Proadrenomedullin NH_2-terminal 20 Peptide Inhibits the Voltage-gated Ca^{2+} Channel Current through a Pertussis Toxin-sensitive G Protein in Rat Pheochromocytoma-derived PC 12 Cells

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Abstract

The effect of proadrenomedullin NH_2-terminal 20 peptide (PAMP) on the voltage-gated Ca^{2+} channel current was investigated using the perforated whole-cell clamp technique on NGF-treated PC12 cells. PAMP inhibited the Ba^{2+} current through N-type Ca^{2+} channels in a concentration dependent manner. Injection of GDPβS into the cell abolished the inhibition while injection of GTPγS into the cell made the inhibition irreversible, indicating that the PAMP-induced inhibition of the voltage-gated Ca^{2+} channel was mediated by a G protein. The inhibition was abolished by pretreating the cells with pertussis toxin, indicating that a pertussis toxin-sensitive G protein was involved in the signal transduction mechanism of PAMP. The present study revealed that the inhibition of catecholamine secretion from sympathetic nerve endings by PAMP could be explained by the inhibition of N-type Ca^{2+} channels, which was mediated by pertussis toxin-sensitive G protein. (*J. Clin. Invest.* 1996. 98: 14–17.) Key words: N-type Ca^{2+} channel • ω-conotoxin • perforated whole cell clamp • NGF • hypertension

Introduction

Proadrenomedullin NH_2-terminal 20 peptide (PAMP)[1] is a newly identified peptide (1, 2) which has a hypotensive effect in vivo (3). This peptide resides in the amino terminus of the precursor peptide, proadrenomedullin, and is cleaved from this precursor by proteolysis (1, 2). This peptide is found in plasma and tissues including adrenal medulla, right atrium, kidney, and brain (4). This distribution indicates its physiological role in the circulation control. Recently, PAMP was found to induce a remarkable hypotension through the inhibition of catecholamine release from peripheral sympathetic nerve endings (3). The inhibition of catecholamine release was not mediated through the interaction with nicotinic or α_2 receptor, suggesting a direct action of PAMP on catecholamine-releasing cells. To investigate whether PAMP has a direct effect on catecholamine-secreting cells, we selected a pheochromocytoma-derived PC12 cell line which possess characteristics common to noradrenergic sympathetic neurons when treated with NGF (5). It is possible that PAMP affects voltage-gated Ca^{2+} channels in these cells because Ca^{2+} influx through these channels has an essential role in regulating intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Thus we investigated whether PAMP has a direct effect on the voltage-gated Ca^{2+} channels in PC12 cells using electrophysiological techniques. In the case of recording Ca^{2+} channel currents with the conventional whole cell clamp technique, wash-out of intracellular soluble substrates is a serious problem. In this study we used the perforated whole cell clamp technique in order to avoid this wash-out.

Methods

Cell culture. PC12 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS). Cells were cultured in a humidified air containing 5% CO_2 at 37°C. Cells were subcultured every week and medium change was done every fourth day. For experiments, cells were seeded on 35-mm plastic dishes and cultured in DMEM containing 10% FCS and 2.5 S nerve growth factor (NGF) (10 ng/ml) for 7 d. We selected cells with the neurite outgrowth for electrophysiology because NGF-treated PC12 cells showing this characteristic is known to be differentiated into sympathetic neuron-like cells (5, 6).

Electrophysiology. The perforated whole-cell clamp technique (7) was used. Ba^{2+} ion was used as a charge carrier through the voltage-gated Ca^{2+} channels. Voltage-gated Na^+ channels were blocked by 1 μM tetrodotoxin. K^+ currents were blocked by intracellular Cs^+ and extracellular Ba^{2+}. The standard patch electrode solution contained (in mM): 95 Cs aspartate, 47.5 CsCl, 1 $MgCl_2$, 0.1 ethyleneglycol-bis(β-aminoethylether)-*N,N,N′*,N′-tetraacetic acid (EGTA) (tetramethylammonium [TMA] salt), and 10 Hepes (TMA salt, pH 7.2). The standard external solution was (in mM): 129 NaCl, 5 KCl, 1 $MgCl_2$, 1 $BaCl_2$, and 10 Hepes (Na salt, pH 7.4). During the experiments the extracellular solution was continuously superfused by a peristaltic pump. Agents were applied by changing the perfusing solution. Liquid junction potential between the standard extracellular solution and the internal solution was measured using a 3 M KCl electrode as a reference, and all the data were corrected for the liquid junctional potential (−4 mV). A List EPC-7 amplifier was used for recording the membrane current and potential. All experiments were performed at room temperature (22–25°C). Glass capillaries of 1.5

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Received for publication 28 November 1995 and accepted in revised form 24 April 1996.

1. *Abbreviations used in this paper:* NGF, nerve growth factor; PAMP, proadrenomedullin NH_2-terminal 20 peptide.

J. Clin. Invest.

0021-9738/96/07/14/04 $2.00
Volume 98, Number 1, July 1996, 14–17

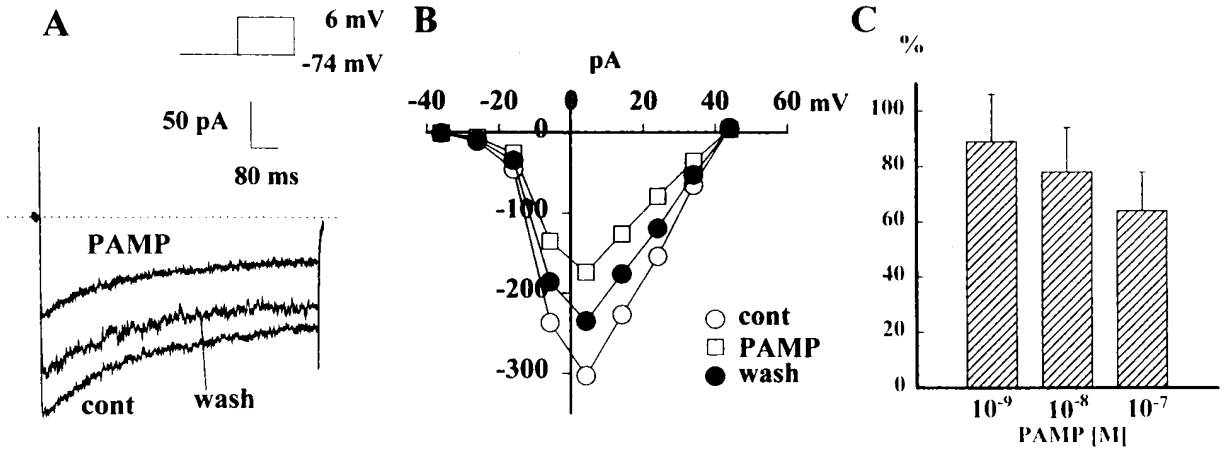

Figure 1. Effect of PAMP on voltage-gated Ca^{2+} channel current. Currents were recorded under voltage-clamp with the perforated whole-cell clamp technique. Ba^{2+} ion was used as a charge carrier. (*A*) The Ba^{2+} current evoked by the pulse step to 6 mV from the holding potential of −74 mV (cont). Application of PAMP inhibited the current (PAMP) with little change in the kinetics and partly recovered by washing out PAMP (wash). Dotted line indicates the zero current level. (B) I-V relationship of the Ba^{2+} current. The peak amplitude of the Ba^{2+} current at each pulse step is plotted. Squares indicate the Ba^{2+} current in the presence of 10^{-7} M PAMP, open circles the control, and closed circles that after washing out PAMP. (*C*) concentration-dependent effect of PAMP on the Ba^{2+} current. The ordinate indicates percent of the Ba^{2+} current as compared to the control. PAMP inhibited the Ba^{2+} current in a concentration dependent manner. Each bar is the mean of five experiments and brackets indicate 1 SD.

mm diameter with a filament were used to make patch electrodes. The resistance of the patch electrodes were between 5 and 8 MΩ. For the perforated whole-cell clamp experiments, a fresh stock solution of nystatin was made in dimethylsulfoxide (50 mg/ml) daily. Shortly before recording, the stock solution was diluted with the patch electrode solution (final nystatin concentration, 200 μg/ml). Details of the perforated whole-cell clamp technique have been reported elsewhere (8). Voltage clamp recordings were made after the series resistance fell below 10 MΩ. Because the amplitude of the current was < 300 pA, the errors caused by the series resistance were ignored.

Microinjection of GDPβS and GTPγS. Guanosine 5′-*O*-2-thiodiphosphate) (GDPβS) and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) were injected into the cell by microinjection. The details of the method for microinjection have already been reported elsewhere (9). GDPβS was dissolved in 150 mM KCl at the concentration of 100 mM and GTPγ was dissolved in 150 mM KCl at the concentration of 10 mM. The solution was microinjected through microcapillaries (Femtotips, Eppendorf) by pressure injection (110 hPa, 0.1 s). At the time of injection, a slight swelling of the cell was observed. During early stage of the experiments, we co-injected fluorescein-conjugated dextran together with the compounds to confirm the injection using fluorescence microscope. The volume of the injected solution was about 100 fl which was estimated by the decrease of the solution after multiple injections. The cells with input resistance of more than 1 GΩ after the microinjection was used for the experiment.

Drugs. Nystatin was obtained from Sigma Chemical Co. (St. Louis, MO), ω-conotoxin GVIA, from Molecular Probes (Eugene, OR), pertussis toxin and NGF (2.5 S) from Funakoshi Chemicals (Tokyo, Japan), GDPβS and GTPγS from Boehringer Mannheim (Mannheim, Germany).

Results

PAMP-induced inhibition of the voltage-gated Ca^{2+} channels. Fig. 1 *A* (cont) shows Ba^{2+} current through voltage-gated Ca^{2+} channels recorded under voltage clamp. Holding potential was −74 mV and the test pulse step to 6 mV was applied. The Ba^{2+} current showed a distinct inactivation process but the steady current remained. This Ba^{2+} current first appeared at a potential step to −34 mV. As the depolarizing steps became greater, the amplitude of the Ba^{2+} current increased. The current-voltage (I-V) relationship of the Ba^{2+} current is shown in Fig. 1 *B* (*open circles*).

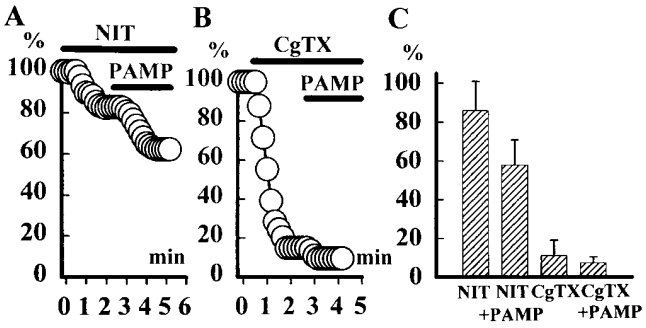

Figure 2. N-type Ca^{2+} channel current inhibited by PAMP. (*A*) Effect of L-type Ca^{2+} channel blocker, nitrendipine (NIT), and PAMP on the Ba^{2+} current. The Ba^{2+} current was evoked by the pulse step to 6 mV from the holding potential of −74 mV. The amplitude of the peak current was measured at every 10 s. The ordinate indicates percent of the Ba^{2+} current as compared to the control and the abscissa, the time course of the application of 5 μM NIT and 10^{-7} M PAMP. After NIT attained its maximal effect, PAMP was applied. (*B*) effect of N-type Ca^{2+} channel blocker, ω-conotoxin GVIA (CgTX), and PAMP on the Ba^{2+} current. The Ba^{2+} current was evoked by the same protocol. After ω-CgTX (1 μM) attained its maximal effect, PAMP (10^{-7} M) was applied. (*C*) Summary of the experiments. The ordinate indicates percent of the Ba^{2+} current as compared to the control. Each bar indicates the mean of five experiments and the brackets indicate 1 SD. "NIT" indicates the amplitude of the Ba^{2+} current after NIT treatment, "NIT+PAMP," that after application of NIT and PAMP, "CgTX," that after ω-CgTX treatment and "CgTX+PAMP," that after application of ω-CgTX and PAMP.

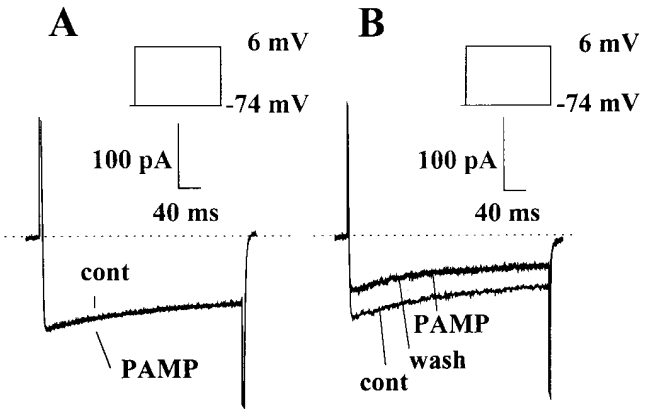

Figure 3. Effect of microinjection of GDPβS and GTPγS into the cell. (*A*) The Ba^{2+} current evoked by the pulse step to 6 mV from the holding potential of −74 mV (cont) in a GDPβS-injected cell. Application of PAMP (10^{-7} M) did not inhibit the Ba^{2+} current (PAMP). (*B*) The Ba^{2+} current in a GTPγS-injected cell (cont). Application of PAMP (10^{-7} M) inhibited the current, but washing out PAMP from the extracellular solution for 20 min did not recover the current (wash). Dotted lines indicate the zero current level.

Fig. 1 *A* (PAMP) shows the effect of PAMP (10^{-7} M) on the Ba^{2+} current at a potential step to 6 mV. Application of PAMP decreased the amplitude of the Ba^{2+} current. The I-V relationship of the Ba^{2+} current in the presence of PAMP is shown in Fig. 2 *B* (*squares*). PAMP decreased the amplitude of the BA^{2+} current at any potential, indicating that the PAMP-induced inhibition of the Ba^{2+} current was not voltage-dependent. The inhibition was reversible ("wash" in Fig. 1 *A* and closed circles in 1 *B*). Fig. 1 *C* summarizes the amplitude of the Ba^{2+} current inhibited by various concentrations of PAMP. The amplitude of the control current was normalized as 100% in each record. It is clear that PAMP inhibited the Ba^{2+} current in a concentration-dependent manner.

To examine what type of voltage-gated Ca^{2+} channels was inhibited by PAMP, nitrendipine (NIT, 5 μM), an L-type Ca^{2+} channel blocker, and ω-conotoxin GVIA (ω-CgTX, 1 μM), an N-type Ca^{2+} channel blocker, were used. Fig. 2 *A* shows the sequential effect of NIT (5 μM) and PAMP (10^{-7} M) on the Ba^{2+} current. In this cell, application of NIT decreased the current by ∼17% of the control. Additional application of PAMP inhibited the current by additional 19.5% of the control. Fig. 2 *B* shows the sequential effect of ω-CgTX (1 μM) and PAMP (10^{-7} M) on the Ba^{2+} current. In this cell, application of ω-CgTX decreased the current by ∼90% of the control. Additional application of PAMP inhibited the current only by ∼3% of the control. Fig. 2 *C* summarizes the data of these experiments. Application of NIT inhibited the Ba^{2+} current to 86±15% (mean±SD, $n = 5$) of the control and additional application of PAMP inhibited it to 59±13% ($n = 5$) of the control. Application of ω-CgTX inhibited the Ba^{2+} current to 11±8% ($n = 5$) of the control and additional application of PAMP inhibited it to 7±3% ($n = 5$) of the control. These results indicate that in NGF-treated PC12 cells, the Ba^{2+} current was mainly carried through N-type Ca^{2+} channels and that these channels were inhibited by PAMP.

Involvement of a G protein. To evaluate the signal transduction mechanism of PAMP, we microinjected GDPβS and GTPγS into the cells and examined the effect of PAMP. Fig. 3 A shows the effect of PAMP on the Ba^{2+} current in a GDPβS-injected cell. PAMP did not decrease the Ba^{2+} current in the GDPβS-injected cells ($n = 6$). Fig. 3 *B* shows the effect of PAMP on a GTPγS-injected cell. Application of PAMP decreased the Ba^{2+} current (PAMP). However, the inhibition was not reversed by washing out PAMP from the extracellular solution for 20 min (wash). The irreversible inhibition of the Ba^{2+} current by PAMP was observed in four additional GTPγS-injected cells. These data indicate that the inhibition of the Ba^{2+} current by PAMP was mediated by a G protein. To examine whether this G protein was sensitive to pertussis toxin, we pretreated the PC12 cells with 100 ng/ml pertussis toxin for 20 h. Fig 4 *A* shows the effect of PAMP (10^{-7} M) on a pertussis toxin-treated cell. PAMP did not inhibit the Ba^{2+} current. The I-V relationship before and after the application of PAMP are plotted in Fig. 4 *B*. Similar findings were reproduced in three additional cells. These data indicate that the effect of PAMP on the voltage-gated Ca^{2+} channels was mediated by a pertussis toxin-sensitive G protein.

Discussion

PAMP is a newly identified peptide possessing a hypotensive action. One mechanism underlying this hypotensive effect is the inhibition of catecholamine secretion from sympathetic nerve terminals (3). The inhibition of catecholamine secretion

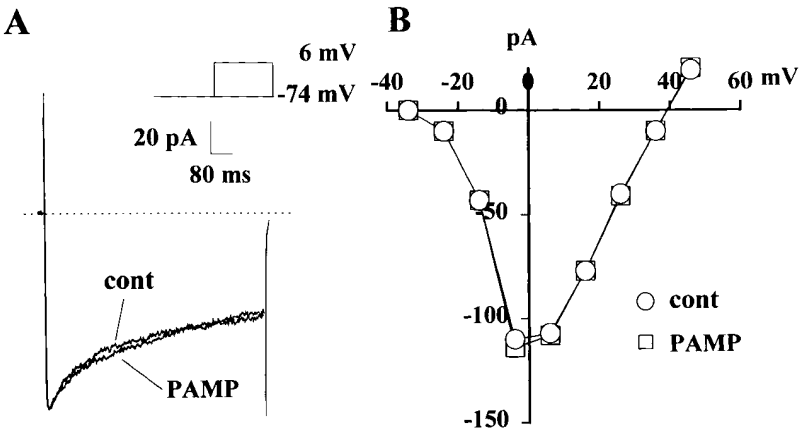

Figure 4. Elimination of the PAMP-induced inhibition of the Ba^{2+} current after pertussis toxin treatment (*A*) Records of the Ba^{2+} current evoked by the pulse step to 6 mV from the holding potential of −74 mV. "cont" indicates the control current and "PAMP," the Ba^{2+} current after application of 10^{-7} M PAMP. The cell was pretreated with 100 ng/ml pertussis toxin for 20 h. Dotted line indicates the zero current level. (*B*) I-V relationships of the Ba^{2+} current before (*circles*) and after (*squares*) application of 10^{-7} M PAMP. The cell was the same as in *A*. The holding potential was −74 mV.

from sympathetic nerve terminals by PAMP is not mediated by interfering with nicotinic or α_2 receptors, suggesting a direct effect of PAMP on sympathetic nerve terminals. In this paper, we investigated the direct effect of PAMP on Ca^{2+} channel currents in NGF-treated PC12 cells. PC12 cells used in this study showed the neurite outgrowth, and $\sim$ 90% of voltage-gated Ca^{2+} channel currents were composed of ω-CgTX-sensitive N-type Ca^{2+} currents. An L-type Ca^{2+} channel blocker, nitrendipine, had small effect on Ca^{2+} channels. These characteristics were consistent with those of differentiated PC12 cells which have characteristics similar to noradrenergic sympathetic neurons including the development of N-type Ca^{2+} channels (5, 6, 10–12). PAMP inhibited this N-type Ca^{2+} channels. The inhibition was in a concentration-dependent manner and was clearly observed at the concentration of 10^{-9} M. At this concentration PAMP significantly decreased the catecholamine secretion from sympathetic nerve endings (3). Inhibition of the voltage-gated Ca^{2+} channels by PAMP results in the inhibition of Ca^{2+} influx through these channels and thereby reduces $[Ca^{2+}]_i$. Because Ca^{2+} influx through N-type Ca^{2+} channels is closely related to the catecholamine secretion (13, 14), the inhibition of N-type Ca^{2+} channels could explain the PAMP-induced inhibition of catecholamine secretion. Microinjection of GDPβS into the cell abolished the PAMP effect and microinjection of GTPγS made the PAMP response irreversible, indicating that the inhibition by PAMP is mediated by a G protein. This G protein was sensitive to pertussis toxin suggesting that Gi or Go is involved in the signal transduction (15). Several neurotransmitters and neuromodulators also inhibit voltage-gated Ca^{2+} channels through a pertussis toxin-sensitive G protein (16, 17). This is the first report implying that the PAMP receptor may be a member of the G protein-coupled receptors.

According to Katoh et al., PAMP acts as a nicotinic cholinergic antagonist on bovine chromaffin cells, inhibiting nicotinic-stimulated Na^+ influx at concentrations higher than 10^{-7} M (18). This may be an alternative mechanism of PAMP-induced hypotension especially when the PAMP concentration is relatively high.

The hypotensive effect of PAMP is prominent, qualifying PAMP as a candidate for anti-hypertensive drugs. For treating hypertension dihydropyridine derivatives have been frequently used. These agents act mainly on L-type Ca^{2+} channels in vascular smooth muscle cells (19). In the present study we revealed that the PAMP-induced hypotensive effect was mediated by another mechanism, which is the inhibition of N-type Ca^{2+} channels in sympathetic neurons. Secretion of noradrenaline from sympathetic neurons in insensitive to L-type Ca^{2+} channel blockers but sensitive to ω-CgTX (14). PAMP can therefore offer a new choice in treating hypertension, especially for those which are refractory dihydropyridines, or which are resulting from the increased sympathetic nerve activity. Because PC12 cells are derived from rat pheochromocytoma cells, it may also be clinically used for controlling hypersecretion of catecholamines from human pheochromocytomas.

Acknowledgments

We thank Dr. K. Kangawa for providing proadrenomedullin NH_2-terminal 20 peptide.

This work was supported by grants from Uehara Memorial Foundation, and from the Ministry of Education of Japan.

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