Proadrenomedullin N-Terminal 20 Peptide Hyperpolarizes the Membrane by Activating an Inwardly Rectifying K⁺ Current in Differentiated PC12 Cells

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Abstract—The mechanism of proadrenomedullin N-terminal 20 peptide (PAMP)—induced inhibition of catecholamine release from adrenergic nerve was investigated in nerve growth factor–treated PC12 cells that have differentiated characteristics somewhat similar to noradrenergic neurons. The effect of PAMP on the excitability of these cells was investigated with the use of perforated whole-cell clamp. PAMP hyperpolarized the membrane by increasing a K⁺ conductance in a dose-dependent manner. The current-voltage relationship (I-V) relationship of the PAMP-induced K⁺ conductance exhibited inward-going rectification. The activation was abolished by microinjecting GDPβS into the cells or pretreating the cells with pertussis toxin. These results indicate that a pertussis toxin–sensitive G protein is involved in the signal transduction. The PAMP-induced activation of the K⁺ conductance was attenuated by microinjecting antibody against the carboxyl terminus of Goia, but it was not influenced by microinjecting antibody against the common carboxyl termini of Goβi and Goγi, which indicated that the G protein coupling the PAMP receptor to the inwardly rectifying K⁺ current is Goia. The PAMP-induced hyperpolarization may inhibit the catecholamine release from the neurons by attenuating the action potential frequency. (Circ Res. 1999;84:445-450.)

Key Words: channel • PC12 cell • hypertension

Materials and Methods

Cell Culture
PC12 cells were maintained in DMEM that contained 10% FCS. Cells were cultured in humidified air that contained 5% CO₂ at 37°C. Cells were subcultured every week, and the medium was changed every 4 days. For electrophysiological experiments, cells were seeded on 35-mm plastic dishes and cultured in DMEM that contained 10% FCS and 2.5S NGF (0.75 nmol/L) for 7 days. We selected cells with the axon outgrowth for electrophysiology because this morphology indicates that the cell has differentiated into sympathetic neuronlike cells.

Electrophysiology
The perforated whole-cell clamp technique was used in most of the experiments. The standard patch electrode solution contained (in mmol/L) K aspartate 95, KCl 47.5, MgCl₂ 1, EGTA TMA salt 0.1, and HEPES 10 (TMA, pH 7.4). The 20 mmol/L K⁺ solution and 40 mmol/L K⁺ solutions were made by the substitution of NaCl in the standard extracellular solution with iso-osmotic KCl. Na⁺-free extracellular solution was made by replacing Na⁺ in standard solution with iso-osmotic TMA⁺. Na⁺-free solution with 300 mmol/L BaCl₂ was made by isosmotically replacing TMACl with 300 mmol/L BaCl₂. In the experiments that investigated the involvement of second messengers, the conventional whole-cell clamp technique was used. The patch pipette solution for the conventional whole-cell experiments contained (in mmol/L) K aspartate 95, KCl 47.5, MgCl₂ 1,
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CaCl₂ 0.013, EGTA TMA salt 2.0, ATP (Na⁺ salt) 2, GTP (Mg salt) 0.1, and HEPES 10 (TMA salt, pH 7.2). The calculated pCa (pCa = −[log Ca²⁺]) of the pipette solution was 6.8.

During the experiments, the extracellular solution was supplied continuously by a peristaltic pump. Selected pharmacological agents were applied by changing the superfusate. The liquid junction potentials between the standard extracellular solution and other solutions used (internal and external) were measured with a 3 mol/L KCl electrode as a reference, and all the data were corrected for the liquid junctional potential (−8 to −2 mV). A EPC-7 amplifier (List) was used to record the membrane current and potential. All experiments were performed at room temperature (22°C to 25°C). Glass capillaries of 1.5-mm diameter that contained an inner filament were used to make patch electrodes. The resistance of the patch electrodes was between 5 and 8 MΩ. For the perforated whole-cell clamp experiments, a fresh stock solution of nystatin was made in DMSO (540 mmol/L) daily. Shortly before recording, the stock solution was diluted with the patch electrode solution (final nystatin concentration, 0.22 mmol/L). Details of the perforated whole-cell clamp technique have been reported elsewhere. For the current-clamp recordings were started after the series resistance fell to <50 MΩ. Voltage-clamp recordings were made after the series resistance fell to <10 MΩ. Because the amplitude of the current was less than 200 pA, the errors caused by the series resistance were ignored.

Microinjection of GDPβS and Antibodies

Guanosine 5'-O-2-thiodiphosphate (GDPβS) as injected into the cell by microinjection. The details of the method for microinjection have been reported elsewhere. GDPβS was dissolved in 50 mmol/L KCl at concentration of 100 mmol/L. For the microinjection, 2 kinds of polyclonal antibodies against the carboxyl terminus peptides of Goα were used. They were (1) anti-Goαi3, Goαi3 antibody (2.8 mg/mL, affinity purified; No. 371723, Calbiochem), which was developed against a peptide that represented amino acids 345 to 354 of the common carboxyl termini of Goαi3 and Goαi10–13; (2) anti-Goαi1 antibody (4.2 mg/mL, affinity purified; No. 371729, Calbiochem), which was developed against a peptide that represented amino acids 345 to 354 of the carboxyl terminus of Goαi1 and which is monospecific to Goαi1,12,13 The antibodies were diluted ×200 by the internal solution used for perforated patch. The solution was microinjected through microcapillaries (Femtotips, Eppendorf) by pressure injection (110 hPa, 0.1 second). At the time of injection, a slight swelling of the cell was observed. The volume of the injected solution was ~100 fL, which was estimated by the decrease of the solution after multiple injections. Only cells with input resistance of >1 GΩ after the microinjection were used for the electrophysiological experiment.

Drugs

Nystatin was obtained from Sigma; pertussis toxin (PTX) and NGF (2.5S) from Funakoshi Chemicals; and GDPβS from Boehringer Mannheim. cAMP, GMP, IP₃, and PMA were obtained from Calbiochem, and BAPTA from Molecular Probes. PAMP was kindly supplied by Dr Kenji Kangawa at National Cardiovascular Center Research Institute (Osaka, Japan). A scrambled peptide of PAMP was supplied by Dr. Kenji Kangawa at the National Cardiovascular Center Research Institute (Osaka, Japan). A scrambled peptide of PAMP was synthesized and purified by HPLC (>95%) by Sawady Co Ltd (Tokyo, Japan). The amino acid sequence of the scrambled peptide was AFRSSWQNKKSRKTAKDRLLW-NH₂.

Results

PAMP-Induced Hyperpolarization

Figure 1A shows a membrane potential record under the current clamp in the standard extracellular solution from an NGF-treated PC12 cell. This cell had a resting potential of ~−60 mV, and application of PAMP (100 nmol/L) hyperpolarized the membrane by ~10 mV. The membrane potential partially recovered slowly by washing out PAMP from the extracellular solution. Hyperpolarization was observed in all the cells examined (n = 11), and the amplitude of hyperpolarization by PAMP (100 nmol/L) was 10 ± 2 mV (mean ± SD, n = 11). To investigate the ionic mechanism of PAMP-induced hyperpolarization, voltage clamp experiments were performed. Figure 1B shows a current record under the voltage clamp at the holding potential of −48 mV. Membrane conductance was monitored by applying a 60-mV hyperpolarizing pulse step (0.8 seconds) every 20 seconds.

Application of PAMP (100 nmol/L) induced an outward current accompanied by an increase in membrane conductance in each of the 20 cells investigated. When the cell was superfused with a scrambled peptide of PAMP (1 μmol/L), there was no change in the membrane current.

Ionic Mechanism of PAMP-Induced Outward Current

To determine which ions are involved in the PAMP-induced outward current, the reversal potential of the PAMP-induced current was investigated. Figure 2A shows the basal membrane currents under the voltage clamp in the standard extracellular solution. The holding potential was −48 mV, and test potentials were −78, −88, −98, −108, −118, and −128 mV. The I-V relationship of the control current is plotted in Figure 2B (closed circle). The basal current showed a distinct inward rectification. To analyze components of the basal membrane conductance, the extracellular solution was changed to Na⁺-free (TMA⁺-substituted) solution. The membrane conductance was decreased in the Na⁺-free extracellular solution [Na⁺(−)] but some residual conductance remained. To determine whether K⁺ conductance sensitive to low concentration of BaCl₂ was involved in the residual membrane conductance, the extracellular solution was changed to Na⁺-free extracellular solution containing 300 μmol/L BaCl₂. The membrane current did not change by...
BaCl$_2$ ([Na$^-$] + BaCl$_2$). These data indicated that the basal current consists of a conductance dependent on extracellular Na$^+$ and a residual conductance that is insensitive to 300 μmol/L BaCl$_2$. The I-V relationships of the membrane current in Na$^+$-free solution (open circle) and Na$^+$-free with 300 μmol/L BaCl$_2$ (open triangle) are plotted in Figure 2B.

Figure 3A shows the membrane currents under the voltage clamp before and after the application of PAMP (10 nmol/L). The holding potential and the test potentials are the same. The PAMP-induced current (“subtracted” in Figure 3A) was obtained by subtracting the control currents from those after the application of PAMP. Application of PAMP (10 nmol/L) changed the membrane current at −78 mV to the outward direction and changed the membrane currents to the inward direction at potentials more hyperpolarized than −88 mV. The amplitude of the PAMP-induced current was more prominent at hyperpolarized potentials than depolarized potentials. The reversal potential, where there is no effect on the membrane current, was about −90 mV, which was close to the equilibrium potential of K$^+$. This suggests that the PAMP-induced current is a K$^+$ current. To determine whether the PAMP-induced current was carried by K$^+$, reversal potentials of the PAMP-induced current at various extracellular K$^+$ concentration were measured. Figure 3B shows the membrane currents before (cont) and after (PAMP) the application of PAMP (10 nmol/L) in the extracellular solution containing 40 mmol/L K$^+$. The membrane currents shifted to the inward direction at −124 mV but did not shift at −34 mV by the application of PAMP, which indicated that the reversal potential of the PAMP-induced conductance shifted with the change of extracellular K$^+$ concentration. These data suggest that PAMP-induced current is a K$^+$ current with inward rectification. We investigated the effect of low concentration of BaCl$_2$ on the effect of PAMP on the membrane currents. Figure 3C shows the membrane currents before (cont) and after (PAMP) the application of PAMP (10 nmol/L) when the extracellular solution contained 300 μmol/L BaCl$_2$. The effect of PAMP was abolished at both the outward limb and inward limb of the current.

Figure 4A shows the current-potential (I-V) relationship of the PAMP-induced current at 5 mmol/L (○) and 40 mmol/L (○) extracellular K$^+$ concentrations. B, Reversal potentials at various extracellular K$^+$ concentration plotted against the extracellular K$^+$ concentration. C, Concentration dependency of the PAMP-induced conductance. PAMP-induced conductances were calculated by dividing the difference of the membrane currents at potentials −118 mV and −88 mV by the potential difference (30 mV). All points are the mean of 5 data. Bar indicates SD.
Signal Transduction of PAMP-Induced Activation of the Inwardly Rectifying K⁺ Current

To investigate the signal transduction of PAMP response, we microinjected GDPβS, a nonhydrolyzable GDP analogue into the cells and applied PAMP (10 nmol/L). As is shown in Figure 6A, the membrane currents were not changed by PAMP (n=6). When the cells were pretreated with 100 ng/mL PTX for 24 hours, application of PAMP (10 nmol/L) did not change the membrane currents (Figure 6B, n=7). These data indicate that the activation of the inwardly rectifying K⁺ current by PAMP was mediated by a PTX-sensitive G protein. To identify the PTX-sensitive G-protein subtype, the antibody against the common carboxyl terminal amino acid sequence of Goi₁ and Goi₂ (anti-Goi₁,₂) or antibody against the carboxyl terminal amino acid sequence of Goi₃ (anti-Goi₃) was microinjected into the cells. Figure 6C shows that PAMP induced a membrane current in a cell microinjected with anti-Goi₁,₂ antibody. On the other hand, PAMP did not induce a membrane current in a cell microinjected with anti-Goi₃ antibody (Figure 6D). To confirm that this attenuation of the PAMP-induced response is due to the binding of the injected antibody to the respective G protein, we neutralized anti-Goi₃ by mixing it with an excess amount of the antigen peptide and injected it into the cells. Application of PAMP on the cell injected with the neutralized anti-Goi₃ activated the membrane conductance (Figure 6E), which indicated that the attenuation of PAMP response was due to the binding of injected antibody to the respective G protein. Figure 6E shows the summary of these experiments.

Figure 5. A-E, Membrane currents under the voltage clamp evoked by test potentials more depolarized than −90 mV. The holding potential was −48 mV, and the test potentials were to −48, −58, −68, −78, and −88 mV. The control currents (A), currents after the application of PAMP (B, 1 nmol/L; C, 10 nmol/L), PAMP (10 nmol/L)-induced currents (D), and currents after washing out PAMP (E) are plotted. PAMP-induced currents are calculated by subtracting the control currents from the currents after the application of PAMP (10 nmol/L). Dotted line indicates zero current level. F, Concentration dependence of the PAMP-induced conductance. PAMP-induced currents are calculated by dividing the difference of the membrane currents at potentials −48 and −88 mV by the potential difference (40 mV). Each value is the mean of 6 data points. Bar indicates SD.

Figure 6. Signal transduction of PAMP-induced response. A, Membrane currents before (cont) and after (PAMP) the application of PAMP (100 nmol/L) on a cell microinjected with GDPβS. B, Membrane current before (cont) and after (PAMP) the application of PAMP (100 nmol/L) on a PTX-pretreated cell. PTX (100 ng/mL) was treated for 24 hours. C, Membrane currents before (cont) and after (PAMP) the application of PAMP (100 nmol/L) in a cell microinjected with anti-Goi₁,₂. D, Membrane currents before (cont) and after (PAMP) the application of PAMP (100 nmol/L) in a cell microinjected with anti-Goi₃. E, Summary of the results of antibody-microinjection experiments. PAMP-induced conductances in cells microinjected with anti-Goi₁,₂ and anti-Goi₃ are summarized. The measurement of PAMP-induced conductances were the same as in Figure 3C. “anti-Goi₁,” “anti-Goi₃ 178,” and “178 neutr. anti-Gi₃” indicate data from cells microinjected with the indicated antibodies and the neutralized anti-Goi₃. The PAMP-induced conductances were significantly different (P<0.01 by ANOVA). All points are the mean of 5 data. The bar indicates 1 SD. F, Summary of the results of experiments on the involvement several second messengers. PAMP-induced membrane conductance was calculated in cells voltage-clamped with the conventional whole-cell clamp technique (column A). PAMP-induced conductances were measured in cells introduced with cAMP (B), GMP (C), or IP₃ (D) and cells treated with PMA (E). The PAMP-induced conductance was not significantly changed in cells where intracellular Ca²⁺ was chelated low by high concentration of BAPTA (F). The PAMP-induced conductances were not significantly different (by ANOVA). All points are the mean of 5 data. The bar indicates 1 SD.
Anti-\(\alpha_\text{i3}\) attenuated PAMP-induced response. These data indicate that the G protein that mediates the PAMP-induced activation of the inwardly rectifying \(K^+\) current was \(\alpha_\text{i3}\). The signal transduction mechanism was investigated further with the conventional whole-cell clamp technique. The PAMP-induced activation of the inwardly rectifying \(K^+\) current was observed and was comparable to that observed with the perforated whole-cell clamp technique (Figure 6F, column A). The involvement of several second messengers was investigated by introducing these second messengers in the cell through the patch pipette. The pipette was filled with an intracellular solution that contained either cAMP (2 mmol/L), GMP (2 mmol/L), or IP\(_3\) (1 mmol/L). To investigate the involvement of PKC, the cell was treated with a phorbol ester, PMA (1 \(\mu\)mol/L), for 30 minutes. To evaluate the involvement of intracellular \(Ca^{2+}\), the intracellular \(Ca^{2+}\) concentration was reduced by chelating with BAPTA. EGTA was replaced iso-osmotically with a high concentration of BAPTA (20 mmol/L). The PAMP-induced conductance was calculated by the same method in Figure 3C. As is shown in Figure 6F, the PAMP-induced conductances were not significantly changed in cells introduced with cAMP, GMP, or IP\(_3\), or in cells treated with PMA. The PAMP-induced conductance was not significantly changed in cells where intracellular \(Ca^{2+}\) was chelated low by a high concentration of BAPTA.

**Discussion**

PAMP is a newly identified peptide that has a hypotensive action. One mechanism that underlies this hypotensive effect is the inhibition of catecholamine secretion from sympathetic nerve terminals. The inhibition of catecholamine secretion by PAMP is not mediated by interfering with nicotinic or \(\alpha_\text{2}\) receptors, which suggests a direct effect of PAMP on sympathetic nerve terminals.

In this paper, we investigated the direct effect of PAMP on NGF-treated PC12 cells. We used PC12 cells that showed prominent axon outgrowth since these characteristics were consistent with those of differentiated PC12 cells that have characteristics similar to noradrenergic sympathetic neurons. In the previous study, we analyzed the voltage-gated \(Ca^{2+}\) currents (VGCC) of these NGF-treated PC12 cells and found that these cells with prominent axon outgrowth express N-type currents as a major component of the VGCC. This is also characteristic to PC12 cells differentiated into sympathetic neuronlike cells.

PAMP hyperpolarized the membrane by inducing an outward current due to the activation of an inwardly rectifying \(K^+\) conductance. The activation of the \(K^+\) conductance was significant, reversible, and concentration dependent at the outward limb of the inwardly rectifying \(K^+\) current that regulates membrane potential at voltage more depolarized than \(-88\) mV. The activation of the \(K^+\) current by PAMP was concentration dependent and was observed at the concentration of 1 mmol/L at which PAMP decreased significantly the catecholamine secretion from sympathetic nerve endings. As is shown in Figure 2B, the basal current showed a steep inward rectification. The basal membrane current consisted of a conductance dependent on extracellular \(Na^+\) and a residual conductance that is insensitive to 300 \(\mu\)mol/L \(BaCl_2\). PAMP-induced current was abolished in extracellular solution that contained 300 \(\mu\)mol/L \(BaCl_2\), which is inconsistent with the observation that the PAMP-induced current is an inwardly rectifying \(K^+\) current. This also suggests that the PAMP-induced inwardly rectifying \(K^+\) conductance was not induced by augmenting the basal inwardly rectifying \(K^+\) conductance because the residual membrane conductance in \(Na^+\)-free extracellular solution was not sensitive to 300 \(\mu\)mol/L \(BaCl_2\).

The latency of the PAMP-induced outward current in Figure 1B was slow in part because it took \(\approx 2\) minutes to change the solution with the perfusion system we used. Other apparatus, such as puff application of the peptide, will be necessary to obtain the accurate latency of the response.

In a physiological situation in which the sympathetic neurons are firing because of excitatory inputs, hyperpolarization by PAMP results in the inhibition of action potential firing or decrease in firing frequency. The inhibition of action potentials reduces \(Ca^{2+}\) influx through the VGCC and thereby decreases [\(Ca^{2+}\]). Because \(Ca^{2+}\) influx through N-type \(Ca^{2+}\) channels is closely related to the catecholamine secretion, the inhibition of \(Ca^{2+}\) influx through N-type \(Ca^{2+}\) channels could explain the PAMP-induced inhibition of catecholamine secretion.

Microinjection of GDP\(\beta\)S into the cells or PTX treatment abolished the action of PAMP, which indicates that a PTX-sensitive G protein is involved in the response. By microinjecting antibody against the carboxyl terminal sequence of G protein \(\alpha\) subunit, this G protein was determined to be \(G_\text{ii}\). Several neurotransmitters and neuromodulators, such as somatostatin and dopamine, also activate inwardly rectifying \(K^+\) current through a PTX-sensitive G protein. The signal transduction mechanism was investigated further with the conventional whole-cell clamp technique. PAMP-induced conductances were not significantly changed in cells introduced with cAMP, GMP, or IP\(_3\), or in cells treated with PMA.

In our previous study, PAMP inhibited N-type \(Ca^{2+}\) current at concentrations higher than 1 mmol/L. Because PAMP significantly decreased catecholamine secretion from the sympathetic nerve endings at concentrations >1 mmol/L, the effect of PAMP on the VGCC and on the inwardly rectifying \(K^+\) current may have physiological significance. The concentration dependence of the inhibition of N-type \(Ca^{2+}\) current was similar to that of the PAMP-induced activation of the inwardly rectifying \(K^+\) current. Because the outward limb of the inwardly rectifying \(K^+\) current is responsible for the regulation of membrane potential, the PAMP-induced conductance was calculated at the outward limb of the I-V curve. As is shown in Figure 5, PAMP-induced conductance at the outward limb of the I-V curve was also concentration dependent and was comparable to the concentration dependence of the PAMP-induced inhibition of the VGCC and PAMP-induced inhibition of catecholamine secretion.
cretion. The inhibitory effect appears to be mediated by a PTX-sensitive G protein. The inhibition of the N-type Ca<sup>2+</sup> channels and hyperpolarization by PAMP cooperatively inhibit the Ca<sup>2+</sup> influx through the VGCC and reduce [Ca<sup>2+</sup>]<sub>i</sub>, and thus decrease the catecholamine secretion. The inhibition of VGCC together with the activation of an inwardly rectifying K<sup>+</sup> current through some PTX-sensitive G proteins are modulations of ionic channels shared by other inhibitory neurotransmitters, such as somatostatin and dopamine. 21–24 These characteristics of ion channel modulation by PAMP may nominate PAMP as a candidate for inhibitory neuromodulator or neurotransmitter.

The use of NGF-treated PC12 cells instead of primary-cultured sympathetic neurons offer the advantage that we can analyze the biochemical effect of PAMP, such as changes in the second messenger levels, with the use of numerous cells. We are planning to investigate these effects of PAMP and compare the signal transduction mechanism to that of PAMP-induced modulation of the ion channels in future studies.

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References
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