Developmental Changes in the Levels of Substrates for Cholera Toxin-Catalyzed and Pertussis Toxin-Catalyzed ADP-Ribosylation in Rat Cardiac Cell Membranes

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Abstract—Developmental changes in the substrates for cholera toxin (CTX)- and pertussis toxin (PTX)-catalyzed ADP-ribosylation in cardiac (ventricular) cell membranes were studied in fetal (16- to 20-day), neonatal (0- to 20-day) and adult (2- to 3-month) rats. The CTX and PTX substrates were determined by the method of CTX-catalyzed and PTX-catalyzed ADP-ribosylation of the α-subunit of GTP-binding (G) proteins, respectively. As early as fetal day 16, three substrates (45-, 47- and 52-kDa proteins) were identified for CTX-catalyzed ADP-ribosylation and one substrate (41-kDa protein) for PTX-catalyzed ADP-ribosylation. The levels of the three CTX substrates (fmol/mg tissue) increased with development between fetal day 16 and neonatal day 16, and then they decreased to their adult levels. The level of the one PTX substrate (fmol/mg tissue) changed as follows: the substrate decreased between fetal day 16 and the day of birth, increased abruptly for 4 days neonatal and increased slowly thereafter until neonatal day 16, and then decreased to the final adult level. The PTX substrate seems to reach a nearly maximum level earlier than the CTX substrates. This information is essential for understanding the developmental changes in the transmembrane signaling system between membrane receptors and their effectors which are coupled with the stimulatory and inhibitory G proteins.

A family of GTP-binding (G) proteins play an important role in transferring signals from membrane receptors to their effectors (1-4). For example, the stimulatory (G_s) and inhibitory G (G_i) proteins are involved in the transferring process for stimulation and inhibition, respectively, of adenylate cyclase by various neurotransmitters and hormones. The α-subunit of G proteins (which are hetero-trimers of α-, β- and γ-subunits) contains a site for ADP-ribosylation. Therefore, cholera toxin (CTX) and pertussis toxin (PTX) have been used to ADP-ribosylate the α-subunit of G_s and G_i proteins, respectively.

CTX was reported to induce the ADP-ribosylation of one 42-kDa protein (5) or three other proteins (45-, 47- and 52-kDa) (6) in the rat heart. CTX-induced ADP-ribosylation was also reported to occur on two proteins in the rabbit heart (42- and 46-kDa) (7) and the bovine heart (45- and 52-kDa) (8). PTX was reported to induce the ADP-ribosylation of one 41-kDa protein (6) in the rat heart, two proteins (40- and 41-kDa) (7) in the rabbit heart and two proteins [39- and 41-kDa (9) or 39- and 42-kDa (10)] in the chick heart.

There are many reports on the developmental changes in the adenylate cyclase-cyclic AMP system (11-14), which couples with β-adrenergic and muscarinic receptors through the G proteins. However, there has
been no systematic study on the ontogenesis of the Gs and Gi proteins. The purpose of the present experiments was to do a systematic study on the developmental changes in the levels of the substrates for CTX-catalyzed and PTX-catalyzed ADP-ribosylation in cardiac cell membranes from fetal, neonatal and adult rats.

Materials and Methods

Animals: Two- to three-month old rats (Wistar strain) weighing 200-300 g were used. For breeding, female rats during estrus were mated with male rats overnight. The finding of sperm in vaginal smears on the next morning was considered positive evidence for pregnancy, and the embryonic age was calculated based on this as the 0-day of gestation. The fetuses were removed from the uterus of their mother under pentobarbital anesthesia (30 mg/kg, i.p.), when required.

Preparations of cardiac cell membranes: Cardiac cell membranes from fetal (16- to 20-day), neonatal (0- to 20-day) and adult (2- to 3-month) rats were prepared at 0-4°C (ice-cold). The ventricles were minced and then homogenized at 0°C for 60 sec with a Polytron-type homogenizer in 25 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl2 and 5 mM EDTA. The homogenate was centrifuged at 1,000 x g for 10 min, and the supernatant was further centrifuged at 50,000 x g for 20 min. The resultant pellet was suspended, for 1 hr at 4°C, in 25 mM Tris-HCl buffer (pH 8.0) containing 1 % sodium cholate, 1 mM dithiothreitol (DTT) and 1 mM EDTA. After centrifugation at 15,000 x g for 3 min at 4°C, the supernatant was used as the preparation for ADP-ribosylation with either CTX or PTX.

ADP-ribosylation of the membrane with CTX or PTX: To activate CTX or PTX, either 100 μg/ml CTX or 10 μg/ml PTX was added to the reaction buffer containing 20 mM DTT (in the case of CTX) or 5 mM ATP/5 mM DTT (in the case of PTX), and was incubated at 30°C for 30 min. For radiolabelling of membrane proteins with [32P]NAD, either pre-activated CTX (100 μg/ml) or PTX (10 μg/ml) was added to the reaction buffer containing 50 μg protein, 2.5 μM [32P]NAD (8–12 Ci/mmol), 10 mM thymidine, and 0.5 mM GTP (in the case of CTX) or 0.5 mM GDP (in the case of PTX), and was incubated 30°C for 60 min. The reaction was stopped by adding 10% trichloroacetic acid to the reaction mixture and heating at 100°C for 5 min.

SDS-PAGE, autoradiography and ADP-ribosylation assay: After centrifugation of the above reaction mixture at 15,000 x g for 3 min, the resultant pellet was suspended in Laemmli’s sample buffer, and it was subjected to SDS-PAGE according to Laemmli’s method (15, 16). The gels were stained with Coomassie Blue, destained and dried. Autoradiography was then performed by exposing the dried gels to Kodak X-AR film at -80°C for 6 to 24 hr. The radioactive parts of the gels were cut out, and were treated overnight with 1 ml H2O2 at 50°C. The radioactivity (β-radiation) was measured using a liquid scintillation spectrometer. To obtain the specific radioactivity, the activities in the molecular marker gel at 41-, 45-, 47- and 52-kDa (i.e., the background activity) were subtracted from the total activities in the protein-containing gel bands at these molecular weights, respectively.

Drugs: [Adenylate-32P]NAD was purchased from New England Nuclear (Boston, MA, U.S.A.); CTX and PTX from Funakoshi Chemical Co., Ltd. (Tokyo, Japan); ATP, GDP, GTP and NAD from Boehringer Mannheim (Mannheim, F.R.G.); sodium cholate from Sigma (St. Louis, MO, U.S.A.); and EDTA, thymidine and DTT from Nakarai Chemicals (Tokyo, Japan).

Results

Developmental changes in the G proteins in cardiac (ventricular) cell membranes were studied in fetal (16- to 20-day), neonatal (0- to 20-day) and adult (2- to 3-month) rats. Figure 1 shows autoradiographs of the substrates for CTX-catalyzed and PTX-catalyzed ADP-ribosylation at different developmental stages. As can be seen from the figure, there were three protein substrates (45-, 47- and 52-kDa) for CTX-catalyzed ADP-ribosylation (CTX substrates), and there was only one protein substrate (41-kDa) for PTX-catalyzed ADP-ribosylation (PTX substrate) in the fetal, neonatal and adult cardiac cell membranes.
Fig. 1. Autoradiographs of the substrates for cholera toxin (CTX; upper panel) and pertussis toxin (PTX; lower panel)-catalyzed ADP-ribosylation in rat hearts (ventricles) of different developmental stages. The arrows indicate the radiolabeled bands of the 45-, 47- and 52-kDa proteins (upper panel) and the 41-kDa protein (lower panel). The A-subunit of CTX and the S-1-subunit of PTX were self-ADP-ribosylated (autophos). The ages (in days) of the fetal, neonatal and adult rats are given at the top of the gel lanes.

These four substrates were identified as early as fetal day 16 (the earliest fetal day examined).

To compare the developmental changes in the CTX and PTX substrates with other related parameters reported in the literature, the levels of the substrates were expressed as a unit of pmol/mg protein and a unit of fmol/mg tissue. Figure 2 depicts the developmental changes in the levels of the CTX substrates at different developmental stages. When the substrate levels were expressed as a unit of pmol/mg protein (Fig. 2A), the developmental changes in their levels were different among the substrates: the 45-kDa protein initially remained almost constant and then decreased to its adult level; the 47-kDa protein essentially decreased; and the 52-kDa protein initially increased and then decreased to its adult level. When the substrate levels were expressed as a unit of fmol/mg tissue (Fig. 2B), the developmental changes in their levels were almost the same: roughly speaking, the levels of the 45-, 47- and 52-kDa proteins increased with development between fetal day 16 and neonatal day 16, and decreased thereafter to their adult levels.

Figure 3 represents the developmental change in the level of the PTX substrate at different developmental stages. When the substrate level was expressed as a unit of pmol/mg protein (Fig. 3A), the level of the 41-kDa protein was highest on fetal day 16, and then decreased during the remaining fetal period. The level of this protein increased for 4 days after birth, and declined gradually thereafter to its adult level. When the substrate level was expressed as a unit of fmol/mg tissue (Fig. 3B), the level of the 41-kDa protein decreased during the fetal period. The level of this protein increased abruptly for 4 days after birth and increased slowly thereafter until neonatal day 16, and then decreased to its adult level.
Concerning the developmental changes in the CTX and PTX substrates, there are some general trends in their changes in the present experiments. When the levels of both substrates were expressed as a unit of pmol/mg protein, in general, the substrates decreased to their final adult levels with development except the following phases: an increasing phase of the 52-kDa protein between fetal day 16 and neonatal day 4 and an increasing phase of the 41-kDa protein between neonatal days 0 and 4. In contrast, when the substrate levels were expressed as a unit of fmol/mg tissue, in general, the CTX and PTX substrates...
increased with development between fetal day 16 and neonatal day 16, and then decreased to their adult levels except for a decreasing phase of the 41-kDa protein during the fetal period.

**Discussion**

In order to obtain some information about the developmental changes in the transmembrane signaling system between membrane receptors and their effectors (which are coupled with G proteins), a systematic study was done on the ontogenetic changes in the CTX and PTX substrates in cardiac cell membranes from fetal, neonatal and adult rat ventricles. The present results revealed that there are
three protein substrates (45-, 47- and 52-kDa) for CTX-catalyzed ADP-ribosylation and that there is one protein substrate (41-kDa) for PTX-catalyzed ADP-ribosylation. These findings are well consistent with the previous report on adult rat hearts by Murakami and Yasuda (6).

As described in the Introduction, several kinds of CTX substrates were identified in cardiac cell membranes: 45-, 47- and 52-kDa proteins in rats (6), 42- and 46-kDa proteins in rabbits (7) and 42- and 52-kDa proteins in bovines (8). Thus, there is variability among the CTX substrate for these three species. Several kinds of PTX substrates were also identified in cardiac cell membranes: 41-kDa protein in rats (6), 40- and 41-kDa proteins in rabbits (7) and 39- and 41 (or 42)-kDa proteins in chicks (9, 10). The 41-kDa protein seems to be a common PTX substrate among these three species.

The discrepancy of the findings on the developmental changes in the CTX and PTX substrates between the different unit expressions (see the last paragraph of the Results) may largely come from the fact that the ratio of mg protein/mg tissue increases with development: 0.044 on fetal day 16, 0.070 on the day of birth, 0.129 on neonatal day 20 and 0.157 at adult ages of 2–3 months (the present data). The developmental decreases of the CTX and PTX substrate levels observed after neonatal day 16 are likely due to a decreasing proportion of sarcolemmal membrane protein to cell protein with age because the ratio of surface area/cell volume decreases (17). Concerning the declining phase of the 41-kDa protein during the fetal period, there is a possibility that the radioactive part of the gels, corresponding to the 41-kDa protein, is contaminated by near 41-kDa protein(s) which decrease(s) during the fetal period and disappear(s) on the day of birth.

At present, we think that the developmental changes in the CTX and PTX substrates are more consistent with the changes in physiological or pharmacological parameters when the substrate levels are expressed in units of fmol/mg tissue, rather than units of pmol/mg protein (see below). The PTX substrate seems to reach a nearly maximum level earlier than the CTX substrates, especially after birth. It should be noted that a factor (ARF) required for the CTX-dependent ADP-ribosylation has been identified in the liver and brain (18, 19). Therefore, the present developmental changes in the CTX substrates may be explained in terms of the developmental changes in the ARF activity or combination of the CTX substrate levels and the ARF activity.

Since β-adrenergic and muscarinic receptors are coupled with the catalytic unit of adenylate cyclase through the $G_s$ and $G_i$ proteins, respectively, (2, 4, 20), it is important to know the developmental changes in the β-adrenergic/muscarinic receptor-adenylate cyclase-effector system. The following are some representative data on rat hearts (for other data on the developmental changes, see ref. 14). The number of β-adrenergic receptors (3H-dihydroalprenolol binding site, fmol/mg tissue) increased with development between fetal day 20 and neonatal day 6 and remained almost constant thereafter (M. Kojima et al., unpublished data, see also ref. 21). The number of muscarinic receptors (3H-quinuclidinyl benzilate binding site, fmol/mg tissue) increased with development between fetal day 20 and the day of birth, remained almost constant until neonatal day 15, and then gradually decreased to the adult level (M. Kojima et al., unpublished data, see also ref. 22). The cyclic AMP content (pmol/mg tissue) increased prenatally and for the first 10 days postnatally, and then declined slightly thereafter (11). The number of calcium channels (3H-nitrendipine binding site, fmol/mg protein) increased with development between fetal day 17 and neonatal day 9, and remained constant thereafter (23). Isoproterenol (ISO) did not increase $V_{max}$ (the maximum rate of rise, an indicator of calcium current) of calcium-dependent slow action potentials during the fetal period, but began to increase the $V_{max}$ progressively after birth; the effects of ISO reached a maximum level at about 2 weeks postnatally (M. Kojima et al., unpublished data). Acetylcholine (ACh), applied to the ISO-preexposed preparations, was ineffective in reducing $V_{max}$ of the slow action potentials during the fetal period, but became effective thereafter: its effects became maximal about 4 days after birth (M. Kojima et al., unpublished data).
Our findings on the developmental changes in the CTX and PTX substrates, β-adrenergic and muscarinic cholinergic receptors, and ISO-ACh actions on calcium current suggest that the numbers of both receptors reach a maximum level earlier than the CTX and PTX substrate levels and the ISO-ACh actions on calcium current, and there is a good developmental coupling between the CTX substrate levels and the effect of ISO on calcium current, and between the PTX substrate level and the effect of ACh on calcium current, especially during the neonatal days 0–16.

The present report is the first presentation of the developmental changes in both CTX and PTX substrates in the rat heart (ventricle) [although there have been two reports on the ontogenesis of the G₁ protein in the chick heart (9, 10)]. It is now under intensive investigation what kinds of physiological or pharmacological functions each of these substrates participates in (1, 3, 24–26). In addition to the coupling of G proteins with the receptor-adenylate cyclase/phospholipase C-effector system (1–5, 24–30), it has been recently found that the G proteins directly couple with potassium channels in atrial cells (31–33), sodium channels in atrial membranes (34) and calcium channels in ventricular cells (35) (see also refs. 36–38). The present information may provide some important clues for understanding how the G₁ and G₁ proteins participate in transferring signals from membrane receptors to their effectors in the process of development in cardiac tissues.

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