (not fura loaded) significantly increased myocyte adherence and viability during the 20-min assay without providing any detectable interference on the calcium responses to cADPR. The intracellular free calcium concentration ([Ca2+]i) was determined as previously described in detail (30).

Acetylcholine (ACh) was added to the perfusion chamber at 50 μM final concentration, and the induced calcium increase was measured by a calcium imaging system (IMSTAR, Geneva, Switzerland) assembled on a Zeiss Axiosvert inverted microscope. Fura 2 emission was stimulated by a very fast monochromator that switched between 340- and 380-nm wavelengths and was positioned along the light beam of a 1,000-W xenon source. Fluorescence images were then acquired, and a ratio was calculated for the resulting calcium image every 1.5 s. The calibration procedure was similar to the protocol previously described (30, 33).

Tracheal smooth muscle contractility studies. The physical characteristics, weight, and resting and maximal force of the 22 strips from the 9 animals used in this study were similar among the experimental groups. Mucosa-free tracheal strips (~10 × 2.3 mm, mean weight 29.7 ± 11.0 mg) were mounted in 25-ml water-jacketed glass tissue baths containing aerated PSS at 37°C and 10−6 M indomethacin to block spontaneous production of prostaglandins. The lower end of each strip was anchored to a stationary hook via a silk string while the upper end was connected by a silk string to a force transducer (Grass Medical Instruments model FT03D) mounted on a micromanipulator. Isometric force was continuously recorded (Gould TA4000 strip-chart recorder).

Muscle strips were contracted for 30 s at 5-min intervals by electrical field stimulation (0.5-ms pulse duration, 25 Hz, and 25 V) provided by a direct current amplifier (Section of Engineering, Mayo Clinic, Rochester, MN) and triggered by an electric stimulator (Grass Medical Instruments model S44) via a pair of vertically mounted parallel platinum plate electrodes (1 × 4 cm). Over a 2-h period, each muscle strip was progressively stretched until the length at which it developed maximal isometric force was determined. This optimal length was maintained throughout the studies. All strips were incubated for 20 min with PSS containing 10−6 M tetrodotoxin to block neurotransmission and spontaneous release of ACh and then contracted with cumulatively increasing concentrations of ACh (10−9 to 10−4 M, in half-log increments). After baseline concentration-response curves to ACh were completed, the strips were washed with PSS until the resting forces were reestablished. The mean resting and maximal forces recorded before incubation with any of the cyclic nucleotides were 1.4 ± 0.6 and 30.9 ± 11.5 g, respectively. Thereafter, strips were incubated for 18 h with 100 μM cADPR (n = 6), 10 nM 3-deaza-cADPR (n = 6), or 10 μM 8-NH2-cADPR (n = 3) while another set of strips incubated with PSS only served as controls to account for the effect of time on contractility. Concentration-response curves to ACh were then repeated. At the end of the studies, all strips were blotted dry, excess tissue was removed, and the strips were weighed. Active force is expressed as a percentage of the maximal force recorded on a baseline concentration-response curve and corrected for the effect of time with the equation:

\[ R_{corrected} = R_{baseline}(R_{after}/R_{baseline}) - (C_{after}/C_{baseline}) + 1 \]

where R is the force developed by muscles incubated with cADPR, 3-deaza-cADPR, or 8-NH2-cADPR and C is the force of the control muscles.

Determination of NAD+ efflux from tracheal mucosa. This was performed with an adaptation of a procedure that was previously developed for cultured cells to maximize NAD+ release by maintaining a near-zero extracellular concentration of NAD+ (33). Briefly, mucosal tissue freshly dissociated from the muscle layer was cut into small pieces and divided into four samples (~2 mg each). One sample used for measuring the starting levels of NAD+ was immediately homogenized by sonication (30 s) in 0.5 ml of carbonate buffer (100 mM Na2CO3, 20 mM NaHCO3, and 10 mM nicotinamide, pH 10) in ice and frozen in liquid nitrogen. The other samples were rapidly washed three times for 30 s each at 4°C with 1 ml of PBS-glucose to remove the intracellular rapidly exchangeable NAD+. Repeated washings of cultured cells have been previously demonstrated to result in a progressive release of NAD+ from cells (33), thus making the dinucleotide efflux measurable. Therefore, one of the washed samples was immediately extracted and frozen as described above to allow determination of the residual NAD+ content. The two remaining washed samples were incubated for 4 h at 37°C, one with and the other without medium changes (1 ml of PBS-glucose) every hour. The corresponding medium samples were immediately centrifuged at 5,000 g for 30 s, TCA extracted as described in Assay of enzyme activities, and alkanized with a one-tenth volume of 10× carbonate buffer. At the end of the incubations, the two tissue samples were extracted in carbonate buffer, homogenized, and frozen as described above. Tissue extracts were then thawed, sonicated, and centrifuged at 50,000 g for 20 min, and the NAD+ content of the extracts and medium supernatants was determined by a sensitive enzymatic cycling assay as described previously (33). To calculate the percentage of cell lysis, hexokinase activity was assayed in aliquots of unextracted supernatants and in sonicated tissue lysates as previously described (33).

Effects of mucosal tissue on TSM [Ca2+]i. Isolated TSMs (1.0-ml suspension) were incubated as described in Cell isolation with fresh strips of mucosal tissue (10-mg total weight) for 2–4 h at 37°C in the presence and absence of either NAD+ase (0.5 U/ml final concentration) or 8-NH2-cADPR (10 μM). Fura 2-AM loading was performed as described in Fluorometric calcium measurements. At the end of the incubation, the mucosal pieces were removed, and the basal [Ca2+]i of isolated myocytes was determined as described in Fluorometric calcium measurements.

Statistical analyses. Concentration-response curves to ACh of the same strips before and after nucleotide incubation were tested for significance by a two-factor repeated-measures ANOVA with Duncan’s post hoc test for multiple com-
parisons. Enzyme activities were tested by paired t-test. \([Ca^{2+}]_i\) values were analyzed with one-way ANOVA and two-sided Dunnett’s t-test. \(P\) values < 0.05 were considered significant.

RESULTS

Expression of ADP-ribosyl cyclase in tracheal tissue.

Cytofluorometric analyses of freshly prepared TSMs revealed that as many as 70% of them express immunoreactive CD38 on their outer surface (data not shown). Moreover, both major cell components of tracheal tissue, mucosa and smooth muscle, were found to express the enzyme activities of CD38, i.e., NAD \(^+\)-ase, GDP-ribosyl cyclase, and cADPR hydrolase (Table 1). These activities, measured on intact cells to confirm their ectocellular localization (see EXPERIMENTAL PROCEDURES), were significantly higher in epithelial cells than in myocytes (\(P < 0.05\)). Analysis of the cyclase on the corresponding Western blots was carried out with an anti-human CD38 MAb (IB4). A 46-kDa band was obtained from both mucosa and smooth muscle as well as from CD38-positive red blood cell membranes (positive control; Fig. 1), but its intensity was much greater in the mucosa, in agreement with a correspondingly higher cyclase activity present in the epithelium compared with smooth muscle. No immunoreactive band was detectable from native, CD38-negative murine 3T3 fibroblasts (30).

Thus on the basis of its catalytic properties, molecular mass, and immunoreactivity with an anti-CD38 MAb, the cyclase or hydrolase activity expressed on both cell components of tracheal tissue was identified as CD38.

Effect of cADPR on basal and ACh-stimulated \([Ca^{2+}]_i\) in intact myocytes. To investigate whether cADPR, the enzymatic product of CD38, could affect calcium homeostasis in TSM cells, intact TSMs were incubated at 37°C with extracellular cADPR, its non-hydrolyzable analog 3-deaza-cADPR, or its antagonist 8-NH\(_2\)-cADPR. After a 2-h incubation of intact bovine TSMs in the presence of cADPR at different concentrations, the \([Ca^{2+}]_i\) increased from 111 ± 4 (control) to 130 ± 8 (1 \(\mu\)M cADPR), 143 ± 9 (10 \(\mu\)M) and 160 ± 4 (100 \(\mu\)M) nM (Fig. 2). At 0.1 \(\mu\)M cADPR, the \([Ca^{2+}]_i\) levels were indistinguishable from the control level (data not shown). The cADPR analog 3-deaza-cADPR elicited a still higher effect than cADPR on myocyte \([Ca^{2+}]_i\) (172 ± 5 nM) but still at nanomolar concentrations. Incubation of intact TSMs with 10 \(\mu\)M 8-NH\(_2\)-cADPR resulted in a significant decrease (40%) in \([Ca^{2+}]_i\) compared with that in control cells. Finally, incubation of myocytes with a mixture of 100 \(\mu\)M cADPR and 100 \(\mu\)M 8-NH\(_2\)-cADPR did not result in any appreciable change in \([Ca^{2+}]_i\) compared with that

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**Table 1. Ectocellular enzyme activities of smooth myocytes and mucosal cells from tracheal tissue strips**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>NAD(^+)-ase, nmol ADPR-min(^{-1})-mg(^{-1})</th>
<th>GDP-Ribosyl Cyclase, nmol cGDPR-min(^{-1})-mg(^{-1})</th>
<th>cADPR Hydrolase, nmol ADPR-min(^{-1})-mg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated myocytes</td>
<td>0.773 ± 0.062</td>
<td>0.242 ± 0.15</td>
<td>0.155 ± 0.012</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>5.864 ± 0.375</td>
<td>2.012 ± 0.181</td>
<td>0.513 ± 0.043</td>
</tr>
</tbody>
</table>

Values are means ± SD from 6 experiments. ADPR, ADP-ribose; cGDPR, cyclic GDP-ribose; cADPR, cyclic ADPR. Assays of NAD \(^+\)-ase, GDP-ribosyl cyclase, and cADPR hydrolase activities were performed on intact cells as described in EXPERIMENTAL PROCEDURES.

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**Fig. 1.** Immunochemical detection of CD38 in epithelial mucosa and smooth muscle cells. Epithelial mucosa cells (lane B), isolated tracheal smooth myocytes (TSMs; lane C), and 3T3 murine fibroblasts (negative control; lane D; Ref. 30) were prepared and lysed as described in EXPERIMENTAL PROCEDURES. Human red blood cell (RBC) membranes (positive control; lane A) were prepared as described in Ref. 31. Samples were subjected to SDS-PAGE (10%) and Western blot analysis. Detection of CD38 was obtained with an anti-CD38 monoclonal antibody (MAb; IB4) and ECL Plus. Results are from a representative experiment (6 performed with different animals). No. on left, molecular mass.

**Fig. 2.** Changes in basal intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) induced by extracellular cyclic ADP-ribose (cADPR) and its analogs in TSMs. Isolated intact myocytes were incubated for 2 h in the presence and absence of different extracellular cyclic nucleotides as described in EXPERIMENTAL PROCEDURES, and basal [Ca\(^{2+}\)]\(_i\) was measured as previously described (30). Bar 1, control (no addition); bars 2–4, cADPR-treated cells (1, 10, and 100 \(\mu\)M, respectively); bar 5, 3-deaza-cADPR (10 nM)-treated cells; bar 6, cells treated with cADPR (100 \(\mu\)M) and 8-NH\(_2\)-cADPR (100 \(\mu\)M) together; bar 7, 8-NH\(_2\)-cADPR (10 \(\mu\)M)-treated cells. Data are means ± SD from 7 different experiments with different animals. The differences among all treated myocytes and the untreated group were significant (\(P < 0.01\)), with the exception of the group of myocytes treated with both cADPR and 8-NH\(_2\)-cADPR (\(P > 0.08\)).
in control myocytes. These data indicate that intact TSMs are intrinsically permeable and functionally responsive to each of the three cyclic nucleotides, the natural one and the two analogs. The concentration dependence of cADPR effects on myocyte $[Ca^{2+}]_i$ suggested the use of a $100 \mu M$ concentration of this HPLC-purified cyclic nucleotide in all subsequent experiments to avoid its possible consumption by cADPR hydrolase activity.

Next, the effect of extracellularly added cADPR on the ACh-induced calcium response in intact TSMs was explored. cADPR-preincubated cells ($100 \mu M$ for 2 h at $37^\circ C$) showed an increased calcium response to ACh ($50 \mu M$) after 5 s, with maximal $[Ca^{2+}]_i$ values of $950 \pm 71$ and $550 \pm 42$ nM in cADPR-treated and control myocytes, respectively ($P < 0.05$; Fig. 3). Comparable results were obtained on incubation of TSMs with 10 nM 3-deaza-cADPR, whereas a 40% inhibition of ACh-induced calcium response was observed with 8-NH$_2$-cADPR ($10 \mu M$)-preincubated cells (Fig. 3).

**Extracellular cADPR enhances ACh-stimulated TSM contractility.** Because ACh-induced calcium release is responsible for muscle contraction, we investigated whether the ACh-induced contraction of TSM strips was affected by extracellularly added cADPR and its analogs. Preliminary experiments indicated that the optimal incubation time to induce consistent changes in TSM contractility was $>12$ h, although a time interval of as low as 2 h was sufficient to elicit increases in $[Ca^{2+}]_i$, both at baseline and after ACh stimulation. This long incubation time is probably required to allow diffusion of cADPR within the tissue pieces to reach the target myocytes; during the incubation time, only minor consumption of cADPR ($<5\%$) occurred due to the hydrolase activity expressed by the whole TSM strip ($0.0011 \pm 0.0002$ nmol ADPR·min$^{-1}$·mg$^{-1}$). After 18 h of incubation with either $100 \mu M$ cADPR (Fig. 4A) or $10 \mu M$ 3-deaza-cADPR (Fig. 4B), the concentration-response curves to ACh were shifted to the left compared with the baseline curves ($P < 0.02$ for both). Increments of contractile force were significant for ACh concentrations ranging between $10^{-7}$ and $10^{-5}$ M with cADPR and between $5 \times 10^{-8}$ and $10^{-4}$ M with 3-deaza-cADPR ($P < 0.05$). Resting force was $1.4 \pm 0.3$
0.9 g after cADPR and 1.3 ± 0.8 g after 3-deaza-cADPR, which were not significantly different (P > 0.08) from paired baseline values (2.1 ± 0.8 and 1.3 ± 0.8 g, respectively). It is interesting to note that the presence of extracellular cADPR reduced by approximately one-half the ACh concentration that results in 50% of maximal force compared with the baseline trace (Fig. 4A).

After 18 h of incubation with 10 μM 8-NH₂-cADPR (Fig. 4C), the concentration-response curve to ACh was shifted to the right compared with the baseline curve (P < 0.05). Decrements of contractile force were significant for ACh concentrations ranging between 10⁻⁷ and 10⁻⁴ M (P < 0.05). Resting force after 8-NH₂-cADPR was 0.6 ± 0.6 g, which was not significantly different from baseline (1.2 ± 0.6 g; P = 0.8).

**NAD⁺ efflux from tracheal mucosa.** The decrease in both basal and ACh-stimulated TSM [Ca²⁺]i, induced by the cADPR antagonist 8-NH₂-cADPR suggested a role for endogenous cADPR in regulating myocyte calcium homeostasis in both conditions. However, the cyclase activity expressed on myocytes was found to be low compared with that expressed on the epithelial mucosa that overlays the myocytes “in vivo.” These quantitatively different cADPR-generating activities in the two cell types prompted us to investigate whether the mucosa itself could be responsible for a functionally significant and myocyte-targeted cADPR production. Because availability of NAD⁺ to ectocellular cyclase would be a prerequisite for the extracellular generation of cADPR to occur, we sought a release of intracellular NAD⁺ from the cyclase-positive mucosal epithelial tissue using a procedure that had demonstrated efflux of NAD⁺ from cultured cells (33). The NAD⁺ content of fresh mucosal tissue immediately after extraction was 868 ± 75 pmol/mg and decreased to 370 ± 28 pmol/mg when tissue fragments were subjected to a cycle of three rapid washings in 1 ml of PBS-glucose at 4°C (see EXPERIMENTAL PROCEDURES). The rationale for removing the rapidly exchangeable NAD⁺ pool (498 ± 47 pmol/mg) was to create a steady-state condition of outward, gradient-directed flow of the dinucleotide. This allowed us to quantitate the subsequent, sustained NAD⁺ release from intact cells and to maximize this process in the washed cells over the unwashed ones by continuous removal of extracellular NAD⁺. In fact, further efflux of NAD⁺ from epithelial mucosa took place when washed tissue pieces were incubated for 4 h at 37°C in PBS-glucose, and the release was considerably enhanced by repeated medium changes as shown in Fig. 5. Thus the NAD⁺ content in each medium supernatant ranged between 18 and 35 pmol/mg (referred to the amount of protein in the incubated tissue). The total amount of NAD⁺ released over the incubation period reached 144 ± 13 pmol/mg when the medium was replaced every hour (Fig. 5), whereas it was as low as 53 ± 4 pmol/mg in the supernatant of the tissue sample incubated for the same time (4 h) but without a change of medium (Fig. 5). Correspondingly, the NAD⁺ content in the tissue extract dropped from 370 ± 28 to 130 ± 12 pmol/mg after four medium changes and remained high (at 350 ± 29 pmol/mg) in the sample incubated without medium changes. A percentage of cell lysis < 5% was calculated from the ratio of hexokinase activity released into the supernatants to the total enzyme activity present in the tissue lysates. Accordingly, the decrease in NAD⁺ from mucosal tissue was not determined by cell lysis during incubation.

These data demonstrate that, as previously observed in cultured cells (33), a remarkable release of intracellular NAD⁺ also takes place from intact tracheal mucosa, thus indicating the occurrence of a transmembrane NAD⁺-transporting system in tracheal epithelium, the function of which was maximized in our experimental conditions. Efflux of NAD⁺ is likely to be, in fact, underestimated because part of the released dinucleotide may be converted to cADPR and ADPR at the surface of CD38-positive epithelial cells.

**Tracheal mucosa affects [Ca²⁺]i in isolated smooth myocytes.** To establish whether production of ectocellular cADPR by cyclase activity expressed on tracheal mucosa could affect the [Ca²⁺]i of intact TSMs, we performed experiments in which freshly isolated TSMs were incubated with intact mucosal strips. Addition of tracheal mucosa to isolated smooth myocytes for 2–4 h caused an increase in basal intracellular calcium in TSMs up to 147 ± 12 nM compared with a [Ca²⁺]i of 111 ± 4 nM observed in control myocytes incubated alone (P < 0.05). This mucosa-dependent increase in TSM [Ca²⁺]i was completely abrogated by addition either of NAD⁺-ase (0.5 U/ml) or of 8-NH₂-cADPR (10 μM) to the incubation mixture. The two effects indicate the involvement of both extracellular NAD⁺ and cADPR in the myocyte calcium response to epithelial mucosa. Exposure of isolated TSMs for 2 h to 1 mM NAD⁺ in the absence of epithelial mucosa resulted in a lower, although still significant, [Ca²⁺]i increase (124 ± 6 nM; P < 0.05). These results, in agreement with the higher cyclase activity expressed on the mu-
cossa compared with TSMs (Table 1), indicate the functional prevalence of mucosa-produced cADPR in mediating the calcium response of myocytes.

**DISCUSSION**

Porcine tracheal smooth myocytes after permeabilization respond to extracellularly added cADPR with an increase in [Ca$^{2+}$]$_i$ (24). In the present study, we tried to complete the picture by addressing two issues: 1) whether and where cADPR is generated in the respiratory smooth muscle tissue and 2) whether extracellular cADPR has any effect on [Ca$^{2+}$]$_i$ and contractility of intact myocytes. The second point underlines a substantial difference from the experimental design of Prakash et al. (24) because it addresses the possibility of cADPR influx into intact myocytes as previously shown for other cell types (6, 11, 23).

Both major components of the tracheal tissue, mucosa and muscle, were found to express ecto-cyclase activity. The enzyme was identified as CD38 by its catalytic properties (NAD$^+$-ase, GDP-riboyl cyclase and cADPR hydrolase activities; Table 1), molecular mass by SDS-PAGE, immunoreactivity with an anti-human CD38 MAb (Fig. 1), and localization to the plasma membrane (10, 16, 25, 26, 32, 33). Moreover, most of the cyclase activity expressed in the trachea was found to reside on the mucosa rather than on myocytes (Table 1).

Therefore, tracheal cells are catalytically competent to metabolize cADPR at their outer surface, thus suggesting possible functional roles for cADPR itself in the intact trachea. In agreement with this hypothesis, intact TSMs were found to be responsive to extracellular cADPR, which increased both basal [Ca$^{2+}$]$_i$ and the ACh-induced calcium release (Figs. 2 and 3). A calcium-induced calcium release (CICR) mechanism, triggered by the higher basal [Ca$^{2+}$]$_i$ in cADPR-treated compared with control myocytes (Fig. 2), probably accounts for the latter effect (8, 9, 15), at least partially (see below). Indeed, canine TSMs have been recently reported to feature classical CICR mechanisms (13). The different kinetic patterns of [Ca$^{2+}$]$_i$ increase elicited by external cADPR on permeabilized (24) and intact TSMs (rapid and sustained, respectively) can be related to different ways of exposure of ryanodine-sensitive receptors to the cyclic nucleotide. When cells are permeabilized, receptors become instantly available to cADPR, whereas in the intact TSMs, occupancy of the same receptors is apparently delayed by the rate-limiting process of cADPR influx across the plasma membrane.

Intact TSMs were also permeable and responsive to 3-deaza-cADPR (a strong agonist and nonhydrolyzable analog) and 8-NH$_2$-cADPR (a cADPR antagonist). The inhibition afforded by the last compound, which induced a substantial decrease in both basal and ACh-stimulated [Ca$^{2+}$]$_i$, in freshly dissociated TSMs, demonstrates the presence of functionally active endogenous cADPR in TSMs. The concentrations of cADPR, its analog, and its antagonist used in this study with smooth myocytes and strips have been recently demonstrated to also be functionally significant in another model system represented by human hematopoietic progenitors (23).

The different [Ca$^{2+}$]$_i$ values induced by the three nucleotides on isolated myocytes proved to correlate with their effect on the ACh-induced contraction of TSM strips. Thus incubation of TSM strips with cADPR or 3-deaza-cADPR caused a leftward shift of the concentration-response curve to ACh, indicating an enhanced contractile response. Conversely, incubation with 8-NH$_2$-cADPR caused a rightward shift of the concentration-response curve to ACh, indicating a reduced contractile response. The changes in basal [Ca$^{2+}$]$_i$ after incubation of isolated intact TSMs with cADPR, 3-deaza-cADPR, and 8-NH$_2$-cADPR were not paralleled by significant changes in the resting force in TSM strips. Furthermore, we failed to record a significant increase in ACh-induced contraction of TSM strips by extracellular NAD$^+$ (data not shown), which conversely elicited a limited increase in [Ca$^{2+}$]$_i$ in intact smooth myocytes due to the cADPR-transporting ability of myocyte CD38, with consequent cADPR influx (7). Our functional studies were limited to isometric force production. Thus any effect of [Ca$^{2+}$]$_i$, variations on other contractile properties of smooth muscle, i.e., extent and velocity of shortening, could not be evaluated. Despite this limitation, the effects of cADPR and 3-deaza-cADPR on TSM contraction described in this study were significant, particularly in increasing myocyte responsiveness to low ACh concentrations (5 × 10$^{-8}$ to 10$^{-7}$ M), which are within the physiological range. Inspection of Fig. 4 suggests that the ACh concentration required to induce 50% of maximal contraction may be reduced by approximately one-half after incubation of TSM strips with cADPR and by two-thirds with 3-deaza-cADPR. Although the physiological significance of these changes cannot be established, these data suggest a role for cADPR in the regulation of airway smooth muscle tone in intact tissue.

On the contrary, incubation of TSM strips with extracellular 8-NH$_2$-cADPR resulted in a significantly decreased contraction compared with control samples. This finding correlates with the decrease in both basal and ACh-elevated [Ca$^{2+}$]$_i$ in isolated myocytes elicited by the same cADPR antagonist. Thus endogenous cADPR plays a dual parallel role in regulating basal calcium homeostasis and in upgrading the ACh-induced calcium mobilization, probably via a CICR mechanism.

The long time course required for externally added cADPR to enhance the ACh-induced contraction of TSM strips compared with the shorter time needed for increasing [Ca$^{2+}$]$_i$ in isolated myocytes seems to mostly reflect a difficult and limited physical accessibility of the cyclic nucleotide to its target smooth myocytes. The opposite effects of 8-NH$_2$-cADPR on contraction of TSM strips under the same experimental conditions and especially at equally long time intervals leave little doubt as to the antagonism between cADPR and...
localized downstream. rightful, still cADPR-dependent yet long-lasting, events result from a combination of short-term effects via a cADPR-upregulated CICR mechanism and of subsequent, still cADPR-dependent yet long-lasting, events localized downstream.

A major outcome of this study is that the \( [Ca^{2+}]_i \) increase in myocytes could be induced by addition either of extracellular cADPR or of tracheal mucosa to the intact myocytes. This effect of epithelial cells is mediated by release of \( Na^+ \) from intact cells, possibly through the same dinucleotide transporter that has recently been identified in other cell types (23) and by conversion of extracellular \( Na^+ \) to cADPR, catalyzed by ectacellular cyclase activity expressed on epithelial cells and, to a lesser extent, on myocytes themselves. In this paracrine process between two different cell types, the mucosa supplies both \( Na^+ \) and cADPR to the adjacent myocytes that, like other previously recognized cell types (6, 11, 23), prove to be permeable to this cyclic nucleotide; thus the released \( Na^+ \) plays a hormonelike role, whereas extracellularly generated cADPR can be viewed as a second messenger eliciting intracellular calcium mobilization in the target myocytes. In this process, myocyte CD38 could also be directly involved in the production and active transport of cADPR across the plasma membrane to reach the responsive calcium stores (7). However, its role in affecting myocyte \( [Ca^{2+}]_i \), is probably less relevant compared with that of mucosa-generated cADPR as demonstrated by \( [Ca^{2+}]_i \) values that were higher in myocytes incubated with \( Na^+ \)-exporting mucosa (147 ± 12 nM) than with \( Na^+ \) alone (124 ± 6 nM). The increases in \( [Ca^{2+}]_i \) levels in TSMs after incubation with epithelial mucosa are comparable in extent to those recorded after incubation of the intact myocytes directly with 10 \( \mu \)M cADPR (Fig. 2), i.e., 147 ± 12 and 143 ± 9 nM, respectively. This is to some extent unexpected because the measured rate of \( Na^+ \) released by the mucosa (Fig. 5), together with actual levels of ectacellular cyclase activity of mucosa and TSMs (Table 1), would suggest extracellular cADPR concentrations of approximately two to three orders of magnitude lower. On the other hand, conditions other than extracellular concentrations of cADPR may optimize its effects on TSMs, e.g., close contact between mucosa and myocytes favoring influx in the latter cells or cofactors reducing the threshold of cADPR activity in TSMs.

At any rate, disruption of the mucosa–myocyte paracrine network by the addition of extracellular \( Na^+ \)-ase prevents the cADPR-mediated \( [Ca^{2+}]_i \), increase in myocytes and underscores the central role of \( Na^+ \). Whatever the specific contribution of mucosa versus myocyte CD38 in generating cADPR, the eventual result of this intercellular exchange of functionally active nucleotides is a calcium-related potentiation of ACh-induced TSM contraction. This adds to the intracellular trafficking of \( Na^+ \) and cADPR, which has been demonstrated to regulate the calcium homeostasis in cells expressing both the dinucleotide transporter and CD38 (5, 33). Occurrence of the \( Na^+ \) transporter and of cyclase activity as well in other cell types with trophic functions in the hemopoietic environment (23) and in astrocytes (Verderio C, Bruzzone S, Zocchi E, Matteoli M, and De Flora A, unpublished data) suggests that the paracrine process identified in bovine trachea may prove to be a common mechanism of cADPR signaling in several mammalian tissues.

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REFERENCES

13. Janssen LJ, Betti PA, Netherton SJ, and Walters DK. Superficial buffer barrier and preferentially directed release of


