Modulation of the Neuronal Nicotinic Acetylcholine Receptor-Channel by the Nitromethylene Heterocycle Imidacloprid

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ABSTRACT

Nitromethylene heterocycle insecticides are known to act on the nicotinic acetylcholine receptor-channel. The effects of the nitromethylene heterocycle, imidacloprid, on the nicotinic acetylcholine receptor-channel of clonal rat phaeochromocytoma (PC12) cells were studied using whole-cell and single-channel patch clamp methods. Imidacloprid suppressed carbachol-induced whole-cell currents in a dose-dependent manner, and this compound itself generated small currents. Multiple conductance states of single-channel currents were also evoked by imidacloprid at the nicotinic acetylcholine receptor-channels. The most frequently generated single-channel currents showed two conductance states, 25.4 and 9.8 pS, which were identical to the conductance states of acetylcholine-generated currents. The mean open time and burst duration of the main conductance currents induced by imidacloprid were shorter than those induced by acetylcholine. Co-application of imidacloprid and acetylcholine caused some interactions at the two conductance states. Mean open time and mean burst duration of the main conductance state currents evoked by acetylcholine were decreased by the co-application of imidacloprid as compared with those induced by acetylcholine alone. In conclusion, imidacloprid has both multiple agonist and antagonist effects on the neuronal nicotinic acetylcholine receptor-channels.

The nicotinic AChR is known to be the target of various chemicals including nicotine, carbachol, d-tubocurarine, general anesthetics and several natural toxins (Benson, 1992; Leech et al., 1991; Sattelle et al., 1989; Benson, 1992; Zwart et al., 1992, 1994). Thus, a question arises as to whether these conflicting observations result from multiple effects of nitromethylene heterocycles at the channel level, or from different subtypes of nicotinic AChR of preparations.

Recently, we have demonstrated that imidacloprid potentially induced subconductance state single-channel currents mediated by nicotinic AChR channels (Nagata and Narahashi, 1995; Nagata et al., 1996a). We now report the results of whole-cell and single-channel patch clamp experiments that have unveiled more detailed mechanisms of imidacloprid action on neuronal nicotinic AChR in PC12 cells. Imidacloprid suppressed the carbachol-induced whole-cell currents and generated currents at nicotinic AChR. Contrary to the main conductance state currents induced by ACh, imidacloprid almost exclusively induced the subconductance state currents. When imidacloprid was co-applied with ACh, the mean open time and burst duration of main conductance state currents were decreased compared with the control in which ACh was applied alone. These results help to explain the multiple effects of imidacloprid on ACh-induced whole-cell currents and some of the results reported previously.

Materials and Methods

Culture of PC12 cell line. The PC12 cell line was kindly provided by Drs. Edson X. Albuquerque and Edna F. R. Pereira of the

ABBREVIATIONS: ACh, acetylcholine; AChR, acetylcholine receptor.

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University of Maryland School of Medicine, Baltimore, MD. Cells were cultured in Dulbecco’s modified Eagles’ medium containing fetal bovine serum (0.1 mg/ml, Sigma Chemical Co., St. Louis, MO) at 36°C in an air + CO₂ (90 + 10%, by volume). For patch clamp experiments, cells were plated on glass cover-slips coated with poly-l-lysine and cultured for 2 to 7 days. PC12 cells without nerve growth factor treatment expressed the nicotinic AChR.

Whole-cell current recording. Membrane currents were recorded using the whole-cell patch clamp technique (Hamill et al., 1981) at room temperature (22°C). Pipette electrodes were made from 0.8 mm (I.D.) borosilicate glass capillary tubes and fire-polished before use. The electrodes had resistances of 2 to 3 MΩ when filled with standard pipette solution. The membrane potential was clamped at -90 mV, and a 5- to 10-min period was allowed after rupture of the membrane to equilibrate the cell interior with pipette solution. Currents through the electrode were recorded by an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered at 10 kHz, and stored on an LSI 11/73 computer (Digital Equipment, Pittsburgh, PA). The data were transferred to a microcomputer (PowerBook 520c, Apple Computer, Cupertino, CA) for further analysis. Currents were continuously monitored by a chart recorder.

Cell-attached single-channel current recording. Single-channel currents were recorded using the cell-attached variation of the patch clamp technique (Hamill et al., 1981) at room temperature (22°C). Pipette electrodes were made by the same method as described above, coated by SigmaCote (Sigma) to minimize the background noise, and fire-polished. The electrodes had resistances of 10 to 12 MΩ when filled with standard pipette solution. The membrane was hyperpolarized to various potentials from the resting potential. Currents through the electrode were recorded using an Axopatch 200A amplifier filtered at 3 kHz, and stored at 88 kHz on a video cassette recorder via an analog-to-digital converter (VR10B, Instrument Corp. Elmont, NY). Current records were analyzed by the pClamp version 6.0 software (Axon Instruments). Only those events greater than 200 μsec of data were considered as accurate in the analysis. Opening and closing of the channels were detected using the 50% threshold criterion (Colquhoun and Sigworth, 1995). Amplitude histograms were fitted by a sum of Gaussian functions using the least-square methods. For the analysis of burst duration, each conductance level was manually chosen and analyzed separately. The interburst interval was determined by the methods of Colquhoun and Sakmann (1985).

Data are expressed as the mean ± S.D. and n represents the number of experiments. For the single-channel data, n represents the number of events which were used for estimating the values including the means of amplitude, open time, closed time and burst duration.

Solutions. The external bath solution for both whole-cell and cell-attached patch clamp experiments contained (in mM): NaCl 165, KCl 5, CaCl₂ 2 and N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid 5. Tetrodotoxin (1 μM) was also added to eliminate sodium channel currents. The pH was adjusted to 7.3 with NaOH, and the osmolarity was adjusted to 330 mOsm by d-glucose. The internal pipette solution for the whole-cell recording contained (in mM): CsCl 80, CsF 80, ethylene glycol bis(β-aminoethylether)-N, N’,N’-,N-tetraacetic acid 10, and N-2-hydroxyethylpiperazine-N’ acid 10. The pH was adjusted to 7.3 with CsOH, and the osmolarity was adjusted to 330 mOsm by d-glucose.

Chemicals. Acetylcholine and carbachol were first dissolved in distilled water to make stock solutions. Imidacloprid was dissolved in dimethylsulfoxide. These stock solutions were then diluted with the internal pipette solution for the cell-attached and with the standard external solution for the whole-cell patch clamp experiments. The final concentrations of dimethylsulfoxide in test solutions were .3% (v/v) or less which had no effect on the activity of ACh- and carbachol-induced currents.

Drug application. For whole-cell experiments, test solutions were applied to the cell using a locally developed application system (Nagata and Narahashi, 1994). The application was controlled by a computer-operated magnetic valve. Using this application system, the external solution surrounding the cell could be completely changed within 100 msec.

Results

Effects of imidacloprid on carbachol-induced currents. Imidacloprid suppressed currents evoked by carbachol. When carbachol was applied for 25 sec at a concentration of 100 μM, a transient inward current was generated and decayed to a very low level (fig. 1Aa). This concentration was somewhat less than the ED₅₀ value (fig. 2), and was chosen to be able to observe the effect of a test compound clearly. Imidacloprid (30 μM) coapplied with 100 μM carbachol suppressed the peak current amplitude (fig. 1Ab) to 70% of the control, and the effect was completely reversible after washing with imidacloprid-free solution. Further experiments showed that imidacloprid suppressed the ACh-induced current in a dose-dependent manner (fig. 1B).

Imidacloprid-generated whole-cell currents. Imidacloprid generated currents when applied alone (fig. 1Ac). At a concentration of 30 μM, imidacloprid induced a small transient inward current that reached approximately 10% of the

![Fig. 1. Effects of imidacloprid on the nicotinic ACh receptor in PC12 cells.](image)
current produced by 100 μM carbachol. The peak amplitude of currents generated by 30 μM imidacloprid was 51.9 ± 8.4 pA (n = 5, mean ± S.D.). The dose-response relationship of imidacloprid-generated current is shown in figure 2B. The minimum effective concentration to induce currents was 1 μM and the currents reached maximum at 30 μM.

**Single-channel currents induced by acetylcholine.** Single-channel currents were recorded by the cell-attached patch clamp technique with the recording electrode containing 10 μM ACh (fig. 3A). The membrane was hyperpolarized by 40 mV from the resting potential which was close to -90 mV. The inward single-channel currents were generated by activation of the nicotinic AChR by ACh (Nagata et al., 1996a). In addition to currents that represented the main conductance state, there were small currents of a subconductance channel (fig. 3A). The two most frequently observed multiple conductance states. The two most frequently observed multiple conductance states were 2.81 ± 0.47 pA (n = 6) and 0.88 ± 0.16 pA (n = 6). Imidacloprid-induced single-channel currents were blocked by 30 μM d-tubocurarine indicating that they were the result of activation of the nicotinic AChR (Nagata et al., 1996a). Imidacloprid first induced currents at a high frequency and with relatively short closings within a long opening forming a burst. This pattern repeated as a cluster of bursts for several tens of seconds followed by quiescence for a few minutes before starting new cluster of bursts. The current-voltage relationships of 10 μM imidacloprid-induced currents are plotted in figure 4B. Two conductances were estimated to be 25.4 and 9.8 pS, and are virtually identical to the main conductance and subconductance of ACh-induced currents (fig. 4A). Thus, imidacloprid opens the main conductance and subconductance channels which are the same as those opened by ACh in addition to at least two other conductance levels which were not analyzed.

**Comparison of single-channel current parameters evoked by ACh and Imidacloprid.** The open time distri-
butions for ACh- and imidacloprid-induced main conductance currents are shown in figure 5A and B, respectively. The time axis is drawn on a logarithmic scale so that the effective bin width increases exponentially from left to right. This displays a multiexponential distribution as a series of skewed bells whose peaks overlie the time constants of several exponential components (Sigworth and Sine, 1987).

The open time distribution for 10 μM ACh- and 10 μM imidacloprid-induced main conductance currents clearly indicated multiexponential components (fig. 5A and B). There were at least three components. The slowest component of the open time distribution for 10 μM ACh-induced currents had a time constant of 42.3 msec (21% of total 221 events, three separate patches combined), the next component had a time constant of 9.9 msec (56%), and the fastest component had a time constant of 0.7 msec (23%) (fig. 5A). For the open time distribution of the imidacloprid-induced main conductance state currents, there were at least four components. The slowest component had a time constant of 11.5 msec (12.2% of total 625 events, three separate patches combined), the next component had a time constant of 2.7 msec (52.7%), the next faster component had a time constant of 0.7 msec (35.1%) and the fastest component had a time constant of 0.7 msec (35.1%) (fig. 5B). When the time constants of three components were compared between ACh-induced main conductance currents and imidacloprid-induced currents, there is no significant difference in the slope conductances between ACh-induced and imidacloprid-induced currents for each conductance state.

The closed time distribution for 10 μM ACh- and 10 μM imidacloprid-induced main conductance state currents shown in figure 6A and B, respectively. The time axis is drawn on a logarithmic scale so that the effective bin width increases exponentially from left to right. This displays a multiexponential distribution as a series of skewed bells whose peaks overlie the time constants of several exponential components (Sigworth and Sine, 1987).

The open time distribution for 10 μM ACh- and 10 μM imidacloprid-induced main conductance currents clearly indicated multiexponential components (fig. 6A and B). There were at least three components. The slowest component of the open time distribution for 10 μM ACh-induced currents had a time constant of 42.3 msec (21% of total 221 events, three separate patches combined), the next component had a time constant of 9.9 msec (56%), and the fastest component had a time constant of 0.7 msec (23%) (fig. 5A). For the open time distribution of the imidacloprid-induced main conductance state currents, there were at least four components. The slowest component had a time constant of 11.5 msec (12.2% of total 625 events, three separate patches combined), the next component had a time constant of 2.7 msec (52.7%) and the fastest component had a time constant of 0.7 msec (35.1%) (fig. 5B). When the time constants of three components were compared between ACh-induced main conductance currents and imidacloprid-induced currents, there were some differ-
ences in the distribution pattern. The slow component (5050 msec) for imidacloprid-induced currents was not found in ACh-induced currents. The component of 295 msec time constant in ACh-induced current was faster than the 440 msec component of imidacloprid-induced current. The 22.5 msec time constant component of ACh-induced currents did not exist in imidacloprid-induced currents.

For the imidacloprid-induced main conductance state currents, the slowest component had a time constant of 12.2 msec (13.7% of total 173 events, combined three separate patches combined), the next component had a time constant of 3.8 msec (57.9%) and the fastest component had a time constant of 0.9 msec (2.8%) (fig. 7B). There were some differences in the distribution pattern between 10 μM ACh-induced main conductance currents with those for 10 μM imidacloprid-induced currents. The two slower components of burst duration for imidacloprid-induced currents were smaller than those for ACh-induced currents.

To examine the characteristics of imidacloprid-induced subconductance state currents, we chose the data which show only subconductance state currents. We could not analyze the ACh-induced subconductance state currents because of their low frequency. The open time distribution for 10 μM
imidacloprid-induced subconductance state currents clearly indicated multieponential components (fig. 8A). There were at least three components. The slowest component of the open time distribution had a time constant of 29.7 msec (38.9% of 636 total observations), the next component had a time constant of 3.0 msec (56%) and the fastest component had a time constant of 0.6 msec (23%). The closed time distribution for 10 μM imidacloprid-induced subconductance state currents clearly indicated three components (fig. 8B). The slowest component had a time constant of 0.5 msec (25.7%) and the fastest component had a time constant of 0.1 msec (51.5% of total observations), the next component had a time constant of 1.1 msec (25.7%) and the fastest component had a time constant of 0.5 msec (49.3%). The distribution of burst duration indicated three components with time constants of 136 msec (43.5% of total 192 events), 2.6 msec (17.3%) and 0.5 msec (39.1%) (fig. 8C).

**Fig. 8.** Distributions of open time (A), closed time (B) and burst duration (C) for subconductance state currents induced by 10 μM imidacloprid to cell-attached membrane patches clamped at a membrane potential 40 mV more positive than the resting potential. The distributions are shown on a logarithmic time axis. The best fit of exponential functions is shown. A, Time constants were estimated to be 0.6 msec (51.5% of total observations), 3.0 msec (9.5%) and 29.7 msec (38.9%). B, Time constants were estimated to be 0.5 msec (49.3%), 1.1 msec (25.7%) and 406 msec (25%). C, Time constants were estimated to be 0.5 msec (39.1%), 2.6 msec (17.3%) and 136 msec (43.5%).

**Coapplication of acetylcholine and imidacloprid.** Coapplication of 10 μM ACh and 10 μM imidacloprid opened the channels exhibiting both the main conductance state and subconductance state (fig. 3C). The current amplitudes were almost the same as those induced by 10 μM ACh or 10 μM imidacloprid, being 2.92 ± 0.36 pA (n = 6, mean ± S.D.) and 1.11 ± 0.43 pA (n = 6) for the main conductance and subconductance states, respectively. The time constants of open time distribution of the main conductance state currents were, 0.9 msec (28.5%), 4.2 msec (37.8%) and 12.1 msec (33.7%) (fig. 5C). The slowest value was closer to that of imidacloprid than ACh. The two faster values were between the values of ACh and imidacloprid.

The time constants of closed time distribution of the main conductance state currents were, 0.7 msec (0.1%), 3.4 msec (5.4%), 273 msec (83.5%) and 1950 msec (10.9%) (fig. 6C). The two faster components were closer to those of imidacloprid than ACh, the second slowest was closer to the slowest component of ACh and the slowest component was reminiscent of that in imidacloprid.

The time constants of burst distribution of the main conductance state currents with coapplication of 10 μM ACh and 10 μM imidacloprid were 0.8 msec (26.5%), 3.0 msec (27.9%) and 12.5 msec (45.5%) (fig. 7C). The distribution was similar to imidacloprid-induced currents. Overall, the open time and burst duration, but not the closed time distribution, for the main conductance state currents with coapplication of ACh and imidacloprid are similar to those of imidacloprid-induced current.

**Discussion**

**Effects of imidacloprid on whole-cell currents of ACh receptors.** Imidacloprid suppressed the whole-cell currents induced by carbachol and generated small whole-cell currents by itself (figs. 1 and 2). Both types of currents were blocked by d-tubocurarine indicating that they were generated at the nicotinic AChR. The inhibitory effects of nitromethylene heterocyclic compounds on the nicotinic AChR were reported by several investigators using different preparations (Bai et al., 1991; Benson, 1989; Cheung et al., 1992; Zwart et al., 1994). Our results are consistent with the previous reports using insect species and are the first with the neuronal AChR of mammals. Most previous reports failed to show the agonistic effect of nitromethylene heterocyclic on the mammalian nicotinic AChRs (Solowey et al., 1978; Liu and Casida, 1993; Zwart et al., 1992, 1994). Zwart et al. (1994) have shown that imidacloprid induced currents in locust thoracic ganglion neurons, but not in N1E-115 and BC3H1 neuroblastoma cells. Because the maximum current induced by imidacloprid in PC12 cells was much smaller than that induced by carbachol, imidacloprid can be considered as a partial agonist.

It is well known that the nicotinic AChRs from various preparations show diverse characteristics including different sensitivities to drugs possibly due to different subunit combinations (Castro and Albuquerque, 1993; Alkondon et al., 1994; Filatov et al., 1993; Garcia-Colunga and Miledi, 1995; Muller et al., 1991; Meeker et al., 1986; Cachelin and Jaggi, 1991; Cachelin and Rust, 1995). The different actions of imidacloprid on different cells may be due to its selectivity on different subunit combinations.
Blocking effect of imidacloprid on carbachol-induced currents. When imidacloprid was co-applied with carbachol, whole-cell currents were suppressed in a dose-dependent manner (figs. 1 and 2). Zwart et al. (1994) proposed that the mechanism of suppression by nitromethylene heterocyclic compounds is due to the acceleration of desensitization of nicotinic AChR. However, channel openings occurring as bursts, which are characteristic of desensitization of channels (Colquhoun and Ogden, 1988; Nagata et al., 1996b), were not observed in the present co-application study (fig. 3C). Thus, it is uncertain whether imidacloprid suppression of carbachol-induced currents is due to receptor desensitization.

Imidacloprid-induced single-channel currents. As we have reported previously (Nagata et al., 1996a), imidacloprid generated single-channel currents of multi-conductance states in PC12 cells. The main conductance and subconductance of currents induced by imidacloprid were identical to those induced by ACh. Cheung et al. (1992) have reported that one of the nitromethylene heterocyclic compounds generated currents of two conductance states in cultured brain cells isolated from American cockroaches. However, they did not perform detailed analysis of the two conductance states. Therefore, it is not clear whether the two conductance states they observed in insects are the same as those observed in PC12 cells.

Imidacloprid induced the subconductance state currents more frequently than the main conductance state currents (Nagata and Narahashi, 1995; Nagata et al., 1996a). The reason for partial suppression of whole-cell currents by imidacloprid is likely due to a shift from main conductance state current to the subconductance state current at the single-channel level. These observations provide an explanation for the small amplitude of whole-cell current induced by imidacloprid (figs. 1 and 2) and the previous observations that the nitromethylene heterocycles suppress whole-cell peak current and accelerate desensitization by nitromethylene heterocycles (Zwart et al., 1994).

Possible mechanisms of the two conductance states. There are three possible mechanisms of subconductance state generated by chemicals. First, the receptor conformation may be modified allosterically through the binding of chemicals to the agonist binding site resulting in reduced conductance (Hamill and Sakmann, 1981; Morris and Montpetit, 1986; Morris et al., 1983,1989). A second hypothesis is that the conductance of fully opened channel is reduced by chemicals through binding to a site within the channel pore thereby reducing ion permeation (Takada and Trautmann, 1984; Trautmann, 1982; Strecker and Jackson, 1989). Third, since the neuronal nicotinic ACh receptor comprises several subunits (Lindstrom, 1996; McGeehe and Role, 1995), chemicals may activate the ACh receptors with different subunits to give rise to different channel conductance levels.

It is known that imidacloprid binds to agonist binding site (Bai et al., 1991; Sattelle et al., 1989; Liu and Casida, 1993). Based on these reports, the following mechanism can be considered. The binding of imidacloprid molecule to the agonist binding site may cause changes in conformation that results in an open channel with reduced conductance as well as an open channel with full conductance. When 10 μM ACh and 10 μM imidacloprid were co-applied, the proportion of main conductance levels was decreased and that of subconductance levels was increased compared with those induced by 10 μM ACh alone. This phenomenon can be explained by assuming that the two compounds bind to the same agonist recognition site of nicotinic AChR.

An alternative explanation is as follows: there may be two different binding sites of imidacloprid, one is at the agonist binding site, and the other is the blocking site that may possibly be located at or near the channel pore. The imidacloprid molecule may bind to the agonist binding site and may compete with other agonists including ACh and nicotine. The binding of imidacloprid to the agonist binding site generates the main conductance state current. In addition, the imidacloprid molecule may bind to a second site which is, say, a partial blocking site, possibly located at the channel pore. This binding may interfere in some way with ion permeation, rendering the channel partially blocked. It was clearly observed that the imidacloprid itself induced the two conductance state currents in a dose-dependent manner, decreased the proportion of the main conductance state and increased that of the subconductance state (Nagata and Narahashi, 1995). This observation can be explained if we assume that the two binding sites may have different affinities to imidacloprid, higher at the agonist binding site and a lower affinity at the blocking site. Further single-channel analyses including more detailed dose dependence and voltage dependence of the effects of imidacloprid are warranted to test these hypotheses.

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