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G-PROTEIN MODULATION OF α_{1A} (P/Q) TYPE CALCIUM CHANNEL EXPRESSED IN GH_3 CELLS

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GH_3 cell lines stably expressing α_{1A} channel were established and the modulation of this channel by G-protein through membrane-delimited pathways were studied. Wild type GH_3 cells were found to express ω-conotoxin MVIIC (MVIIC) sensitive Ca^{2+} current but this component was different from the α_{1A} channel because of its susceptibility to G-protein modulation, suggesting MVIIC also blocks channels other than P/Q type. α_{1A} channel expressed in GH_3 cells showed slowing of activation and reduction of current amplitude by the application of carbachol. Both of these effects were pertussis toxin (PTX) sensitive and voltage dependent. α_{1A} channels were also found to be modulated through a PTX insensitive pathway, the modulations observed were similar to those in the PTX sensitive pathway. The results further suggest that these two effects are governed by a different mechanism in both PTX sensitive and insensitive pathways.

Ca^{2+} entry through voltage-gated Ca^{2+} channels in neurons is essential for initiating a variety of physiological processes including synaptic transmission and gene expression (1-3). Ca^{2+} channels are also involved in neuronal cell migration (4). Several types of Ca^{2+} channel are co-expressed in a single neuron and each channel type seems to play a specific role in some functions and a cooperative role in others. For the fine tuning of complex neuronal function involving multiple types of Ca^{2+} channel activation, it is essential to know the similarities and differences of the modulatory mechanism for each type of Ca^{2+} channel. Regulation of voltage-gated Ca^{2+} channels by neurotransmitter receptor/G-protein is one of the essential mechanisms for Ca^{2+} channel modulation (5-7). α_{1A} channels which correspond to P-type and/or Q-type Ca^{2+} channels (8) are widely expressed in brain (9) and are thought to be a major and essential Ca^{2+} channel in the central nervous system (10). Although a substantial amount of studies are focused on the modulation of N-type channels in peripheral neurons, information about the modulation of P-type and Q-type Ca^{2+} channels is limited.

GH_3 anterior pituitary tumor cell lines, in rat, have been shown to be useful for studying the modulation of Ca^{2+} channels by receptor coupled G-proteins (11,12) and to investigate the role of this ion influx pathway in excitation-secretion and excitation-transcription coupling (13,14). In

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the present study, we have established GH_3 cells expressing α_{1A} channels ($GH_3BI2neo7$) and studied the modulation of this channel by G-protein through the membrane-delimited pathway.

MATERIALS AND METHOD

Cell culture: Rat GH_3 cells were obtained from Dr. Priscilla S. Dannies (Yale University School of Medicine). Cells were cultured in Ham's F10 medium containing 2.5 % fetal bovine serum (Hyclone), 15 % horse serum (Sigma), plus penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml) and were incubated in a humidified atmosphere of 5% CO_2/95% O_2, at 37°C. In the experiment with pertussis toxin (PTX), cells were pretreated with 1 μg/ml PTX for 5-28 hours.
Construction and transfection of the expression plasmid: The expression plasmid, pBI-2neo, encoding the α_{1A} channel, was constructed by inserting the 7.5 kb *Hind* III /*Hind* III fragment from pSPCBI-2 (9) into the *Hind* III site of pCDNA3 (Invitrogen). The expression plasmid contains a second transcription unit to direct the expression of the *neo* marker gene. GH_3 cells (2×10^6 in 250 μl of the plain Ham's F10 medium) were transfected by electroporation (1000 μF, 280 V) using Electroporator II (Invitrogen). 10 μg of pBI-2neo was used for transfection. Colonies were selected with G418 (200 μg/ml) starting 5 days after electroporation. 41 cell lines were cloned and tested for the expression of α_{1A} current. The cell line, $GH_3BI2neo7$ was thus established.
Electrophysiological measurements: Ca^{2+} currents were measured using the whole-cell variant of the patch-clamp technique (15). Recordings were made using an EPC-9 amplifier driven by a Pulse program. Data were filtered at 2.5 kHz and sampled at 10 kHz. The linear leakage and capacitive currents were subtracted from the raw data records by the P/4 protocol. Data were analyzed and displayed using the Igor program. The perfusing solution contained: 125 mM NaCl; 10.8 mM $BaCl_2$; 1 mM $MgCl_2$; 5.4 mM CsCl; 10 mM glucose; 3 μM tetrodotoxin and 10 mM HEPES (pH 7.4 with NaOH). Perfusion flow was continuous at ~3 ml/min in all experiments except for toxin experiments where the flow rate was ~1 ml/min. The pipette solution contained: 120 mM CsCl; 1 mM $MgCl_2$; 10 mM EGTA; 3 mM MgATP and 10 mM HEPES (pH 7.4 with NaOH). In the experiment with a peptide toxin, ω-conotoxin MVIIC or ω-conotoxin GVIA, the perfusion solution was supplemented with 0.1 mg/ml cytochrome C to saturate nonspecific peptide-binding sites and Ba^{2+} concentration was reduced to 5 mM.

RESULTS AND DISCUSSION

Characterization of wild type Ca^{2+} channels. Two types of Ca^{2+} channel (L and T type) have been shown to be present in GH_3 cells (16). We have found that N-channel blocker (10 μM ω-conotoxin GVIA (GVIA)) reduced ~20% of the total current (n=4), L-channel blocker (5 μM (±) PN 200-110 (PN)) reduced ~70% of the total current (n=13), 10 μM GVIA blocked ~13% of the non-L current (current insensitive to PN) (n=4) (Fig. 1A), and P/Q channel blocker (5 μM ω-conotoxin MVIIC (MVIIC)) reduced ~69% of the non-L current (n=3) (Fig. 1B). These results taken together suggest the presence of at least four different types of Ca^{2+} channel, based on drug sensitivity, in GH_3 cells. The estimated percentage of each component in the total currents are: α_{1D} subtype (PN and GVIA sensitive) (~16%) together with α_{1C} subtype (PN sensitive but not GVIA sensitive) (~54%) of L current; non-L, GVIA sensitive current (N-like) (~4%); non-L, MVIIC sensitive current (P/Q-like) (~21%) and others (may include T-type) (~5%). Cholinergic agonist, carbachol (CA) has been shown to block ~23% of the total current in GH_3 cells, without changing the activation kinetics, through a muscarinic receptor-$G\alpha_{o1}/\beta_3/\gamma_4$ pathway (12). As shown in Fig.

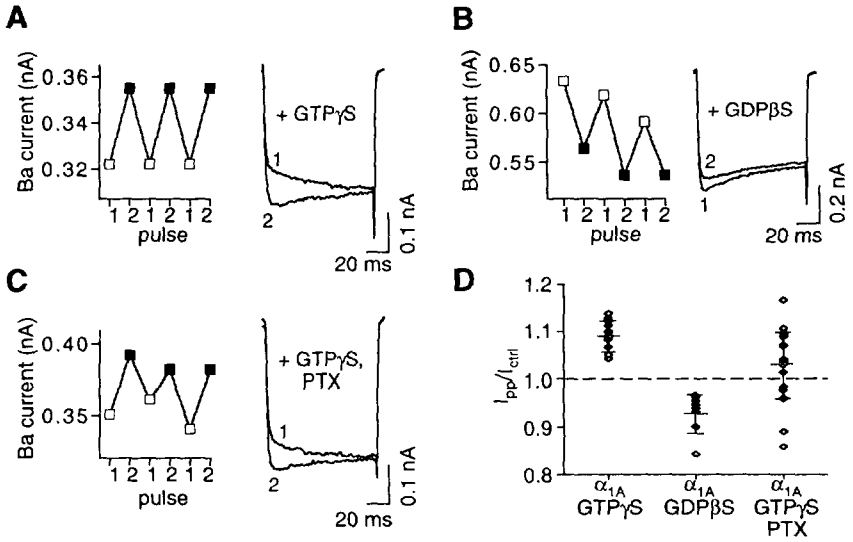

Fig. 1. Modulation of Ba^{2+} currents in wild type GH_3 cells. Time plots showing the effects of application of 5 μM (±) PN 200-110 (PN) and 10 μM ω-conotoxin GVIA (GVIA) (A), 5 μM PN and 5 μM ω-conotoxin MVIIC (MVIIC) (B), 5 μM PN and 10 μM carbachol (CA) (C), on peak Ba^{2+} current. Ba^{2+} currents were evoked at 30 s intervals. In each panel, asterisks mark the peak current values corresponding to the current traces illustrated. Holding potentials were -80 mV; test potentials were -10 mV (A and B) and 0 mV (C). D, Effect of prepulse to the PN insensitive Ba^{2+} currents (non-L currents) in the presence of 0.5 mM GTPγS in the pipette solution. An arrow indicates the point that starts prepulse experiment (top left). Pulse protocols (see ref. 17): every 10 s, a 100 ms test pulse to the potential showing peak current was applied (pulse 1), followed by a 10 s waiting period at a holding potential of -80 mV, a 50 ms prepulse to +70 mV, 10 ms repolarization to -80 mV and then another 100 ms test pulse to the same potential as in pulse 1 (pulse 2). Peak current was plotted for three consecutive series of pulse protocols (bottom left) and each of these three currents in pulse 1 (open square) and pulse 2 (filled square) were averaged and displayed (bottom right). Peak current in pulse 1 and 2 from three to five consecutive series of pulse protocols are averaged and the ratio of averaged current in pulse 2 over averaged current in pulse 1 (I_{pp}/I_{ctrl}) was determined. Individual data points are plotted as open diamonds (overlapping values may appear as single diamond), short horizontal lines indicate the mean ± S.D. and vertical lines indicate the range of S.D. Ratios >1 indicate enhancement of the current amplitude by the prepulse, and ratios <1 indicate that the prepulse produced a net reduction that may be caused by the voltage dependent inactivation (dashed line shows ratio of 1) (top right, n = 4).

1C, 10 μM CA does not block the non-L channels (n=6), suggesting that the CA block of Ca^{2+} current is exclusive to L-type (α_{1C} and/or α_{1D}) channels. Subsequently, the effect of a depolarizing prepulse to the non-L current in the presence of GTPγS in the pipette was examined (17). Inclusion of GTPγS in the pipette caused the facilitation of inactivation in the control current (pulse 1) that may reflect the enhancement of the T-type current (18); however, this does not change the activation kinetics, the prepulse causing a decrease of the Ca^{2+} current (n=4) (Fig. 1D). This suggests that non-L channels in GH_3 cells may not be susceptible to G-protein modulation through the membrane-delimited pathway.

Characterization of α_{1A} channel expressed in GH_3 cells. ~54% of the total Ca^{2+} current in GH_3 cells expressing α_{1A} channels (GH_3BI2neo7) was blocked by 5 μM PN (n=11).

Assuming that the expression of the α_{1A} channel does not have any effect on the expression level of the endogenous Ca^{2+} channels, an estimation of the α_{1A} component in the total non-L current was calculated at ~50%. 5 μM MVIIC blocked ~77% of the non-L current (n=10) (Fig. 2A), suggesting ~85% of the α_{1A} current is blocked by this concentration of toxin (19). 10 μM GVIA blocked ~18% of the non-L current (n=7) (Fig. 2B), suggesting ~23% of the α_{1A} current is blocked by this concentration of toxin. We further tested for effects of CA on the α_{1A} current. Although the non-L currents in wild type GH_3 cells were not sensitive to CA, non-L currents in GH_3BI2neo7 cells were found to be sensitive to this compound. Application of 10 μM CA reduced the amplitude of the current (~32% block of the non-L current, suggesting ~64% block of the α_{1A} current, n=40), and in 58% of cases (23/40 cell), non-L currents also showed slowing of activation. Both of these effects can be partially removed by a strong depolarizing prepulse (Fig. 3A, bottom). The effects of CA on Ca^{2+} current amplitude and kinetics resembled those of G-protein modulation on N-type channels through membrane delimited pathways (5-7) and were quite different from those of G-protein modulation on L-type channels in GH_3 cells (11,12). Current-voltage relations in the control (open circles), presence of 10 μM CA (closed circles) and washout (open triangles) are shown in Fig. 3B. The inhibition of current amplitude by CA was highly voltage dependent, as illustrated by the bell-shaped relation between current inhibition and test pulse potential (n=11) (Fig. 3C). Maximum inhibition occurred over the voltage range of -10 mV to 0 mV. Pretreatment of the cells with Pertussis toxin (PTX) completely eliminated the effects of CA on α_{1A} channels (n=12) (Fig. 3D). These results suggest that the α_{1A} channel is modulated by the muscarinic receptor/PTX sensitive G-protein through the membrane delimited pathway.

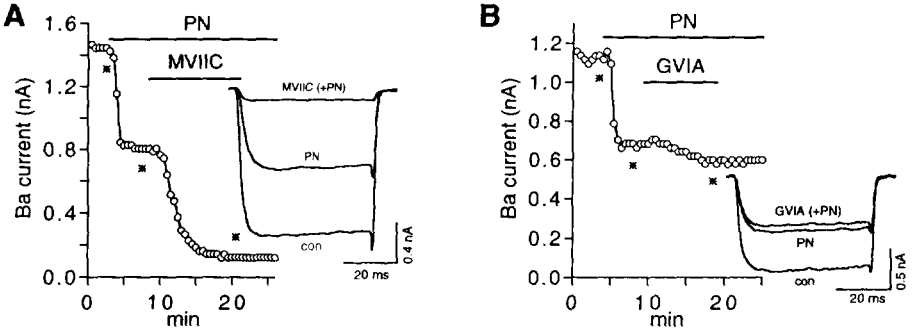

Fig. 2. Block of Ba^{2+} currents by peptide toxins in GH_3BI2neo7 cells expressing the α_{1A} channel. Time plots showing the effects of application of 5 μM PN and 5 μM MVIIC (A), and 5 μM PN and 10 μM GVIA (B), on peak Ba^{2+} current. Ba^{2+} currents were evoked at 30 s intervals. In each panel, asterisks mark the peak current values corresponding to the current traces illustrated. Holding potentials were -80 mV; test potentials were -10 mV.

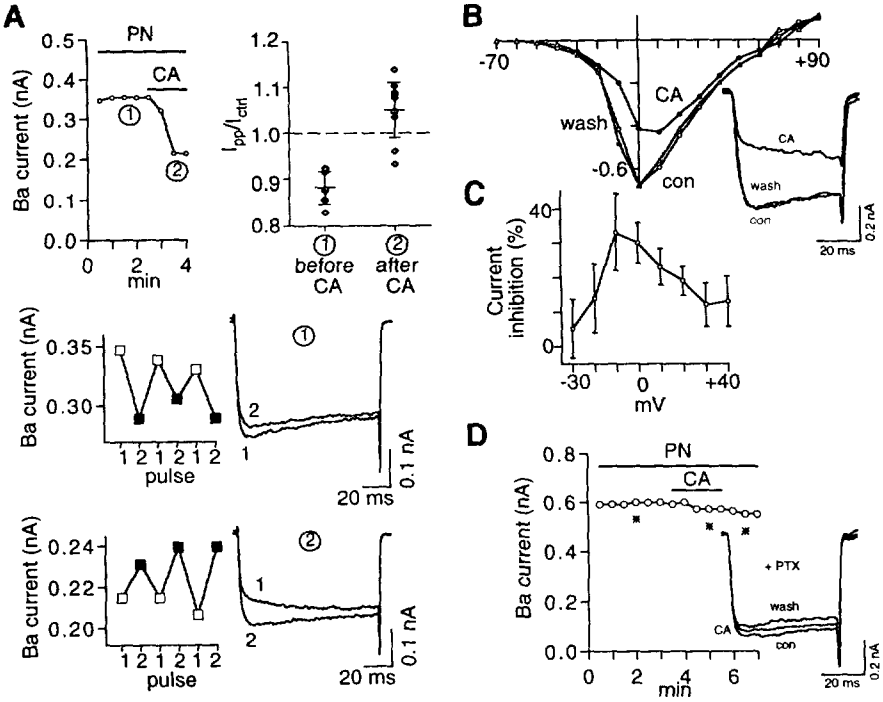

Fig. 3. Modulation of Ba^{2+} currents by carbachol in $GH_3BI2neo7$ cells expressing the α_{1A} channel. A, Time plots showing the effects of application of 10 μM carbachol (CA) on peak Ba^{2+} current in the presence of 5 μM PN (top left). Ba^{2+} currents were evoked at 30 s intervals. Prepulse experiment (see Fig. 1D) was performed before (①, middle) and after exposure of CA (②, bottom) in the presence of PN. No GTP was included in the pipette. Comparison of the ratio of peak current with and without prepulse (I_{pp}/I_{ctrl}) for the current before (n=8) and after (n=11) exposure of CA (top right). B, Peak current-voltage relation for Ba^{2+} current in control Ba^{2+} solution containing 5 μM PN (open circle), exposure to 10 μM CA in the presence of PN (closed circle) and washout with control solution containing PN (open triangle). Current traces illustrated are at 0 mV test potential. C, Carbachol-mediated current inhibition plotted as a function of test potentials. Data are expressed as mean ± SD (n=11). D, Time plots showing the effects of application of 10 μM CA on peak Ba^{2+} current in the presence of 5 μM PN. Cells were pretreated with 1 μg/ml pertussis toxin (PTX) for 7.5 hours. Asterisks mark the peak current values corresponding to the current traces illustrated.

This modulation caused both decrease of the current amplitude and slowing of the channel activation, however, these two effects may be governed by a different mechanism because CA decreased the current amplitude in all the cells tested, but did not change the activation kinetics in 42 % of the cells.

The dialysis effect of the cell interior with the non-hydrolyzable GTP analogues was then examined. Previously we have demonstrated that the α_{1A} channel expressed in dysgenic myotubes displays prepulse enhancement of the current, with a change in activation rate; not only with GTPγS but also with GDPβS (with much lower efficacy), suggesting this channel is modulated through a novel membrane-delimited pathway that may not involve G-protein activation (17). Although the non-L currents in wild type GH_3 cells did not show the prepulse deblocking effect, the non-L currents in $GH_3BI2neo7$ cells showed prepulse enhancement of the current with GTPγS

in the pipette (13/13 cell), and 69% of these cells (9/13 cell) also showed slowing of activation (Fig. 4A and D). This facilitation effect was similar to that observed in modulated N-type channels with GTPγS in the pipette (20-28). The non-L currents with GDPβS in the pipette did not show the deblocking effect (Fig. 4B and D, n=9), suggesting that the mechanism observed in dysgenic myotubes (17) may not be present in GH_3 cells or a large population of cells may need to be tested because their efficacy of GDPβS compared to GTPγS. Pretreatment of the cells with PTX did not eliminate the effects of GTPγS on α_{1A} channel, in contrast with the effect of PTX on CA modulation. 74% of the cells (17/23 cell) treated with PTX evinced prepulse enhancement of the current and 47% of these cells (8/17 cell) also showed slowing of activation (Fig. 4C and D). Relatively smaller prepulse enhancement effect by GTPγS in PTX treated cells (Fig. 4D) may be because the prepulse enhancement effect observed in control cells is a combined effect, release from both PTX sensitive and insensitive G-protein modulation. These results suggest that the α_{1A} channel is modulated by the PTX insensitive G-protein. The apparent nature of the modulations were similar to those in the PTX sensitive pathway.

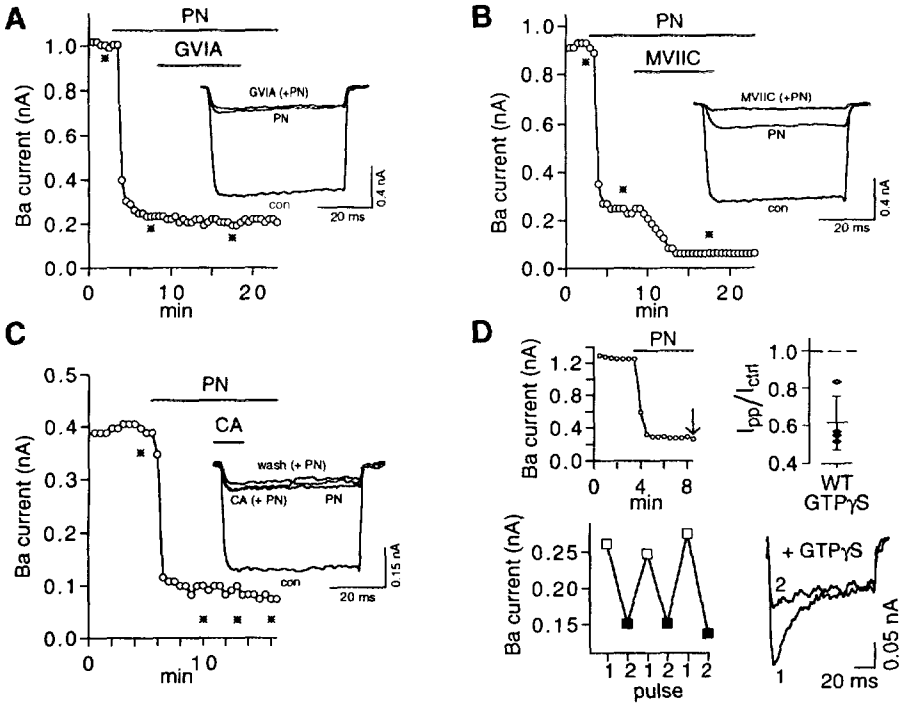

Fig. 4. Modulation of Ba^{2+} currents by GTPγS in GH_3BI2neo7 cells expressing the α_{1A} channel. Prepulse experiments (see Fig. 1D) were performed in Ba^{2+} solution containing 5 μM PN. 0.5 mM GTPγS (A, C) or 0.5 mM GDPβS (B) was included in the pipette solution. In C, cells were pretreated with 1 μg/ml PTX for 9.5 hours. D, Comparison of the ratio of peak current with and without prepulse (I_{pp}/I_{ctrl}) for the currents with GTPγS (n=13), GDPβS (n=9) and GTPγS/PTX (n=23). In GTPγS/PTX, cells were pretreated with 1 μg/ml PTX for 6-28 hours.

In this work, we have shown that non-L channels including MVIIC sensitive Ca^{2+} channel in wild type GH_3 cells are not regulated through membrane delimited pathway of G-protein modulation and that the α_{1A} channel expressed in GH_3 cells was modulated through two independent membrane delimited pathways, one is PTX sensitive and the other is PTX insensitive. Two effects, slowing of channel activation and reduction of current amplitude, are not always observed in concurrence, suggesting these two effects are governed by a different mechanism.

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