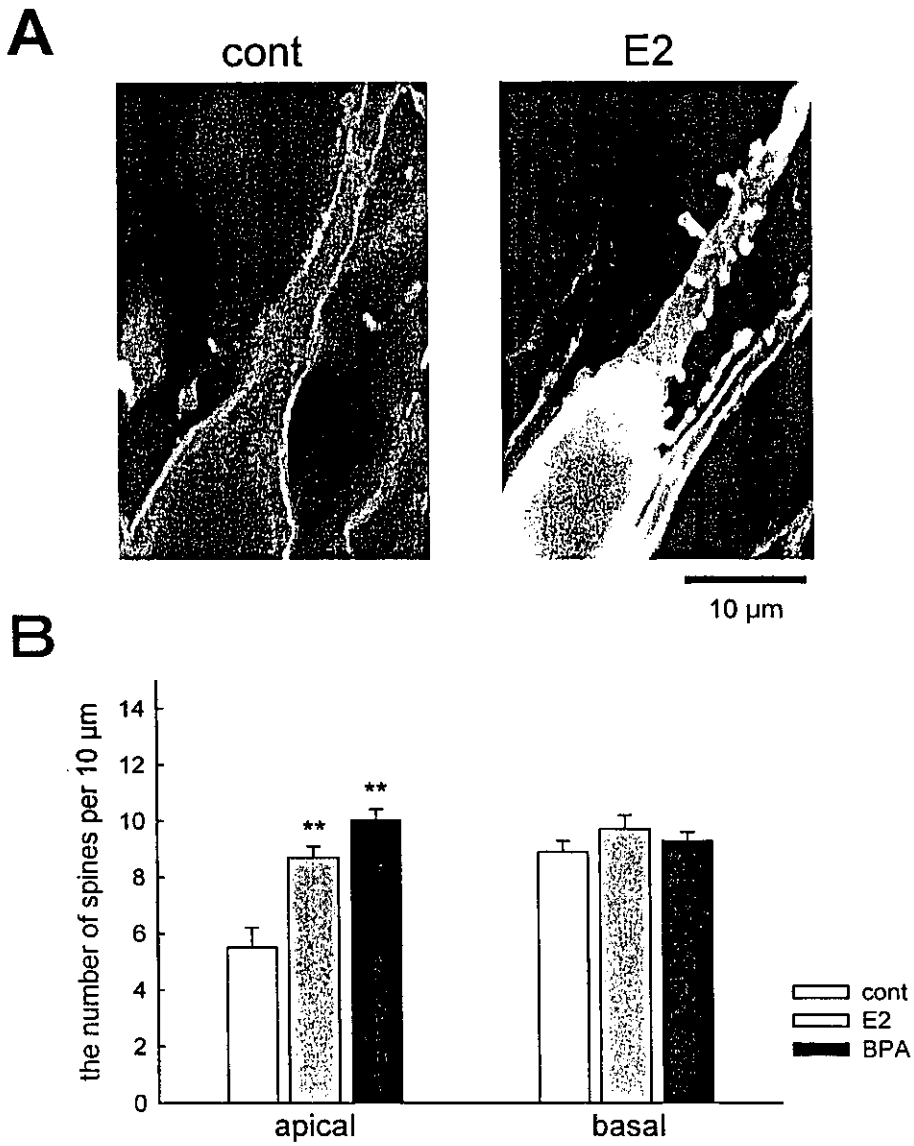
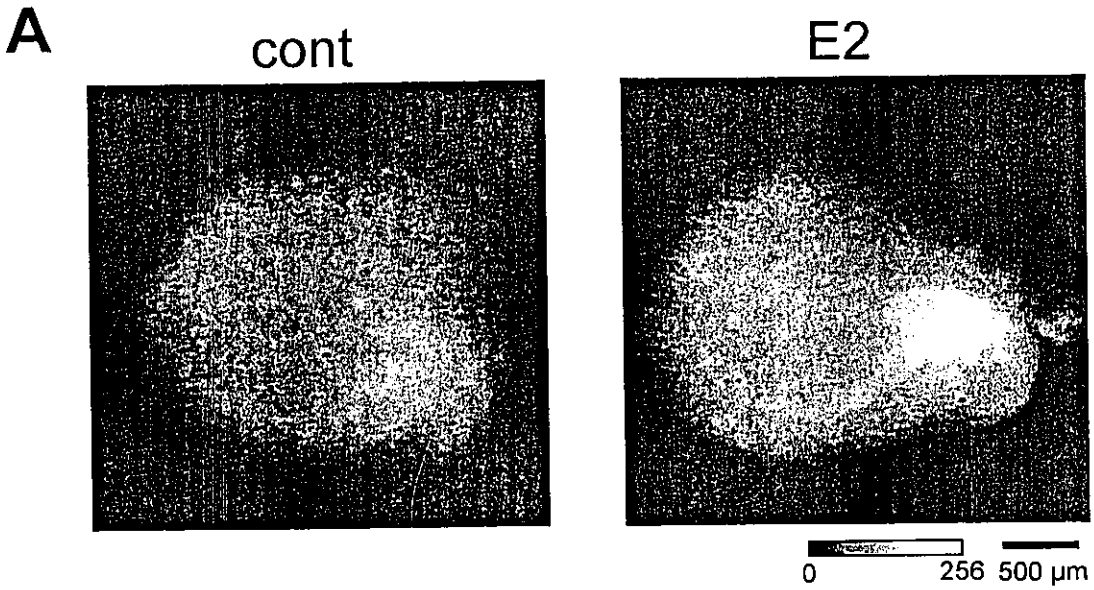

A
cont
E2
10 μm
B
the number of spines per 10 μm
0
2
4
6
8
10
12
14
**
**
apical
basal
cont
E2
BPA

Fig.5


A
cont
E2
0
256 500 μm

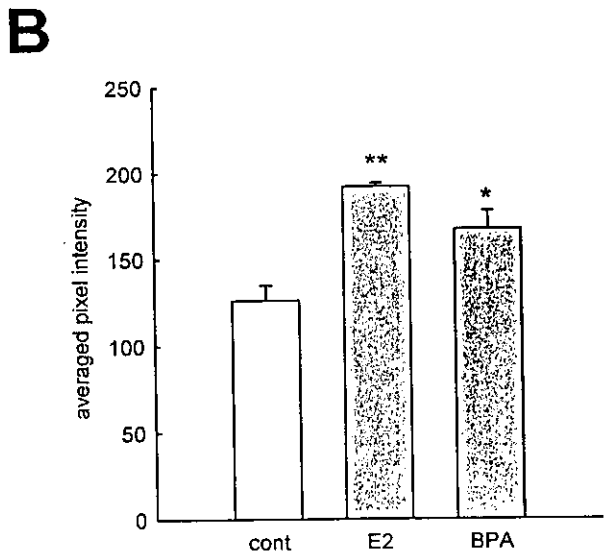

B
averaged pixel intensity
250
200
150
100
50
0
**
*
cont
E2
BPA

Fig. 6

厚生科学研究費補助金（生活安全総合研究事業）
分担研究報告書

分子生物学的手法によるヒト型エストロゲン受容体異種細胞発現系に関する研究

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各種エストロゲン様化学物質のヒトに対する影響を評価する目的で，昨年度までに確立したヒト型受容体を分子生物学的手法により異種細胞に発現させた系を用いて検討を行なった．エストロゲン様化学物質の標的であるヒト型ニコチン様アセチルコリン受容体をアフリカツメガエル卵母細胞を発現させ，エストロゲン様物質の作用の特性を検討した．その結果，作用は受容体を構成するサブユニットに依存し，抑制作用が主作用であるが抑制／増強の両者が現われる場合もあること，その作用はアセチルコリンの結合に対する競合的なものではないこと等が判明した．また，ヒト型 ATP 受容体で同様の試験を行ない，この受容体もエストロゲン様化学物質の標的となるが，作用はアセチルコリン受容体に比べて顕著ではないことが判明した．

A. 研究目的

各種エストロゲン様化学物質のヒトへの影響を評価する系の開発を目的とし，ヒト型受容体を分子生物学的手法により異種細胞に発現させ研究する方法について検討を加える．3年計画の 3 年度である本年度は，昨年度までにエストロゲン様化学物質の標的となることをアフリカツメガエル卵母細胞発現系で示したヒト型ニコチン様アセチルコリン受容体に対する作用の特性を解明し，さらにアセチルコリン受容体とは構造が大きく異なり，かつ相互作用する可能性があることが指摘されているヒト型 ATP 受容体に対する作用を比較検討することを目的とした．

B. 研究方法

ヒト型受容体を発現させる細胞としては，大きく，扱いやすく，安価であるアフリカツメガエル卵母細胞を用いた．ヒト型受容体 cDNA は哺乳動物発現型プラスミドに組み込み，大腸菌を用いて増幅させた．これを鋳型として RNA を in vitro 転写によ

り合成した．ヒト型受容体としては古典的な神経伝達物質であるアセチルコリンのニコチン様受容体，および近年新たに神経伝達物質であることが広く認知された細胞外 ATP の P2X7 受容体を用いた．アフリカツメガエルより卵母細胞を摘出し，コラゲナーゼ処理によりろ胞細胞を除去した．第 IV，V 期の卵母細胞を実体顕微鏡下で選別し，これに RNA を注入した．18℃で 2 - 5 日間の培養後，電気生理学的手法により受容体に対する各種化合物の影響を検討した．

（倫理面への配慮）

1 回に作製する卵母細胞標本の数は通常 20 個以上であり，また，同一のアフリカツメガエルより 5 回程度の卵母細胞の摘出が可能である．よって，1 匹のアフリカツメガエルより多数（100 以上）の標本を作製でき，使用する動物の数を大幅に制限できる．動物を死亡させる際には当研究所の実験動物倫理委員会の規定に従い，与える苦痛が最小限となる方法を用いた．

C. 研究結果

ヒト型アセチルコリン受容体を α3＋β4 というサブユニットの組み合わせで発現させた場合，この受容体を介するイオン電流は 17β-エストラジオール，17α-エストラジオール，17α-エチニルエストラジオール，ジエチルスチルベストロール，ビスフェノール A，p-ノニルフェノール，p-オクチルフェノールにより抑制された．受容体のサブユニット構成を α4＋β2 に替えた場合，ジエチルスチルベストロール，ビスフェノール A，p-ノニルフェノール，p-オクチルフェノールは抑制作用を示したが，17β-エストラジオール，17α-エストラジオールは抑制を示さなかった．また，このサブユニットの組み合わせにおいて 17α-エチニルエストラジオールは低濃度で抑制，高濃度で増強という 2 相性の作用を示した．核内エストロゲン受容体の阻害薬であるタモキシフェンはこれらの作用を阻害せず，自らアセチルコリン受容体抑制作用を示した．抑制，増強のいずれの場合でもアセチルコリン受容体の濃度-作用曲線は平行移動せず最大値が変化した．サブユニットの組み合わせで作用が大きく変化した化合物についてサブユニット交換（α3＋β2，α4＋β4）の影響を調べた結果，作用を決定するのは単独のサブユニットではないことが判明した．ヒト型 ATP 受容体（P2X7）を発現させた場合，これを介するイオン電流は 17β-エストラジオールおよびビスフェノール A で抑制されたが，抑制の程度はアセチルコリン受容体で認めら

れたものに比べて顕著ではなかった.

D. 考察

ヒト型アセチルコリン受容体に対してエストロゲン様物質が影響することは，このアセチルコリン受容体が近年注目されている細胞膜エストロゲン受容体の一種であることを示唆している．エストロゲン様物質の作用はアセチルコリン受容体を構成するサブユニットに依存するが，これは単独のサブユニットに帰するものではなくサブユニットにより受容体が構成された後に現われる性質であると考えられた．作用態度は非競合的であり，アセチルコリンの結合に対して直接的に影響するものではないと推察される．また，エストロゲン様物質の結合部位は核内エストロゲン受容体と異なるものであると考えられる．ヒト型 ATP 受容体に対しては影響が認められたものの，アセチルコリン受容体に比べると顕著ではなかった．このことはエストロゲン様物質の作用はアセチルコリン受容体に対して選択的であることを示している． ATP 受容体がアセチルコリン受容体と構造が大きく異なることを考慮すると，アセチルコリン受容体と類似した構造を有する他の受容体（例えばグルタミン酸受容体，γ-アミノ酪酸受容体）に対する影響の可能性も考えられ，脳，神経系に対する広い影響も懸念される．

E. 結論

本年度の研究により，ヒト型アセチルコリン受容体に対してエストロゲン様物質が非競合的抑制あるいは増強を惹起すること，この作用は ATP 受容体に対する作用に比べ選択的であることが示された．また，これらの研究を通じてアフリカツメガエル卵母細胞がこのようなヒトに対する作用を予見するのに有用であることが示唆された.

F. 研究発表

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G. 知的所有権の取得状況

なし

(資料 1)

Short communication

Size of Side-Chain at Channel Pore Mouth Affects Ca^{2+} Block of $P2X_2$ Receptor

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Abstract

Effects of amino acid replacement at the channel pore mouth of $P2X_2$ receptor/channel on multivalent cation channel block were investigated. When Asn^{333} was replaced with various amino acid residues with neutral side chains (Gly, Ala, Val, Leu and Ile), the block by Ca^{2+} was attenuated according to the sizes of the side chains. The block by La^{3+} was also greatest with the Gly-substituted mutant, but this preference was not found for the block by other multivalent cations tested. The side chain at the channel pore mouth may interfere with the access of Ca^{2+} block by steric hindrance.

Keywords:

$P2X_2$ receptor

ion channel

Ca^{2+}

multivalent cation block

site-directed mutagenesis

Xenopus oocytes

1. Introduction

P2X receptors are ion channel-forming proteins which are activated by extracellular ATP, and its roles in excitatory neurotransmission have been demonstrated in various tissues (Burnstock, 1997; Khah, 2001). To date, at least seven subclasses ($P2X_{1-7}$) have been cloned, and they have been shown to form homo- or heteromeric receptors which act as functional ion channels (North and Surprenant, 2000). The analysis of the hydropathy profiles of amino acid sequences of P2X receptors has shown that each subclass consists of two transmembrane domains (TM1 and TM2) and one long extracellular domain between them (E1). A line of experimental evidence supports the contribution of TM2 to the formation of the channel pore (Rassendren et al., 1997; Egan et al., 1998; Migita et al., 2001), and recent findings have also suggested the contribution of TM1 to the pore formation (Jiang et al., 2001; Haines et al., 2001). An aspargine residue at the position 333 in TM2 of $P2X_2$ receptor (Asn^{333}) is believed to exist near the outer mouth of the channel pore, and serve as a key residue which determines single channel conductance (Nakazawa et al., 1998a) and prevents the dilation of the channel pore upon long-lasting receptor activation (Virginio et al., 1999). Ca^{2+} and other divalent cations (Nakazawa and Hess, 1993; Ding and Sachs, 1999, 2000; Negulyaev and Markwardt, 2000) and trivalent cations (Nakazawa et al., 1997) are known to inhibit ionic current permeating through P2X receptor/channels. In the present study, we replaced Asn^{333} of $P2X_2$ receptor/channel with various amino acids, and investigated the block by Ca^{2+} and other multivalent cations of these mutant channels to elucidate the interaction between these ions and the channel pore mouth.

2. Materials and methods

Mutants of $P2X_2$ receptor constructed from the cloned rat $P2X_2$ receptor (Brake et al., 1994) were kindly supplied by Prof. R. A. North, except for N333I, N333V, N333L and N333I, which were constructed by site-directed mutagenesis in our laboratory as described (Nakazawa et al., 1998b). Channels were expressed in *Xenopus* oocytes and ionic currents permeating through them were measured as previously described (Nakazawa and Ohno, 1996; Nakazawa et al., 1998b). Oocytes were bathed in ND96 solution containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.5 with NaOH). ATP (adenosine 5'-triphosphate disodium salt; Sigma, St. Louis, MO, U.S.A.) was applied by superfusion for about 6 s with a regular interval of 1 min. All the divalent and trivalent cations used were chloride salts of reagent grade. The trivalents cations and Mn^{2+} were dissolved in standard ND96 solution. When block by Ca^{2+} or Mg^{2+} was assessed, these cations were dissolved in Ca^{2+}-free, Mg^{2+}-free ND96 solution. The current amplitude in the presence of trivalent cations and Mn^{2+} was normalized to that in the absence of these cations. Under divalent cation-free condition, *Xenopus* oocytes become electrically too leaky to record current responses to ATP because of the opening of divalent cation-sensitive non-selective cation channels (Arellano et al., 1995; Zhang et al., 1998). Thus, for the current block by Ca^{2+} or Mg^{2+}, the current amplitude was normalized to that in the presence of 0.18 mM Ca^{2+} or Mg^{2+}, respectively.

3. Results

By increasing extracellular Ca^{2+}, ionic current activated by 30 μM ATP was decreased (Fig. 1A). Figure 1B compares the block by Ca^{2+} of ionic current through $P2X_2$ receptor/channel mutants that possess amino acid residues with neutral side chains at position 333 (N333G, N333A, N333V, N333L and N333I). Among these neutral mutants, N333G was the most sensitive to Ca^{2+}, and the sensitivity was lowered almost completely according to the size of the side chains (Gly > Ala ≅ Val > Leu > Ile). The block by La^{3+} was also greatest with N333G; the remaining neutral mutants uniformly exhibited lower sensitivities (Fig. 1D).

Figure 1C compares the block by Ca^{2+} of the channels that possess amino acid residues with negatively polarized (WT and N333Q) or charged (N333D and N333E) residues at the position 333. With introducing aspartic acid at the position 333, the block by Ca^{2+} was enhanced, suggesting that a negative charge at this position increases Ca^{2+} sensitivity. However, such enhancement was not observed with the introduction of glutamic acid. As for the block by La^{3+}, the block was not augmented by the introduction of glutamic acid (Fig. 1E). The effect of La^{3+} on N333D channel was not examined because the ATP-evoked current permeating through this channel became too small to analyze the blocking effect quantitatively in the presence of 1.8 mM Ca^{2+}, as seen in Fig. 1C.

Tests were made to determine the size-dependence found for the Ca^{2+} block was also found for the block by other divalent cations. Figure 2A shows the block by Mg^{2+} of the neutral mutants of $P2X_2$ receptor/channel. When the magnitude of the block by 1 mM Mg^{2+} was compared, the size-dependence was found for Ala, Val, Leu and Ile, but not for Gly; the order was Ala > Val > Leu > Gly > Ile. On the other hand, no size-dependence

was found for the block by Mn^{2+} of these neutral mutants (Fig. 2B).

The effects of various trivalent cations were compared between N333G and N333L to determine whether or not selective block of N333G channel similar to that observed with La^{3+} was found. Among four trivalent cations tested (Al^{3+}, Ce^{3+}, Gd^{3+} and Nd^{3+}), none of them preferentially blocked N333G channel; the cations rather preferentially blocked N333L channel (Fig. 2C).

4. Discussion

By comparing the effects on the mutants possessing neutral amino acid residues at the position 333, we have demonstrated that the size of amino acid residues at this position affects the block by Ca^{2+} of $P2X_2$ receptor/channel. $P2X_2$ receptor/channel is permeable to both Na^+ and Ca^{2+}, but Ca^{2+} provides much smaller conductance than Na^+ does (Nakazawa and Hess, 1993). Ca^{2+} reduces net ionic current through $P2X_2$ receptor/channel by its competition with Na^+ at the channel pore. The block observed in the present study may mainly reflect this competitive inhibition. Thus, the size-dependence of the Ca^{2+} block indicates that larger amino acids at the channel pore mouth interferes with the access of Ca^{2+}. For the mutants with a negative charge at the position 333, N333D, but not N333E, exhibited higher sensitivity to Ca^{2+} than the wild type channel. This difference may due to a smaller size of aspartic acid residues than glutamic acid residues.

The selective block of the glycine-substituted mutant was also found for La^{3+}, but not for other multivalent cations tested, suggesting that the size-dependence is not uniform among cation species. One possible explanation for such diversity is the sizes of cations (or those of their hydrated forms). For example, the Shannon and Prett's ionic radius of Ca^{2+} is 1.14 Å at coordination number of 6, and this is larger than that of Mg^{2+} (0.86 Å) or Mn^{2+} (0.81 Å) (Cotton et al., 1995). Similarly, the ionic radius of La^{3+} of 1.06 Å is larger than those of other trivalent cations tested in the present study (Al^{3+}, 0.68; Ce^{3+}, 1.03; Gd^{3+}, 0.94; Nd^{3+}, 0.99; in Å). Large multivalent cations may be more readily affected by steric hindrance at the position 333.

In addition to size, negative polarity or charge at the position 333 is also a determinant of the magnitude of the Ca^{2+} block because, when comparing among amino acid residues of similar sizes (Val, Asn and Asp; Chothia, 1975), the sensitivity was

increased according to negativity (Val < Asn < Asp; Fig. 1B and C). The sensitivity order of Val < Asn was also found for the block by Mg^{2+}, Mn^{2+} (not shown) and La^{3+} (Fig. 1D and E), and thus, negative polarity at this position may attract multivalent cations regardless of cation species.

The present findings of the roles of the amino acid residue at the position 333 for multivalent cation block may further supports the importance of this position as the entrance of the channel pore, and may provide useful information about the relationship between the channel structure and functions including ion selectivity.

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Figure legends

Fig. 1.

A. Ionic currrent activated by 30 μM ATP in a *Xenopus* oocyte expressing the wild type $P2X_2$ receptor/channel in the presence of 0.18 (left) or 5.4 mM Ca^{2+} (right). The oocyte was held at –50 mV and stepped for 400 ms to –80 mV every 2 s.

B-E. Concentration-response curve for Ca^{2+} (B, C) and La^{3+} (D, E) block on channels with neutral (B, D) or negatively polarized and charged (C, E) amino acid residues at the position 333. Current was measured as in A, and responses at -80 mV were normalized to those in the presence of 0.18 mM Ca^{2+} or in the absence of the La^{3+} (see Materials and methods). Each symbol and bar represent the mean and S.E. obtained from 4 to 6 oocytes tested.

Fig. 2. Block by various multivalent cations of N333 neutral mutants. Current was measured as in Fig. 1A. Each symbol and bar represent the mean and S.E. obtained from 4 to 5 oocytes tested.

A, B. Concentration-response curve for Mg^{2+} (A) and Mn^{2+} block (B) on channels with neutral amino acid residues at the position 333. Current responses at -80 mV were normalized to those in the presence of 0.18 mM Mg^{2+} (A) or in the absence of Mn^{2+} (B).

C. Effects of Al^{3+}, Ce^{3+}, Gd^{3+} and Nd^{3+} on N333G (filled symbols) and N333L (open symbols) mutant channels. Current responses at -80 mV were normalized to those in the absence of trivalent cations.

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Fig. 1

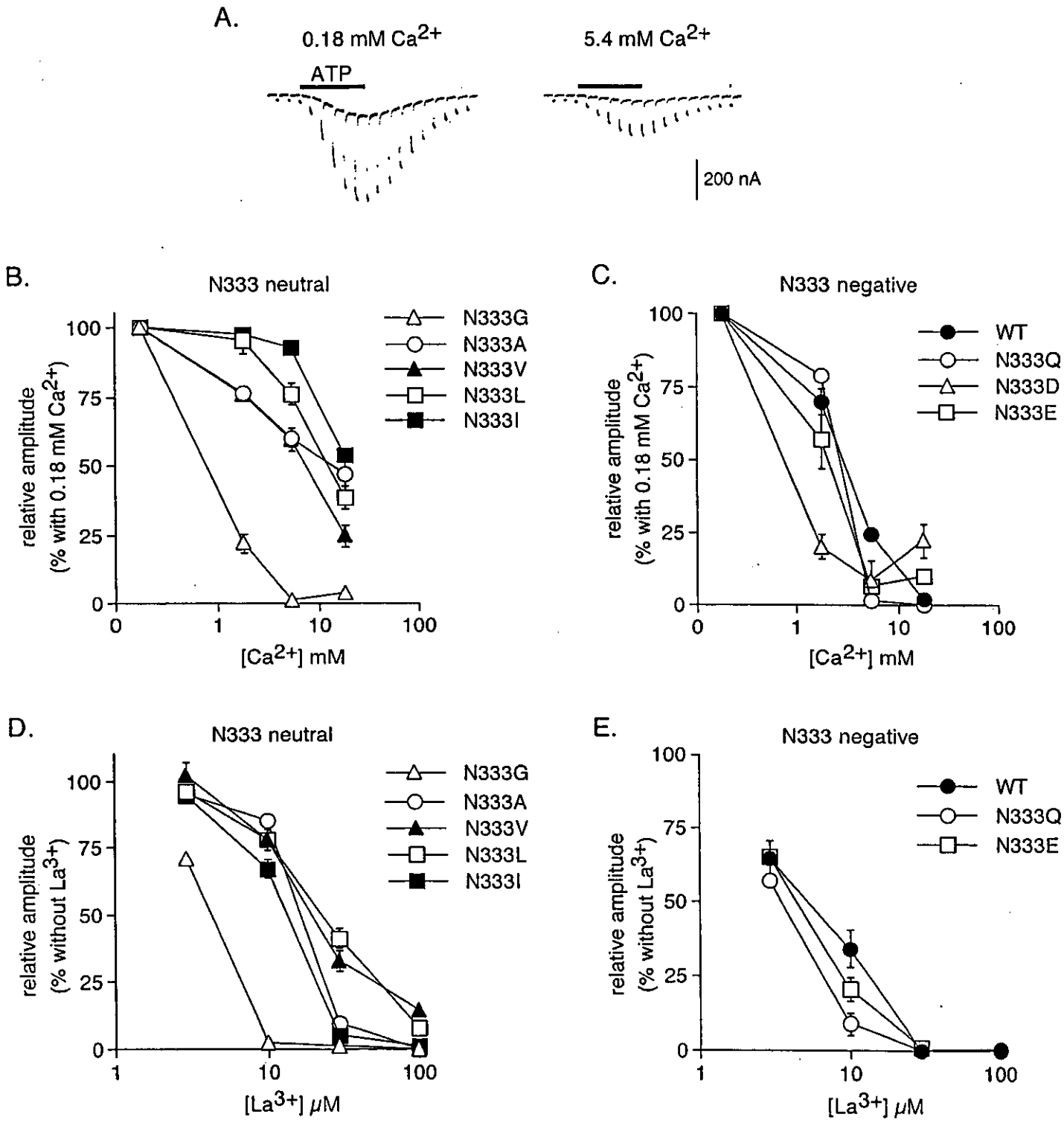

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Fig. 2