Cyclic ADP-ribose (cADP-ribose) is synthesized from β-NAD⁺, an abundant intracellular substrate, by ADP-ribosyl cyclase in sea urchin eggs and in mammalian cells (1, 2). Pharmacological studies suggest that cADP-ribose is an endogenous modulator of ryanodine-sensitive Ca²⁺ release channels. An unsolved question is whether or not cADP-ribose mediates intracellular signals from hormone or neurotransmitter receptors. The first step in this study was to develop a TLC method to measure ADP-ribosyl cyclase, by which conversion of [3H]NAD⁺ to [3H]cADP-ribose was confirmed in COS-7 cells overexpressing human CD38. A membrane fraction of NG108-15 neuroblastoma × glioma hybrid cells possessed ADP-ribosyl cyclase activity measured by TLC. Carbamylcholine increased this activity by 2.6-fold in NG108-15 cells overexpressing m1 or m3 muscarinic acetylcholine receptors (mACHRs), but inhibited it by 30–52% in cells expressing m2 and/or m4 mACHRs. Both of these effects were mimicked by GTP. Pretreatment of cells with cholera toxin blocked the activation, whereas pertussis toxin blocked the inhibition. Application of carbamylcholine caused significant decreases in NAD⁺ concentrations in untreated m1-transformed NG108-15 cells, but an increase in cholera toxin-treated cells. These results suggest that mACHRs couple to ADP-ribosyl cyclase within cell membranes via trimeric G proteins and can thereby control cellular function by regulating cADP-ribose formation.

ADP-ribosyl cyclase seems to be present in both cytosolic and membrane-bound forms (1, 2, 12). The mammalian membrane-bound form of ADP-ribosyl cyclase has been identified as a cell-surface antigen, CD38 (13–19) and BST-1 (20).

Recently, it has been shown that the formation of cADP-ribose is regulated by nitric oxide or cGMP (21–23) and that nitric oxide or cGMP is increased by stimulation with agonists (24, 25). These findings suggest the hypothesis that the regulation of the cADP-ribose level is located far downstream in the signal transduction cascade from receptors (11). An alternative hypothesis is that the ADP-ribosyl cyclase formation is regulated by ADP-ribosyl cyclase through the direct action of G proteins activated by receptors within the surface membrane, as already shown for the formation of cyclic AMP, inositol 1,4,5-trisphosphate, and diacylglycerol (26–28). To test this hypothesis, we used NG108-15 neuroblastoma × glioma hybrid cells (29), in which signal transduction from receptors to effectors has been extensively characterized (29, 30). In particular, in NGPM1-27 cells (31), which overexpress muscarinic acetylcholine receptors (mACHRs), it has been shown that intracellular NAD⁺ or NAD⁺ metabolites are involved in signal transduction from m1 mACHRs to K⁺ channels (32, 33). In this context, such neuronal cell lines have advantages for analyzing receptor-ADP-ribosyl cyclase coupling in detail.

For measurement of ADP-ribosyl cyclase, high pressure liquid chromatography (HPLC) is commonly used to separate cADP-ribose-related compounds (1, 2, 8, 14, 15, 17, 19, 34, 35). However, since it takes 30–60 min to process one sample, it is essential to develop a much more rapid method that can allow processing of multiple samples at once. There are two papers that describe ADP-ribosyl cyclase assay by TLC (21, 36), in which NAD⁺ migrates faster than cADP-ribose. The methods used in those reports seem to be affected by large amounts of radiolabeled substrates. We here developed a TLC method that overcomes this problem and allows separation of cADP-ribose in up to 19 samples within 40–50 min. Our TLC method was first tested on COS-7 cells overexpressing human CD38 and was shown to be applicable for measuring ADP-ribosyl cyclase activity. We demonstrate that crude cell membranes of NG108-15 cells possess ADP-ribosyl cyclase activity and that such activity is activated or inhibited in a mACHR subtype-specific manner in NG108-15 cells overexpressing distinct mACHR subtypes. Furthermore, to ascertain the intracellular role of the catalytic activity of ADP-ribosyl cyclase in neuronal cell membranes, the time course of [NAD⁺]i, after extracellular application of acetylcholine to these cells was investigated.

**EXPERIMENTAL PROCEDURES**

Materials—β-[2,8-adenosine-3H]NAD⁺ (30.5 Ci/mmol) and β-[4-nicotinamide-3H]NAD⁺ (1.03 Ci/mmol) were purchased from NEN Life Sci-
ence Products and Amersham International (Buckinghamshire, United Kingdom), respectively. $[^3]H$cADP-ribose and $[^3]H$ADP-ribose were synthesized from adenine-labeled $[^3]H$NAD$^+$ by the method described previously (5, 12, 37). cADP-ribose was obtained from either Yamasa Shoyu (Choshi, Japan) or Sigma. pBluescript II KS$^+$(+) and pZeoSV were purchased from Stratagene (La Jolla, CA) and Invitrogen (San Diego, CA), respectively. Maltose-binding protein-human CD38 fusion protein was synthesized by the method described previously (34). Silica Gel 60 F$_{254}$ plastic TLC sheets were obtained from Merck (Darmstadt, Germany).

Thin-layer Chromatography—Two-μl aliquots were spotted on silica gel TLC sheets (50 × 10 cm), and the layers were developed in the ascending direction for 40–120 min at room temperature with four different solvents, as listed in Table 1. A mixture of water/ethanol/ammonium bicarbonate (in the ratio 30:70:0.2%) was used most frequently.

Expression of Human CD38 in COS-7 Cells—COS-7 cells were grown as described previously (38). The expression plasmid pZHCDS38 was constructed as follows. The 0.55-kb HindIII (vector/pSpI606) fragment and the 0.36-kb PstI (606/BgII1969) fragment from pSV2-HCD38 (34) were ligated with the 3.0-kb BamHI/HindIII fragment from pBluescript II KS$^+$ (+) to yield pHCD38 (restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5’- and 3’-terminal nucleotide generated by cleavage). The 0.91-kb HindIII (vector/pSpI606) fragment containing the entire protein-coding sequence from pSHCD38 was ligated with the 3.4-kb SpeI/HindIII fragment from expression vector pZeoSV to yield pZHCDS8. COS-7 cells were transfected with pZHCDS8 by the DEAE-dextran method (38). Cells were used for cADP-ribose assay at ~72 h after transfection.

Cell Membrane Preparation from COS-7 Cells—Mock- and CD38-transfected COS-7 (COS-CD38) cells were washed once with Dulbecco’s phosphate-buffered saline without Ca$^{2+}$ and Mg$^{2+}$ (PBS(−)), dissociated in 10 ml of PBS(−), and collected by centrifugation at 300 × g for 5 min. The washed cells were frozen and stored at −80 °C. Immediately before use, the cell pellet was thawed and suspended in 10 ml Tris-HCl solution, pH 7.4, with 5 mM MgCl$_2$ (1 ml for cells from a 75-cm$^2$ flask) at 4 °C for 25 min (39). The suspension was homogenized in a Dounce glass homogenizer with 50 strokes. The resultant homogenate was centrifuged at 4°C for 5 min at 1000 × g to remove unbroken cells and nuclei. Crude membrane fractions were prepared by centrifugation (twice) of homogenates at 105,000 × g for 1 h. The final pellet (100,000 × g) was resuspended, and the final pellet (100,000 × g) was dispersed in 10 ml Tris-HCl solution, pH 6.6. Protein was measured by the Bio-Rad protein assay dye reagent.

ADP-ribosyl Cyclase Assay—Each 20-μl reaction mixture contained 50 molarity Tris-HCl, pH 6.6, 100 mM KCl, 5 mM MgCl$_2$, 0.1 mM EDTA, 2 μM β-NAD$^+$, 0.11 μM β-[2,8-adename]-H Nad$^+$ (0.06 μCi), and 1.2–6 μg of membrane proteins according to a formula reported previously (34), with a slight modification. In experiments in which the activation effect by agonists was measured, MgCl$_2$ and EDTA was replaced with 10 μM CaCl$_2$. Reaction mixtures were incubated for 0.5–16 min at 37 °C. The production of [3H]cADP-ribose and [3H]ADP-ribose was proportional to protein concentration within the range of 1–20 μg of membrane fraction protein/reaction mixture. Reactions were stopped by adding 2 ml of 2 μg of 48% trichloroacetic acid to the reaction mixture. Aliquots were centrifuged for 2.5 min at 14,000 × g, and 2 μl of the supernatant was used for analysis by TLC. The positions of authentic cADP-ribose, ADP-ribose, and NAD$^+$ after UV detection were confirmed in each run. Corresponding positions (~1 × 0.7 cm) were cut, and the radioactivity was counted in a liquid scintillation counter.

Autoradiography of Thin-layer Chromatograms with $[^3]H$NAD$^+$—The same reaction mixture used for the ADP-ribosyl cyclase assay containing 0.06 μCi of [2,8-adename]-H Nad$^+$ was incubated with membranes of COS-CD38 and NGP1-27 cells. Two μl of reaction mixture were spotted on TLC sheets and developed. Autoradiography was carried out after exposure on a Fuji BAS 1000 $^{3}$H imaging plate for 24–36 h.

Cell Membrane Preparation of NG108-15 Cells—Parental NG108-15 cells, which possess endogenous m4 mAChRs (30), and mACHr-transformed NG108-15 cells, such as NGP1-27 (expressing endogenous m4 mAChRs and overexpressing m1 mAChRs), NGP2-105 (m4 and m2 mAChRs), NGRM-309 (m4 and m3 mAChRs), and NGRM-215 (m4, m5, and endogenous m4 mAChRs), were described previously (30–33). Hybrid cells were grown to confluence (32) and collected. Membrane fractions were prepared as described for COS-7 cells.

Mass Spectographic Measurements—Silica gel spots of the enzyme reaction samples corresponding to the migration position of authentic cADP-ribose were removed by shaving. NAD$^+$ metabolites were recovered in water or acidic solutions and concentrated by freeze-drying. The

Table I
Flow rate of NAD$^+$ metabolites on silica gel TLC sheets developed with various solvents

| Composition (ratio) | $R_f$ | Migration distance of cADP-ribose (cm) |
|---------------------|-------|--------------------------------------|
| H$_2$O/H$_2$O/CH$_3$OH/NH$_4$HCO$_3$ (30%:70%:0.2%) | 1.17 | 0.72 |
| H$_2$O/H$_2$O/NaCl (30%:70%:0.2%) | 1.58 | 1.11 |
| H$_2$O/(CH$_3$)$_2$OH/CH$_3$COOH (1:2:1) | 1.20 | 0.92 |
| CH$_3$OH/CH$_2$COONH$_4$ (5:2) | 1.31 | 1.13 |

$^a$ $R_f$ value; $^{ADPR}$ ADP-ribose; $^{cADPR}$ cyclic ADP-ribose.

![Figure 1](image-url)  
**Fig. 1.** Separation of cADP-ribose, ADP-ribose, NAD$^+$, and rection sample by thin-layer chromatography. Two μl each of 10 mM NAD$^+$, 10 mM ADP-ribose (ADPR), 2 mM cADP-ribose (cADPR), and reaction mixture with NGPM1-27 cell membrane (sample) were spotted from the left at the position designated origin. The TLC sheet was developed for 80 min at 21 °C. After drying, the plate was visualized by UV illumination and photographed. Note that there are no corresponding endogenous nucleotides detected as visible spots in the sample lane.

freeze-dried material was dissolved in 20–200 μl of H$_2$O and subjected to fast atom bombardment mass spectral analysis (JMS-DX300, JOEL Inc., Tokyo).

NAD$^+$ Content—NGP1-27 cells were cultured on polycarbonate-coated dishes (35 mm in diameter) for 4 days. The NAD$^+$ content in the supernatant of the heat-inactivated cell homogenate was determined by a slight modification (32) of an enzyme cycling method (40).

RESULTS

Behavior of Authentic cADP-ribose, ADP-ribose, and NAD$^+$ in Thin-layer Chromatograms—Of four different solvents tested (Table I), the best separation was obtained with the mixture of water/ethanol/ammonium bicarbonate (30%:70%:0.2%). In this solvent, cADP-ribose moved slower than ADP-ribose, but faster than NAD$^+$ (Fig. 1). The average flow rates for ADP-ribose, cADP-ribose, and NAD$^+$ were 1.18 ± 0.017 (mean ± S.E.; n = 13), 1.0 (n = 13), and 0.71 ± 0.021 (n = 13), respectively (Table I). Related compounds, ADP and nicotinamide, moved with flow rates of 0.47 (n = 2) and 5.43 (n = 2).

Separation was examined quantitatively by measuring the distribution of each radiolabeled compound in different fractions by TLC (Fig. 2, A–C). The recoveries of [3H]ADP-ribose,
The radioactivity was normalized to the total count applied before TLC development. The values are the means of triplicate determinations. Error bars indicate S.E. D shows the recovery of radioisotope in the cADP-ribose region in the chromatogram. Two μl of [3H]cADP-ribose dissolved in 0.52, 1.05, 2.11, 4.22, 8.44, and 12.66 nmol of unlabeled cADP-ribose were spotted, and the TLC plate was developed for 50 min. Radioactivity in spots corresponding to the migration positions of cADP-ribose was counted. Symbols are the means of duplicate experiments of triplicate determinations. Standard errors are within symbols.

[3H]cADP-ribose, and adenine-labeled [3H]NAD⁺ at their relevant migration positions were 75.5 ± 1.3% (n = 3), 60.0 ± 3.4% (n = 3), and 81.8 ± 2.9% (n = 6), respectively. Contamination of [3H]cADP-ribose in the cADP-ribose fraction caused by tailing was 12.2 ± 0.35% (n = 3). [3H]cADP-ribose recovered from the cADP-ribose position in the chromatogram increased linearly with the concentration of cADP-ribose spotted (up to 12.7 nmol) (Fig. 2D). Based on these quantitative results on separation, our TLC method was applied to measuring ADP-ribosyl cyclase activity in COS-7 cells.

ADP-ribosyl Cyclase in CD38-transfected COS-7 Cells—Crude membrane pellets (100,000 g) of COS-CD38 cells were incubated with 2 μM NAD⁺ and 0.11 μM adenine-labeled [3H]NAD⁺ at 37 °C for 0.5–8 min. The samples were then subjected to TLC. As shown in an autoradiogram (Fig. 3), H counts in the cADP-ribose fraction were immediately increased during the first 2 min and thereafter decreased in COS-CD38 cells, consistent with the previous measurement by HPLC (15, 34, 36). The average activity was 20.6 ± 1.31 nmol/min/mg of protein (n = 3), calculated from the initial rate (Fig. 4A). No such activity was found in nontransfected (data not shown) or mock-transfected (Fig. 4A) COS-7 cells.

Although ³H count accumulation in the ADP-ribose fraction was below the detectable level during the first 1–2 min with COS-CD38 cell membranes, [³H]ADP-ribose formation was gradually increased and exceeded the cADP-ribose level after 8 min (Fig. 3). These results seem to faithfully reflect the two-enzyme reaction of human CD38, from NAD⁺ to ADP-ribose via cADP-ribose, and thereby indicate that our TLC method is sensitive enough to measure ADP-ribosyl cyclase activity.

ADP-ribosyl Cyclase Activity in NGPM1-27 Cells—Using the TLC method, ADP-ribosyl cyclase activity was examined with crude membranes (100,000 g) of NGPM1-27 cells. The ³H count in the cADP-ribose fraction increased linearly during the
first 0.5–6 min and thereafter was maintained at a steady-state level (Fig. 4B) or decreased (Fig. 3). The average activity measured by \(^{3}H\) accumulation in the cADP-ribose fraction was 229.8 ± 29.5 pmol/min/mg of protein (n = 12). In contrast, the \(^{3}H\) count in the ADP-ribose fraction increased linearly over 16 min (Figs. 3 and 4B). The average activity was 296.3 ± 63.5 pmol/min/mg of protein (n = 7). The total rate of NAD\(^+\) utilization was measured under the same conditions, except that 2 \(\mu\)M NAD\(^+\) was mixed with nicotinamide-labeled \(^{3}H\)NAD\(^+\) (0.01 \(\mu\)Ci/sample). The release of \(^{3}H\)nicotinamide catalyzed by NGPM1-27 cell membranes was 460.5 ± 59.3 pmol/min/mg of protein (n = 10). It thus appears that a large part of NAD\(^+\) is converted to either cADP-ribose or ADP-ribose with some other metabolites.

To verify that the above \(^{3}H\) accumulation in cADP-ribose fractions is due mainly to accumulation of \(^{3}H\)cADP-ribose produced by the activity of the ADP-ribose cyclase of NGPM1-27 cells, the compounds collected in the cADP-ribose fraction by TLC were analyzed. The first test carried out was to show whether or not the product is converted to ADP-ribose by heat inactivation (1) or CD38. Extracts from the silica gel were inactivated by treating them for 30 min at 95 °C or were metabolized by cADP-ribose hydrolase in maltose-binding protein-CD38 for 16 min before lyophilization and then rechromatographed. The radioactivity in the ADP-ribose fraction recovered increased with both treatments, and the amount of \(^{3}H\) count in the cADP-ribose fraction was reduced correspondingly. Secondarily, lyophilized samples extracted from the ADP-ribose fraction in the rechromatographed thin-layer chromatogram were analyzed by mass spectrometry. A main peak of mass ions at \(m/z\) 558, a peak unique for ADP-ribose (1), was found, reflecting either the original ADP-ribose or ADP-ribose hydrolyzed from cADP-ribose during processing, or both. These two lines of evidence suggest that the majority, if not all, of the products separated in the cADP-ribose fraction are ADP-ribose, which is very hydrolyzable (1). Armed with this evidence, the receptor control mechanism of membrane-bound ADP-riboyl cyclase was investigated in NG108-15 cells expressing various subtypes of mACHRs.

**Carbamylcholine-induced Changes in ADP-riboyl Cyclase Activity**—After application of 10 \(\mu\)M carbamylcholine (CCh), an increased cyclase activity in NGPM1-27 cell membranes over the control level was observed for 2–4 min (Fig. 5A) and was abolished in the presence of 0.1–1 \(\mu\)M atropine. The average increase was 2.61 ± 0.27-fold (n = 6) (Fig. 6A), which was significantly larger (p < 0.001). The activation by CCh was dose-dependent, with an ED\(_{50}\) value of 26 ± 1.4 nM (n = 3). Addition of 10 \(\mu\)M GTP alone or together with CCh also caused increased activity (Fig. 5B). Membranes washed extensively with 10 mM Tris-buffered saline, pH 6.6, with or without 0.1 mM EDTA (n = 4) retained the cyclase activity, which could be stimulated by 10 \(\mu\)M GTP or GTP\(\gamma\)S (2.97- and 2.27-fold, respectively; n = 2), although no activation by CCh was observed (102.0 ± 5.8% of the control level; n = 4). Cyclic GMP (10 \(\mu\)M), however, had no effect on ADP-riboyl cyclase activity (106.7 ± 9.4%; n = 7) in this membrane system.

In m3 mACHR-transformed NG108-15 cells, a similar amount of activation by 10 \(\mu\)M CCh (2.93 ± 0.29-fold; n = 6) was observed (p < 0.001) (Fig. 6A). In contrast, the effect of CCh was inhibitory in crude membranes of nontransfected (Fig. 5C) and mock-transformed NG108-15 cells that possess endogenous mACHRs of the m4 subtype (31). The cyclase activity was inhibited to 49.7 ± 5.5% (n = 14) and 69.5 ± 5.8% (n = 6) of the control level (p < 0.01) (Fig. 6A), respectively, and this effect was mimicked by 10 \(\mu\)M GTP (Fig. 5D). The IC\(_{50}\) for CCh was ~2.5 nM (n = 3) in NG108-15 cells. Inhibition of 40–82% was produced by 10 \(\mu\)M GTP or GTP\(\gamma\)S, even in well washed membranes (n = 3). Cyclase activity in membranes prepared from NG108-15 cells overexpressing m2 or m4

**Fig. 5.** Activation of ADP-riboyl cyclase by CCh and GTP in NG108-15 and NGPM1-27 cells. Reaction mixtures containing 3.2 \(\mu\)g of membrane preparation of NGPM1-27 cells (A and B) per sample point or 1.5 \(\mu\)g of NG108-15 cells (C and D) were incubated with 10 \(\mu\)M CCh ([]), 10 \(\mu\)M GTP (○), both (△), or neither (●) for the indicated time periods. The values are the means of triplicate measurements of duplicate determinations. cADPR, cyclic ADP-ribose; Con, control.

**Fig. 6.** CCh-induced activation or inhibition of ADP-riboyl cyclase activity in various NG108-15-derived cell lines. ADP-riboyl cyclase activity was estimated from the initial velocity in time course experiments with membranes prepared from parental NG108-15 cells, mock-transfected cells (NGV102), or NG108-15 cells transformed to express m1 (NGPM1-27), m2 (NGPM2-105), m3 (NGRM3-309), and m4 (NGRM4-215) mACHRs. These cells were treated without (A) or with 100 ng/mL CTx (B) or PTx (C). The level of activity was compared in the presence and absence of 10 \(\mu\)M CCh in the reaction mixture. The values are the means of six determinations. * and **, significantly different from control activity (100%, without CCh) at p < 0.01 and p < 0.001, respectively.
mACHRs was also inhibited by CCh (52.5 ± 10.4 and 55.2 ± 9.8% of the control level, respectively; n = 6; p < 0.01) (Fig. 6A). Altogether, these results clearly show that mACHRs of the endogenous m4 subtype or overexpressed m2 and m4 subtypes couple with ADP-ribosyl cyclase in an inhibitory manner, whereas overexpressed m1 and m3 mACHRs acquire a new pathway for activating the cyclase in NG108-15 cells.

We next examined which G proteins mediate these mACHR subtype-specific and GTP-dependent effects. When m1- and m3-transformed cells were pretreated with 100 ng/ml cholera toxin (CTx) for 5–8 h, CCh-induced activation of the cyclase activity was specifically eliminated, and instead, CCh induced a significant inhibition. The CCh-induced inhibition remained in a set of nontransfected, mock-transfected, and m2- or m4-transformed cells that had been treated with CTx (Fig. 6B). Thus, CCh induced an inhibition to 49.7–70.8% of the control level in all types of CTx-treated cells (p < 0.001). In contrast, treating the above set of cells (except for m1- and m3-transformed cells) with 100 ng/ml pertussis toxin (PTx) for 12 h prevented the CCh-induced inhibition (Fig. 6C). The significant activation by CCh was retained in PTx-treated m1- and m3-transformed cells (171 ± 19 and 150 ± 16% of the control level, respectively; n = 6; p < 0.01), although the apparent activation was reduced. The toxin sensitivities suggest that the activation or inhibition signal from each mACHR subtype to ADP-ribosyl cyclase is conveyed by distinct G proteins.

**Agonist-induced Decrease in \([\text{NAD}^+]\)**—To confirm the above agonist effects in vivo, we examined agonist-stimulated changes in substrate levels, as shown previously (32). Fig. 7 shows the time course of \([\text{NAD}^+]\) in NGPM1-27 cells challenged with 10 \(\mu\text{M} \) CCh. \([\text{NAD}^+]\), was significantly decreased for 15–60 s after CCh application. The decrease in \([\text{NAD}^+]\), at 30 s was 17.9 ± 2.3% (n = 9; p < 0.001). \([\text{NAD}^+]\), then recovered to the control level by 120 s. As expected, in CTx-treated NGPM1-27 cells, CCh caused a slight but significant increase in \([\text{NAD}^+]\), (108.8 ± 2.0% of the control value at 30 s; n = 6; p < 0.001).

**DISCUSSION**

The chromatographic procedure used here resulted in a resolution sufficient to measure \(^3\text{H}\) accumulation in the ADP-ribosyl fraction originating from adenine-labeled \(^3\text{H}\)NAD\(^+\), and it was free from interference by the labeled substrate. One possible limitation could be that the ADP-ribosyl fraction was contaminated by ~15% of the newly synthesized \(^3\text{H}\)ADP-ribosyl (Fig. 2A). However, the risk that we measured the ADP-ribosyl count in the ADP-ribosyl fraction seems to be negligible since different patterns of \(^3\text{H}\) accumulation in ADP-ribosyl and ADP-ribosyl fractions were obtained (Fig. 4), reflecting that distinct materials are accumulated with different rates of formation and degradation. The autoradiograms for COS-CD38 and NGPM1-27 cells (Fig. 3) clearly demonstrated the overall fate of \([\text{NAD}^+]\) in that the majority of \([\text{NAD}^+]\) was converted to either ADP-ribosyl or ADP-ribosyl or both in two or more enzyme reaction steps, probably ADP-ribosyl cyclase and ADP-ribosyl hydrolase.

The \([\text{NAD}^+]\) concentration used in our reaction mixture (2 \(\mu\text{M}\)) was much lower than values (~100 \(\mu\text{M}\)) used in other studies (1, 2, 5, 15). However, our concentration is very similar to the estimated \(K_a\) value (3–5 \(\mu\text{M}\)) for ADP-ribosyl cyclase in NG108-15 cell membranes.\(^2\) Under such conditions, \(^3\text{H}\)ADP-ribosyl accumulated linearly for 2–4 min and \(^3\text{H}\)ADP-ribosyl for 16 min, suggesting that it is not an insufficient dose. Thus, our TLC method was used for measurement of ADP-ribosyl cyclase, at least during the first few minutes. We do not know whether or not this TLC method is applicable for measurement of other recently found enzyme reactions in ADP-ribosyl cyclase, such as production of cyclic 2′-phosphoadenosine diphosphoribose (41), nicotinic acid adenine 5′-diphosphate ribose (42), and dimeric ADP-riboside (43). It remains to be tested how our TLC method differs in accuracy from HPLC and fluorometric measurement of cyclic GDP-riboside formation (20, 44, 45).

The results provide the first evidence that cholinergic signals are transduced from mACHRs to ADP-ribosyl cyclase within the membrane in a receptor subtype-specific fashion (Figs. 5 and 6). The finding that the inhibition of ADP-ribosyl cyclase via m2/m4 mACHRs is mediated through a PTx-sensitive G protein resembles the inhibitory signal transduction known for adenylate cyclase in NG108-15 cells (29–31). Coupling of m1/m3 mACHRs to ADP-ribosyl cyclase is relatively resistant to PTx, but highly sensitive to CTx, which seems to be different from the signal pathway to phospholipase C\(\beta\) (30, 31). Thus, the coupling from mACHRs to ADP-ribosyl cyclase via G proteins would be a new mode of signal transduction, in parallel with the known pathways to adenylate cyclase and phospholipase C in NG108-15 cells.

The simplest explanation for our \([\text{NAD}^+]\), data in NGPM1-27 cells (Fig. 7) could be that \([\text{NAD}^+]\) consumption is accelerated by the activation with the agonist of a membrane-bound form of ADP-ribosyl cyclase whose catalytic site resides in the cell interior. This speculation, however, would require a different topology from that proposed for the CD38 cell-surface antigen, whose catalytic sites of ADP-ribosyl cyclase (15, 18, 45), ADP-ribosyl hydrolase (15, 46), and \([\text{NAD}^+]\) glycohydrolase (19) are located at the extracellular side. Thus, ADP-ribosyl cyclase activity detected in the intracellular side in NG108-15 cells could not be the same as CD38, but a neuronal isoform of ADP-ribosyl cyclase. The degree of identity between ADP-ribosyl cyclase in NG108-15 neuronal cells and that in ovotestis of Aplysia (12) or CD38 should be further examined.

\(^2\) H. Higashida, S. Yokoyama, M. Higashida, and M. Noda, unpublished data.
NG108-15 cell membranes. This conclusion, however, does not exclude the regulatory mechanism in the cytoplasm, where cytosolic ADP-ribose cyclase could be activated through the nitric oxide/cGMP-dependent cascade (11). In summary, our results suggest that stimulation of conventional neurotransmitter receptors can regulate cellular function through cADP-ribose as a second messenger, independently from or in concert with other second messengers.

Acknowledgments—We thank Tatsuya Haga for discussion and Hugh Robinson for critical reading of the manuscript.

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