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Mechanism for Modulation of Nicotinic Acetylcholine Receptors That Can Influence Synaptic Transmission

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Only recently has it been appreciated that neuronal nicotinic ACh receptors (NnAChRs) are highly permeable to Ca^{2+} and are modulated by Ca^{2+} in a dose-dependent manner. These findings suggest that Ca^{2+} could have roles in cholinergic synaptic plasticity. We report a possible mechanism for Ca^{2+}-initiated synaptic plasticity that differs from the intracellular Ca^{2+} cascade associated with plasticity of glutamatergic synapses. Rapid changes in external Ca^{2+} modulate cholinergic spontaneous synaptic currents in superior cervical ganglionic sympathetic neurons. Inhibition of cholinergic currents by chlorisondamine, which blocks only open channels and becomes trapped in the pore, showed that the modulation is not by a mechanism that activates a previously unresponsive population of NnAChRs. Rather, single-channel recordings with ganglionic NnAChRs from chromaffin cells indicated that Ca^{2+} directly alters the probability of the channels being open. We hypothesize from the results that activity-dependent decreases in external Ca^{2+}, which occur throughout the nervous system, could directly underlie a rapid negative-feedback mechanism that decreases the responsiveness of NnAChRs at synapses. When external Ca^{2+} is decreased, presynaptic Ca^{2+} currents and transmitter release also are diminished. Thus, several mechanisms could combine to potently and rapidly depress synaptic nicotinic receptors until the external Ca^{2+} concentration recovers.

[Key words: synaptic modulation, calcium, calcium modulation, cholinergic, neuronal ACh receptors, nicotine]

Neuronal nicotinic ACh receptors (NnAChRs) are found throughout the CNS and PNS. It was recently shown that NnAChRs have a high Ca^{2+} permeability (Adams and Nutter, 1992; Mulle et al., 1992a; Vernino et al., 1992, 1994). Permeability ratio measurements indicated that a NnAChR subtype composed of the $\alpha 7$ subunit has an even higher Ca^{2+} permeability, comparable to the permeability of the NMDA subtype of glutamate receptors (Séguéla et al., 1993). At glutamatergic synapses, Ca^{2+} influx through NMDA receptors enhances protein kinase activity, which is an early step in the process of long-term potentiation (Malinow et al., 1988; Malenka et al., 1989; Madison et al., 1991). At highly active cholinergic synapses, Ca^{2+} influx through NnAChRs could activate similar intracellular mechanisms, but such mechanisms have not yet been observed at central cholinergic synapses.

The present study focuses on the mechanism and potential for synaptic plasticity arising from another aspect of Ca^{2+} modulation that is unique to neuronal nAChRs. External Ca^{2+} enhances NnAChR responses in a dose-dependent manner (Mulle et al., 1992a; Vernino et al., 1992). Applications of nicotinic agonist to a cholinoceptive neuron induce progressively smaller responses as external Ca^{2+} is decreased. The modulation is seen in a concentration range of Ca^{2+} from 0 to 10 mM and is strongest at the physiologic level of about 1 mM after correcting for the permeation properties of nAChRs for Ca^{2+} (Decker and Dani, 1990; Mulle et al., 1992b; Vernino et al., 1992). This modulation of NnAChRs by external Ca^{2+} can be of biological importance because high synaptic activity can produce millimolar reductions in external Ca^{2+} (Benninger et al., 1980; Pumain and Heinemann, 1985; Mody and Heinemann, 1986; Heinemann et al., 1990; Livsey et al., 1990). These activity-dependent changes in extracellular Ca^{2+} were measured using electrodes that averaged over a relatively large volume. Because the local density of current in the small cleft of an active synapse can be higher than the average current density in a large volume of neurons, the Ca^{2+} reduction within a synaptic cleft could be significantly greater than estimated using extracellular electrodes. We report here that changes in external Ca^{2+} act on NnAChRs to modify spontaneous cholinergic synaptic currents. Therefore, activity-dependent reductions in external Ca^{2+} could cause a rapid negative feedback onto cholinergic synaptic transmission. Our results indicate that Ca^{2+} does not activate a previously unresponsive population of NnAChRs. Rather, Ca^{2+} increases the likelihood that responsive NnAChRs will be open in the presence of agonist.

Materials and Methods

Cell culture. Sympathetic neurons were isolated from the superior cervical ganglia (SCG) of neonatal rats. During the first few days in culture, these neurons are adrenergic. When grown in coculture with cardiac muscle cells or in conditioned media, the neurons become cholinergic (Hawrot and Patterson, 1979). We grew cardiac cells on the bottom of culture dishes. Hearts from newborn rats were minced, dissociated with divalent-free solutions in 1 mg/ml collagenase, triturated, washed, and plated. The cardiac cells matured and covered the bottom of the dish in a few days. Then, poly-D-lysine/collagen coated cover-glass slips were placed on top of the cardiac cells. SCG neurons were plated onto the cover glass. After 7 or more days in culture, cholinergic/cholinoceptive synapses formed among the SCG neurons on the cover glass. The neurons were obtained from a pair of SCG by

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dissecting away the capsule and cutting the SCG into two pieces in Dulbecco's modified Eagle medium (DMEM). The pieces were incubated in 6 ml of 0.15% trypsin (GIBCO) for 15 min. After several trituration steps, the cells were placed into DMEM with 10% fetal bovine serum (FBS), pelleted, resuspended, and plated onto the cover glass in 10% FBS in DMEM with 25 ng/ml nerve growth factor (NGF 7S, Cambridge Res. Biochem.). After about three days, the cells were treated with 5 μM cytosine arabinofuranoside.

Ganglionic-type nAChR channels also were studied in bovine chromaffin cells that were prepared using standard techniques (Greenberg and Zinder, 1982) as we have described previously (Vernino et al., 1992). Fresh adrenal glands were collected from a slaughterhouse, washed, and perfused by 0.25% collagenase through the adrenal vein. The medulla was dissected from the cortex, minced, and treated with collagenase for a total of 90 min. Several steps of centrifugation through a fetal calf serum/Locke's solution pad (1:1) were used to purify the chromaffin cells. Then the cells were resuspended and plated in DMEM (Hazleton or GIBCO) containing 10% heat inactivated FBS (GIBCO or Hyclone), 100 U/ml of penicillin, 100 μg/ml of streptomycin (Hazleton), and 2 mM glutamine (Hazleton) in an incubator at 37°C with 5% CO_2. The cells were plated onto collagen-coated cover glass and were used 5–20 d after being plated.

Patch-clamp recordings. Whole-cell and single-channel NnAChR currents were measured in sympathetic neurons and chromaffin cells using standard patch-clamp techniques (Hamill et al., 1981) as we have described previously (Amador and Dani, 1991; Vernino et al., 1992). Patch pipettes were pulled in two stages (PP-83, Narishige USA) using glass tubes (Garner Glass Co.). To decrease capacitive noise, the pipettes were coated with Sylgard silicon elastomer. Pipette tips were polished immediately before the experiment using a microforge (Narishige USA) to a final resistance of 2–4 MΩ in our solutions. Currents were amplified and filtered (four-pole Bessel filter) using an Axoclamp patch-clamp amplifier and were collected and analyzed using AXOBASIC and PCLAMP programs (Axon Inst). The single-channel currents were sampled at 10 kHz and were filter at 2 kHz. For all the experiments with synaptically coupled SCG neurons, the holding potential was −70 mV. The spontaneous synaptic currents were judged to be cholinergic if they were blocked by the cholinergic antagonist, hexamethonium. Occasionally very small currents were seen in hexamethonium, but they were rapid and did not have the slow falling phase typical of synaptic currents.

Solutions. For all of the experiments except those conducted on synaptically coupled SCG neurons, the solutions were as follows: external, 150 mM CsCl, 20 mM HEPES, 10 mM glucose, 1 μM atropine, 1 μM TTX, and 0 Ca^{2+} + 1 mM EGTA or 1 mM $CaCl_2$ or 10 mM $CaCl_2$; internal, 140 mM $CsCH_3SO_3$, 5 mM NaCl, 20 mM HEPES, 10 mM BAPTA, with or without 4 mM ATP-Mg. To measure spontaneous synaptic currents (SSCs) from synaptically coupled SCG neurons, the solutions were the same as above except external CsCl was replaced by NaCl, and the external $CaCl_2$ concentration was usually 1, 2, or 5 mM. Since we have shown previously the Ca^{2+} modulation occurs over a range from 0 to 10 mM (Vernino et al., 1992), we chose external Ca^{2+} concentrations that were physiologically reasonable but also were conducive for long-lasting recordings, which were more difficult in Ca^{2+} concentrations below 1 or 2 mM. Also, below 1 mM Ca^{2+}, the cholinergic currents become smaller because of Ca^{2+} modulation, and therefore, measurements cannot be as precise. The pH was adjusted to 7.35 with the base of the main ion, and the osmolality was adjusted to 300 mOsm with NaCl.

Fast solution changes, to change the external Ca^{2+} solution or to apply agonist, usually were made with large outflow tubes (375 μm inner diameter glass pipettes) that were positioned in a row next to each other as we have described previously (Amador and Dani, 1991). The outflow tubes were mounted on a high speed motorized manipulator (Newport Corp.) so that they could be repositioned rapidly for solution changes that were complete in tens of milliseconds.

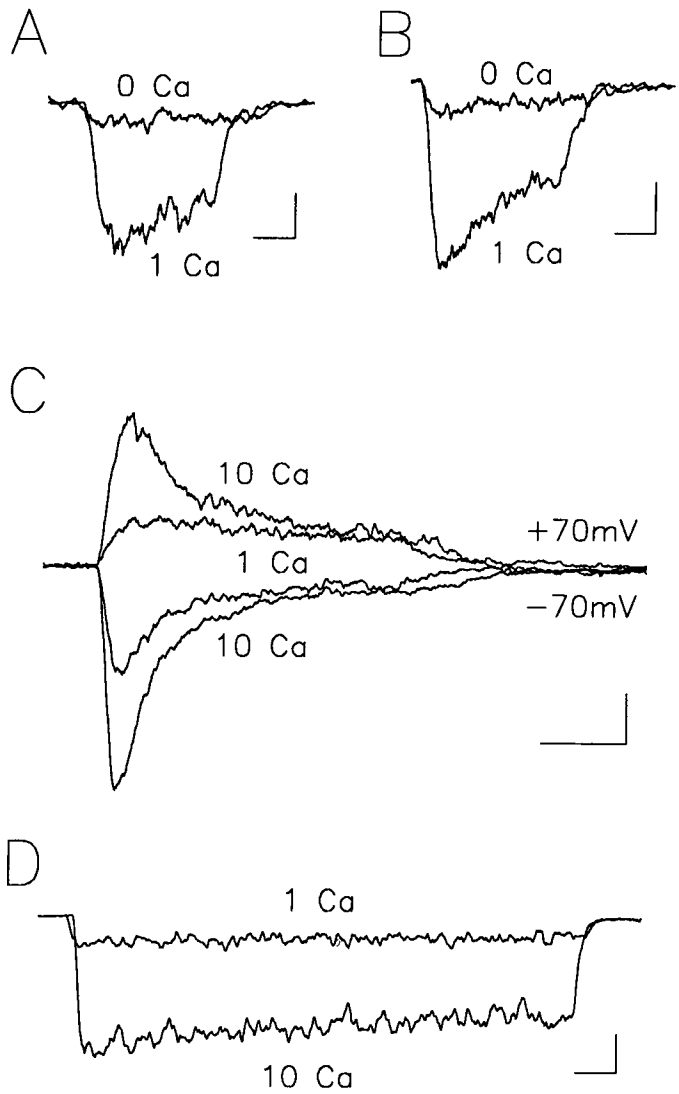

Figure 1. Physiologic concentrations of Ca^{2+} act externally to modulate NnAChRs. *A*, NnAChRs from SCG neurons were activated by whole-cell application of 56 μM DMPP, a specific nicotinic agonist. The agonist-induced current in 1 mM Ca^{2+} is 5.5 times larger than the current activated by the same agonist application in a Ca^{2+}-free external solution (containing 1 mM EGTA). The holding potential was −70 mV. Calibration: 25 pA, 250 msec. *B*, Exactly as in *A*, ganglionic NnAChRs from adrenal chromaffin cells were activated in 0 and 1 mM Ca^{2+}. The current are five times larger in the solution of 1 mM Ca^{2+}. Calibration: 50 pA, 250 msec. *C*, Four currents from an adrenal chromaffin cell activated by 32 μM DMPP are shown. The enhancement in the currents in 10 mM Ca^{2+} as compared with 1 mM Ca^{2+} are similar at both holding potentials: threefold at +70 mV and twofold at −70 mV. For the currents that are shown, the cells were internally perfused with 10 mM BAPTA. Calibration: 200 pA, 250 msec. *D*, In this chromaffin cell, the current in 10 mM Ca^{2+} is 4.1 times larger than in 1 mM Ca^{2+}. Although the agonist (32 μM DMPP) was applied for more than twice as long as in *C*, there is much less desensitization. The holding potential was −70 mV. Calibration: 50 pA, 250 msec.

Results

Ca^{2+} acts externally to modulate NnAChRs

External Ca^{2+} enhances currents induced by exogenous application of the specific nicotinic agonist, DMPP, to a SCG neuron (Fig. 1*A*) or a chromaffin cell (Fig. 1*B*). The currents induced by 56 μM DMPP are larger in a solution containing 1 mM Ca^{2+} as compared with an identical Ca^{2+}-free solution, indicating that Ca^{2+} modulates NnAChRs. The 1 mM Ca^{2+} enhanced currents from chromaffin cells by 5.4 ± 0.7 (SE, n = 3) and from SCG neurons by 5.5, 5.0 (n = 2). Ca^{2+} is not a coagonist, however, because there are small agonist-induced currents even in the Ca^{2+}-free solution.

Consistent with previous work (Mulle et al., 1992b; Vernino et al., 1992), Figure 1 illustrates that Ca^{2+} acts externally to

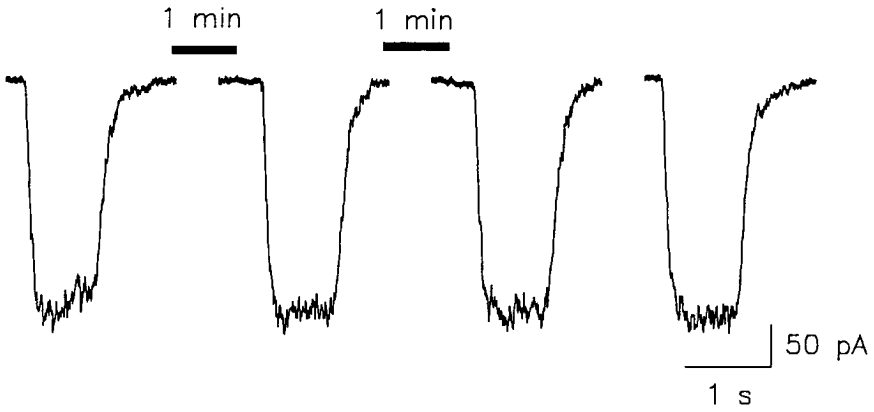

Figure 2. Chlorisondamine does not block closed NnAChRs. Four currents are shown from a SCG sympathetic neuron induced by application of 56 μM nicotine at a holding potential of −70 mV in a solution containing 5 mM Ca^{2+}. The nicotine applications are separated by 1 min, during which the neuron was bathed in 5 μM chlorisondamine as indicated by the *solid bars*. The chlorisondamine was washed off for about 200 msec before the next nicotine application.

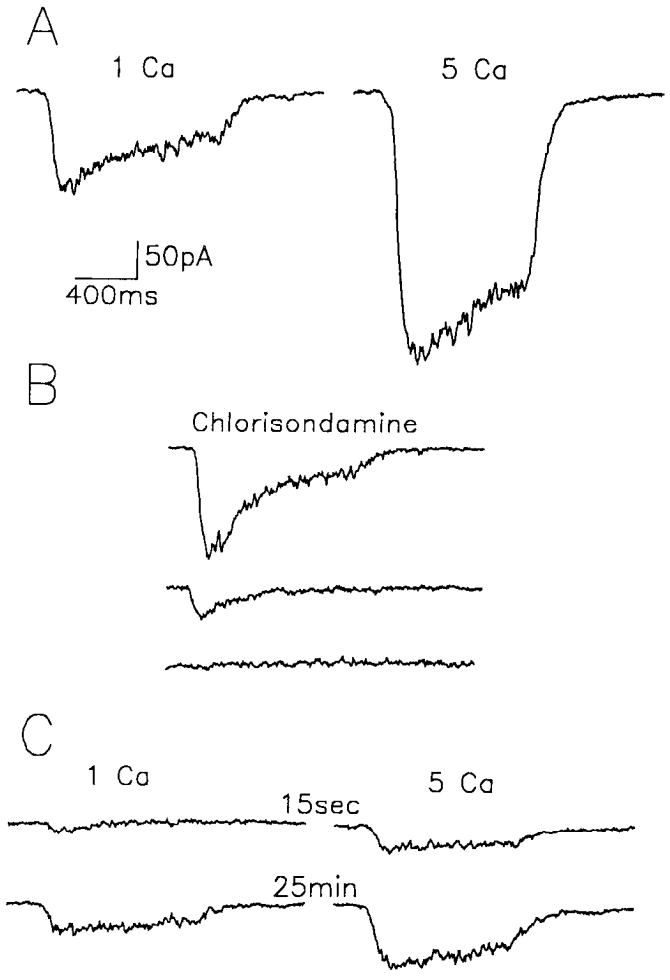

Figure 3. External Ca^{2+} does not enhance NnAChR currents by activating an otherwise unresponsive population of channels. *A*, Currents activated in a sympathetic neuron by 32 μM DMPP are three times larger in 5 mM versus 1 mM Ca^{2+}. *B*, In the 1 mM Ca^{2+} solution, the cell was stimulated by 32 μM DMPP in the presence of the open-channel blocker, 5 μM chlorisondamine, every 12 sec for 3 min. The top record shows the first DMPP application. The falling phase of the current is mostly attributable to chlorisondamine entering and blocking channels after they open. The middle record was obtained after 1 min. The bottom record was the last agonist application after all the channels that open in 1 mM Ca^{2+} were blocked. *C*, After washing the chlorisondamine away for 15 sec, agonist was applied again, first in 5 mM then in 1 mM Ca^{2+}. The current is three times larger in 5 mM Ca^{2+}. After 10 agonist applications and 25 min of washing, the currents have recovered more from chlorisondamine blockade, but the enhancement by 5 mM Ca^{2+} is still threefold. The holding potential was always −70 mV.

modulate NnAChRs. First, Ca^{2+} influx into the cell through nAChRs is strongly voltage dependent (Decker and Dani, 1990; Vernino et al., 1994), but the modulation by Ca^{2+} is not (Fig. 1*C*). At −70 mV there is a large Ca^{2+} influx into the cell, but at +70 mV very little Ca^{2+} enters. If the currents from different chromaffin cells are normalized to their amplitudes in 1 mM Ca^{2+}, then the enhancement of the currents in 10 mM Ca^{2+} is 2.6 ± 0.3 (n = 28) at −70 mV, 3.1 ± 0.2 (n = 32) at −50 mV, and 2.8 ± 0.3 (n = 15) at +70 mV. SCG neurons are similarly modulated at −70 mV: 2.7 ± 0.3 (n = 3). Second, the modulation by Ca^{2+} is unaffected by internal perfusion of the cell with Ca^{2+} chelators. In Figure 1 and in most cases, the cells were perfused with 10 mM BAPTA, but the same enhancement and lack of voltage dependence were seen with 20 mM BAPTA (n = 3), 20 mM EDTA (n = 3), and 10 mM EDTA + 10 mM EGTA (n = 2) with or without 4 mM ATP-Mg. These data were all combined to give the averages listed above. In summary, voltage and intracellular Ca^{2+} buffering do not influence the modulation, indicating that Ca^{2+} is acting externally and not through an intracellular enzyme cascade (Mulle et al., 1992b; Vernino et al., 1992).

Figure 1 also indicates other types of modulation and variability that are seen superimposed on the enhancement of NnAChR currents by external Ca^{2+}. Although the current increase caused by Ca^{2+} is similar in Figure 1, *C* and *D*, the rate and degree of desensitization are much different. Desensitization is thought to arise from intrinsic properties of the receptor channel. Figure 1 shows that for each cell desensitization is greater with increased Ca^{2+} and at more negative potentials, as has been found for muscle nAChRs (Fiekers et al., 1980; Léna and Changeux, 1993). There is, however, much variability from cell to cell. This variability is thought to arise from intercellular enzyme activity, such as phosphorylation speeding the rate of muscle nAChR desensitization (Huganir, 1988). Thus, the currents can differ significantly in appearance while still giving comparable magnitudes of external Ca^{2+} modulation.

Ca^{2+} increases the likelihood of NnAChRs being open and does not activate a previously unresponsive population of NnAChRs

Ca^{2+} could enhance cholinergic responses by activating an otherwise unresponsive population of NnAChRs. There is a precedent for this type of modulation: treatment of ganglionic neurons with cAMP can convert a population of unresponsive NnAChR channels into responsive channels (Margiotta et al., 1987; Berg et al., 1989). The open channel blocker, chlorison-

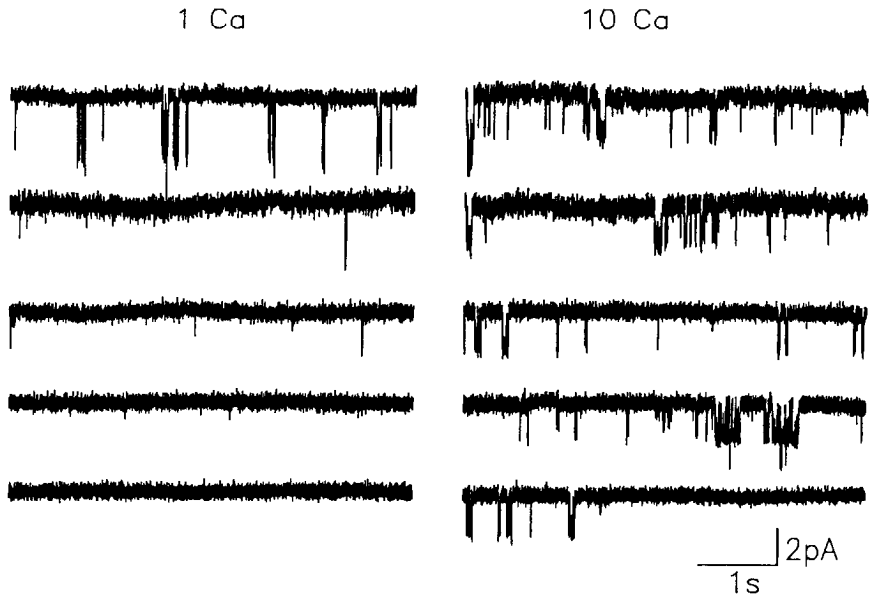

Figure 4. The probability of a NnAChR channel being open is increased in elevated Ca^{2+}. Single-channel currents are shown from an outside/out patch of membrane excised from an adrenal chromaffin cell and exposed to 10 μM DMPP in a solution containing either 1 mM or 10 mM Ca^{2+}. The patch was moved alternately between the two solutions and was held in each solution for 8–47 sec. Each record shows the first 5 sec of single-channel activity for the first 10 solution changes. The holding potential was −70 mV. Consistent with other experiments that are not shown, the single-channel activity runs down with time in both solutions, but more rapidly in 1 mM Ca^{2+}. The amplitude of the single-channel events is larger in 1 mM Ca^{2+}, but the probability of being open is greater in 10 mM Ca^{2+}. Integration of the current over the full length of the recording revealed that NnAChRs are 6 times more likely to be open in the 10 mM Ca^{2+} solution.

damine, was used to determine whether Ca^{2+} modulates already active NnAChRs or converts an unresponsive population of channels. Chlorisondamine enters NnAChR channels after they open and blocks them like a cork in a bottle. Also, chlorisondamine becomes trapped in the pore after the nAChR closes and continues to block until the channel is reopened and chlorisondamine has time to diffuse away (shown with muscle nAChRs by Neely and Lingle, 1986).

Our strategy for testing whether or not Ca^{2+} enhances currents by opening a new population of NnAChRs was as follows. Use chlorisondamine to block all the NnAChR channels that open in a solution of 1 mM Ca^{2+} and, then, see whether large nicotinic currents can still be seen in a solution of 5 mM Ca^{2+}. If large nicotinic currents are seen in 5 mM Ca^{2+}, then that current is passing through a new population of unblocked NnAChRs that did not open in the low Ca^{2+} solution. In separate experiments, chlorisondamine's mechanism of action was examined with NnAChRs to verify the work of Neely and Lingle (1986) on muscle nAChRs. In Figure 2, a SCG sympathetic neuron is stimulated by 56 μM nicotine producing four nicotinic currents each separated by 1 min. The solid bars represent 1 min of exposure to 5 μM chlorisondamine that is terminated 200 msec before the next agonist application. Figure 2 shows that chlorisondamine does not inhibit NnAChRs that have not opened. Therefore, chlorisondamine can be used to identify NnAChRs that have opened by blocking them and becoming trapped within the pore.

In Figure 3*A* application of 32 μM DMPP to a SCG sympathetic neuron induces a current that is three times larger in a solution containing 5 mM Ca^{2+} as compared with the current in 1 mM Ca^{2+}. Then, repeated applications of agonist in the presence of chlorisondamine were made until all the channels that opened in 1 mM Ca^{2+} became blocked (Fig. 3*B*). Next, chlorisondamine was washed away, and the modulation by Ca^{2+} was retested (Fig. 3*C*). After 15 sec of wash, the first application of agonist activates only a small current in 1 mM Ca^{2+}. The enhancement of that current by 5 mM Ca^{2+} is threefold as before, but the size of the current is small. After 10 exposures to agonist and 25 min of recovery from chlorisondamine, the currents are larger, but the modulation by Ca^{2+} remains the same (threefold). Since only the channels that open in 1 mM Ca^{2+} were blocked by chlorisondamine, it is clear that elevated Ca^{2+} does not cause the activation of another population of previously unresponsive channels. If another population of channels had become active, chlorisondamine would not have decreased the extra current originally seen in 5 mM Ca^{2+} (Fig. 3*A*). Similar results giving identical conclusions were obtained in four other cells using 5 μM chlorisondamine with either 32 μM DMPP or 56 μM nicotine. The recovery from blockade by chlorisondamine continued during the course of these experiments, but the recovery was slow because the neurons where always held at −70 mV in these experiments. Recovery was faster and more complete when the holding potential was depolarized to drive chlorisondamine out of the channel.

Although Ca^{2+} does not increase the population of responsive NnAChRs, single-channel recordings in excised patches of membrane indicated that Ca^{2+} does increase the probability of responsive NnAChRs being open. Figure 4 shows single-channel currents induced in a single excised patch of membrane from a chromaffin cell. The agonist concentration, 10 μM DMPP, remained constant as the patch was moved back and forth between solutions containing 1 or 10 mM Ca^{2+}. Each record represents the first 5 sec after a solution change. The fraction of time that a NnAChR channel is open is greater in 10 mM Ca^{2+}. Over the course of the experiment, integration of the single-channel currents for the same length of time indicated that a channel is open six times more often in 10 mM Ca^{2+}. The frequency of channel openings (bursts) increased 4.0 ± 1.2 fold, and the burst length increased slightly, 1.7 ± 0.3 fold. In a total of four patches, integration of the currents gave a 3.4 ± 1.2 fold increase in the probability of a channel being open in 10 mM versus 1 mM Ca^{2+}. This increased probability of opening quantitatively accounts for the Ca^{2+} modulation we see with macroscopic currents. The amplitude of the single-channel currents is larger in low Ca^{2+}, as has been explained previously (Decker and Dani, 1990; Vernino et al., 1992).

Calcium modulation in chromaffin cells appears the same as

for SCG sympathetic neurons (Vernino et al., 1992), but much effort was made to obtain comparable single-channel results with membrane patches excised from SCG neurons. Stable single-channel records were impossible with the SCG neurons, however, because the patches had a low density of channels that "run down" very rapidly. In much less than a minute no channel events could be seen. Therefore, we could not obtain single-channel numbers as described for the chromaffin cells, but channel openings were more frequent and could be followed longer in high calcium with the SCG neurons. It also was not possible to characterize the run down process is SCG neurons because the process was so rapid, but we have characterized the effect in habenula neurons (Lester and Dani, 1994). Mulle et al. (1992b) had similar problems with run down in habenula neurons, where they compared 5–10 sec of NnAChR activity in 0 versus 4 mM Ca^{2+}. Similar to our findings with chromaffin cells, they found a threefold increase in the opening frequency of the NnAChRs in higher Ca^{2+} explained the modulatory effect.

External Ca^{2+} alters cholinergic synaptic transmission consistent with the postsynaptic modulation of NnAChRs

Since external Ca^{2+} modulates NnAChRs, changes in external Ca^{2+} could alter cholinergic synaptic transmission. Figure 5*A* shows cholinergic, nicotinic spontaneous synaptic currents (SSCs) between SCG sympathetic neurons that were grown in culture to form cholinergic/cholinoceptive synapses (Hawrot and Patterson, 1979; Furshpan et al., 1986). In the presence of the NnAChR antagonist, hexamethonium, no large SSCs were ever seen. Propagated excitation was inhibited by blocking voltage-dependent Na^+ channels with 1 μM TTX. The SSCs were obtained at a holding potential of −70 mV while the external solution was alternated 18 times between agonist-free solutions containing either 2 or 5 mM Ca^{2+}. The amplitudes of the SSCs were used to build histograms with bin widths of 3 pA. The most populated bin for each of the two histograms represents the most commonly observe SSC amplitude: 7 pA in 2 mM Ca^{2+} and 10 pA in 5 mM Ca^{2+} (Fig. 5*B*). When all the current amplitudes were averaged together, the bin containing the average-size current was at 17 pA in 2 mM Ca^{2+} and at 24 pA in 5 mM Ca^{2+} (Fig. 5*C*). After hundreds of SSCs were obtained, 32 μM nicotine was exogenously applied to the voltage-clamped neuron in 2 mM Ca^{2+} and then in 5 mM Ca^{2+} (Fig. 5*D*). As described earlier, external Ca^{2+} enhanced the whole-cell current induced by nicotine: the current was 700 pA in 2 mM Ca^{2+} and 1070 pA in 5 mM Ca^{2+}. The ratio 1070/700 can be defined as a modulation factor (f) that accounts for changes in the amplitudes of the SSCs caused by external Ca^{2+} modulating NnAChRs. If the amplitude of the most common SSC in 2 mM Ca^{2+} is multiplied by f, the product equals the amplitude of the most common SSC in 5 mM Ca^{2+}: 7 pA × 1070/700 = 10.7 ≈ 10 pA. The same relationship holds for the average-size SSC: 17 pA × 1070/700 = 26.0 ≈ 24 pA.

The two SSC amplitude distributions are shown in Figure 6*A*. The number of SSCs in the peak bin is normalized to 100 (ordinate). The distribution in 2 mM Ca^{2+} lies at smaller amplitudes than the distribution in 5 mM Ca^{2+}. Then, all the amplitudes of the distribution obtained in 2 mM Ca^{2+} were multiplied by f, and the distributions were plotted again in Figure 6*B*. The two distributions then overlap. The modulation factor accounts for the differences in the amplitudes of the two SSC distributions, indicating that extracellular Ca^{2+} alters the amplitudes of the SSCs by acting postsynaptically to modulate NnAChRs. Such skewed and wide SSC distributions are not seen for mature neuromuscular junctions, but they are common in the central nervous system. Bekkers et al. (1990) investigated the source of the variability for glutamatergic synapses in cultured hippocampal neurons. They reported that the variability of quantal size is the major determinant. Hume and Honig (1991) proposed similar mechanisms to explain the extremely broad amplitude histograms obtained from preganglionic and sympathetic neurons. Those reports also discuss other potential sources of the variability.

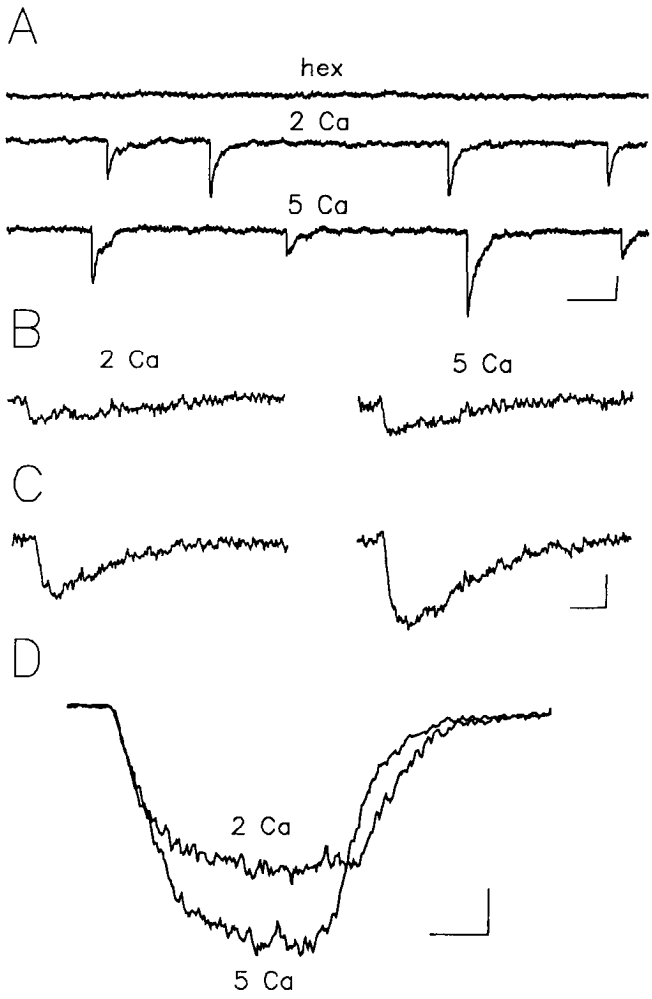

Figure 5. Cholinergic, nicotinic, spontaneous synaptic currents (SSCs) from sympathetic neurons are modulated by external Ca^{2+}. *A*, Three continuous 3.2 sec SSC records are shown for a sympathetic neuron held at −70 mV. When the nicotinic antagonist, 20 μM hexamethonium (*hex*), was present, SSCs were not seen. SSCs were obtained as the external (agonist free) solution was alternated between 2 mM and 5 mM Ca^{2+}. On average the bathing solution was rapidly switched every 80 sec. Calibration: 20 pA, 250 msec. *B*, An example is shown of the most common amplitude of the SSCs obtained in 2 mM or 5 mM Ca^{2+}. *C*, An example of the average-size amplitude of the SSCs obtained in 2 mM or 5 mM Ca^{2+}. Calibration: 10 pA, 10 msec for both sets of examples. *D*, After obtaining many SSCs, 32 μM nicotine was exogenously applied to the sympathetic neuron, which was whole-cell clamped at −70 mV. The current was 700 pA in 2 mM Ca^{2+} and 1070 pA in 5 mM Ca^{2+}. Calibration: 200 pA, 250 msec.

The influence of external Ca^{2+} on the cholinergic SSCs can be fully explained by Ca^{2+} directly modulating the postsynaptic

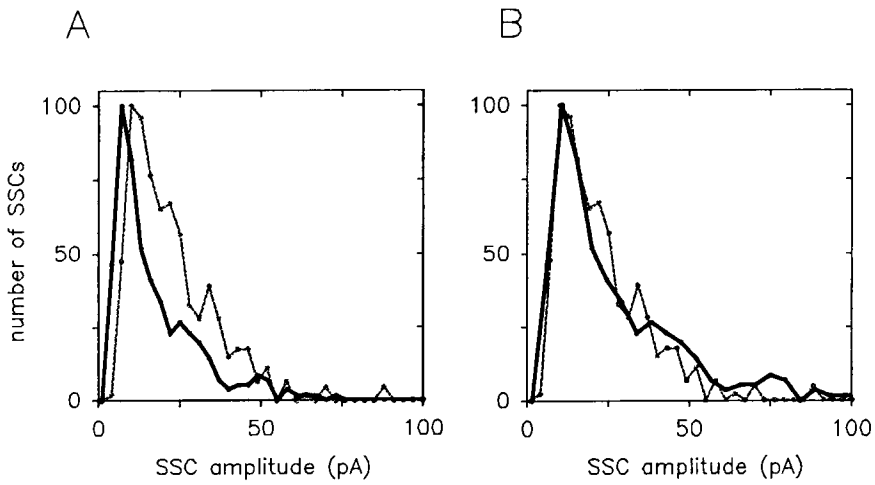

Figure 6. Postsynaptic modulation of NnAChRs by Ca^{2+} accounts for the difference in the amplitudes of the spontaneous synaptic currents. *A*, The amplitude distribution of SSCs is shown for 2 mM Ca^{2+} (*thick line*) and 5 mM Ca^{2+} (*thin line*). The most populated bin is scaled to 100 on the y-axis (number of SSCs), which is plotted against SSC amplitude in pA on the x-axis. The distribution obtained in 2 mM Ca^{2+} falls to the left (smaller amplitudes) of the distribution obtained in 5 mM Ca^{2+}. *B*, When the amplitudes of the SSCs in 2 mM Ca^{2+} are adjusted for Ca^{2+} modulation of NnAChRs by multiplying all the amplitudes by the modulation factor, *f*, the 2 mM Ca^{2+} distribution then falls on top of the 5 mM Ca^{2+} distribution. The distributions contained 286 events in 2 mM Ca^{2+} collected during a total of 627 sec and 333 events in 5 mM Ca^{2+} collected during a total of 788 sec.

NnAChRs. There was no effect on the frequency of SSCs, which was 27 ± 3 events per minute in 2 Ca^{2+} and 25 ± 2 events per minute in 5 Ca^{2+}. Thus, presynaptic factors did not appear to influence the change in the amplitude of the SSCs in the two solutions, which is consistent with previous work (Redman, 1990).

In 4 out of 4 other neurons, the SSC distribution in 1 or 2 mM Ca^{2+} was displaced to lower current amplitudes as compared with the distribution in 5 mM Ca^{2+}. Figure 7 shows an example of another neuron where we were able to determine in 2 and 5 mM Ca^{2+} both the amplitude distributions and the modulation factor, *f*, which was 535/400 at a holding potential of −70 mV using 32 μM nicotine. The average-size currents for the two distributions were 16 pA in 2 mM Ca^{2+} and 21 pA in 5 mM Ca^{2+}. Again, the modulation factor accounted for the difference in the two amplitude distributions, as is exemplified by the average currents: 16 pA × 535/400 = 21 pA. Figure 7 shows the cumulative histograms of SSC amplitudes. The total number of SSCs of a particular size or smaller are added together to produce the cumulative histograms. This procedure diminishes the fluctuations from bin to bin and more clearly shows the shift to lower SSC amplitudes in 2 mM versus 5 mM Ca^{2+} (Fig. 7*A*). When the amplitudes obtained in 2 mM Ca^{2+} are multiplied by the modulation factor (f = 535/400), the two

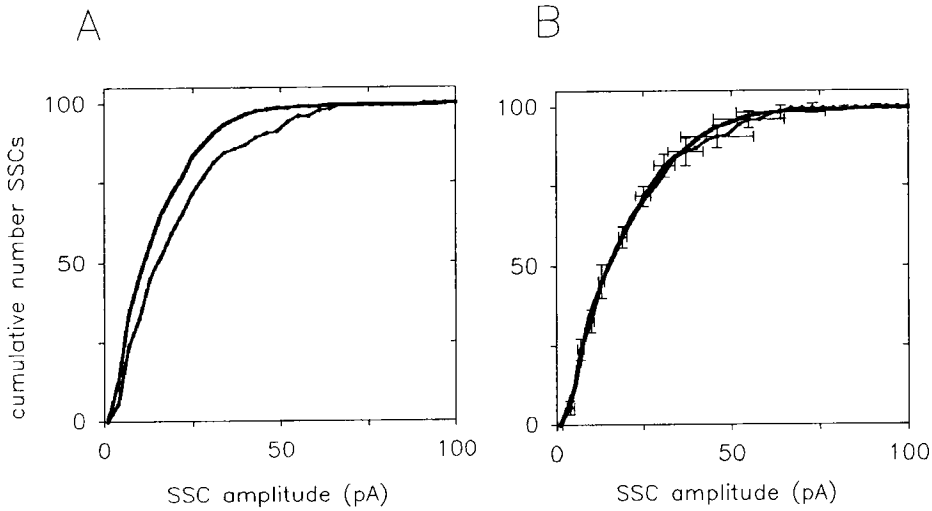

Figure 7. Cumulative histograms also show that postsynaptic modulation of NnAChRs by Ca^{2+} accounts for the difference in the amplitudes of the spontaneous synaptic currents. *A*, The amplitude distribution of SSCs are transformed into cumulative histograms by adding the number of SSCs that are equal to or less than the amplitude bin in question. The total number of SSCs is normalized to 100 for both distributions. The distribution in 2 mM Ca^{2+} (*thick line*) is at smaller amplitudes than the distribution in 5 mM Ca^{2+} (*thin line*). *B*, When the amplitudes of the SSCs in 2 mM Ca^{2+} are adjusted for Ca^{2-} modulation of NnAChRs by multiplying all the amplitudes by the modulation factor, *f*, the 2 mM Ca^{2+} distribution then falls on top of the 5 mM Ca^{2+} distribution. The distributions contained 190 events in 2 mM Ca^{2+} collected during a total of 108 sec and 167 events in 5 mM Ca^{2+} collected during a total of 108 sec. The error bars were calculated by obtaining the deviations of the 2 mM Ca^{2+} distribution from its corresponding 5 mM Ca^{2+} distribution for three separate experiments (i.e., n = 3).

distributions overlap (Fig. 7*B*). The distribution obtained in 5 mM Ca^{2+} is shown with error bars, illustrating that the modulation factor accounts for the shift in the amplitude histograms with very small errors from trial to trial ($n = 3$ comparing 2 mM Ca^{2+} to 5 mM Ca^{2+}).

Discussion

The results document two properties of neuronal nicotinic receptors and synapses. First, modulation of NnAChRs by external Ca^{2+} does not involve activation of a previously refractory or unresponsive population of NnAChRs. Rather, Ca^{2+} acts via a direct mechanism that increases the probability of the channels being open. Mulle et al. (1992B) similarly reported that Ca^{2+} modulation enhances opening frequency of NnAChRs from rat medial habenular neurons. Chromaffin cells and habenular neurons are likely to express different NnAChR subtypes (Role, 1992), but the modulation by Ca^{2+} seems to arise from the same mechanism (i.e., a change in the opening frequency). Vernino et al. (1992) reported this form of Ca^{2+} modulation with four different NnAChR subtypes expressed in oocytes. The accumulation of evidence indicates that this form of Ca^{2+} modulation may be common among many NnAChR subtypes in the nervous system. The second finding of this work is that the modulation by Ca^{2+} alters spontaneous cholinergic synaptic currents. That result shows that synaptic processes can be affected by this type of Ca^{2+} modulation, suggesting that activity-dependent decreases in external Ca^{2+} may negatively affect nicotinic responses to further ACh release.

Biological implications

It is interesting that muscle nAChRs have a lower Ca^{2+} permeability and are not modulated by external Ca^{2+} in the same way as neuronal nAChRs (Decker and Dani, 1990; Vernino et al., 1992). Since the neuromuscular synapse must work well even during extended use, it would not be adaptive to have decreased excitability caused by external Ca^{2+} modulation during times of high activity. The lower Ca^{2+} permeability of the muscle receptor suggests that the nerve–muscle synapse need not undergo the same activity-dependent forms of plasticity that may be required at neuronal cholinergic synapses.

Neuronal nAChRs have a higher Ca^{2+} permeability, and at least one neuronal nAChR subtype has a Ca^{2+} permeability comparable to that of the NMDA subtype of glutamate receptors (Vijayaraghavan et al., 1992; Séguéla et al., 1993). At highly active cholinergic synapses, Ca^{2+} influx through NnAChRs could activate intracellular mechanisms leading to synaptic plasticity in a manner previously reserved for glutamatergic synapses (Malinow et al., 1988; Malenka et al., 1989; Madison et al., 1991). In addition, our results indicate that at nicotinic synapses where high activity has decreased external Ca^{2+} there will be a diminished response of NnAChRs to further ACh release. This mechanism will be accompanied by other processes, such as the influence of external Ca^{2+} on transmitter release and nAChR desensitization (Augustine et al., 1987; Léna and Changeux, 1993). Since NnAChRs have both presynaptic and postsynaptic locations in the CNS (see Sargent, 1993), the influence of this hypothesized rapid negative feedback mechanism could be quite varied. A reasonable scenario that could decrease cholinergic synaptic excitability after high activity is as follows. High synaptic activity decreases extracellular Ca^{2+}. Subsequent excitation of the presynaptic terminal produces a smaller Ca^{2+} influx and a concomitant decrease in ACh release. Ca^{2+} modulation makes the postsynaptic NnAChRs less likely to open in response to ACh. Consequently, cholinergic synaptic transmission is diminished. Since many NnAChRs are located presynaptic where they can enhance release of other neurotransmitters, the mechanism of Ca^{2+} modulation could be widespread, influencing the activity-dependent release of many neurotransmitters. There could be other roles for such a mechanism. One role could be to limit the size of the intracellular Ca^{2+} signal so that intracellular Ca^{2+} does not become dangerously high, as is expected during some forms of excitotoxic neuron death seen at glutamatergic synapses (Regan and Choi, 1991; Choi, 1992).

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