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Imaging of intracellular calcium during desensitization of nicotinic acetylcholine receptors of rat chromaffin cells

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1 The possible role of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in desensitization of nicotinic acetylcholine receptors (AChRs) was investigated in rat cultured chromaffin cells by use of combined whole-cell patch clamping and confocal laser scanning microscopy with the fluorescent dye fluo-3.

2 On cells held at −70 mV, pressure-application of nicotine elicited inward currents with associated $[Ca^{2+}]_i$ rises mainly due to influx through nicotinic AChRs. These responses were blocked by (+)-tubocurarine (10 μM) but were insensitive to α-bungarotoxin (1 μM) or Cd^{2+} (0.1 mM).

3 Pressure applications of 1 mM nicotine for 2 s (conditioning pulse) evoked inward currents which faded biexponentially to a steady state level due to receptor desensitization and were accompanied by a sustained increase in $[Ca^{2+}]_i$. Inward currents evoked by subsequent application of brief test pulses of nicotine were depressed but recovered with a time course reciprocal to the decay of the $[Ca^{2+}]_i$ transient induced by the conditioning pulse.

4 Omission of intracellular Ca^{2+} chelators or use of high extracellular Ca^{2+} solution (10 mM) lengthened recovery of nicotinic AChRs from desensitization while adding BAPTA or EGTA intracellularly had the opposite effect. When the patch pipette contained fluo-3 or no chelators, after establishing whole cell conditions the rate of recovery became progressively longer presumably due to dialysis of endogenous Ca^{2+} buffers. None of these manipulations of external or internal Ca^{2+} had any effect on onset or steady state level of desensitization.

5 High spatial resolution imaging of $[Ca^{2+}]_i$ in intact cells (in the presence of 0.1 mM Cd^{2+}) showed that its level in the immediate submembrane area decayed at the same rate as in the rest of the cell, indicating that Ca^{2+} was in a strategic location to modulate (directly or indirectly) AChR desensitization.

6 The present data suggest that desensitized nicotinic AChRs are stabilized in their conformation by raised $[Ca^{2+}]_i$ and that this phenomenon retards their recovery to full activity.

Keywords: Calcium imaging; nicotinic receptor; desensitization; acetylcholine; nicotine; chromaffin cells; confocal microscopy

Introduction

Although desensitization is a common feature of a wide variety of ligand-gated channels, its underlying mechanisms and modulation by intracellular factors are incompletely understood. In the case of nicotinic acetylcholine receptors (AChRs) early work showed that their desensitization was accelerated by high extracellular Ca^{2+} levels ($[Ca^{2+}]_o$) (Manthey, 1966; Magazanik & Vyskocil, 1970), whereas subsequent studies have suggested that raised intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is actually the important factor to facilitate desensitization on muscle (Miledi, 1980; Chestnut, 1983). Progress in this field has been aided by the observation that desensitization of nicotinic AChRs is a heterogeneous process comprising fast and slow phases (Feltz & Trautmann, 1982; MacOnochie & Knight, 1992), only the latter one being modulated by $[Ca^{2+}]_i$ (Cachelin & Colquhoun, 1989). One important characteristic of desensitization is how quickly receptors can recover from it since this aspect will determine intercellular signalling (Jones & Westbrook, 1996). The use of combined patch clamping and $[Ca^{2+}]_i$ imaging enabled it to be shown that recovery from desensitization is dependent on $[Ca^{2+}]_i$ in the case of glutamate (Medina *et al*., 1996) or adenosine 5′-triphosphate (ATP) (Khiroug *et al*., 1997) receptors. Since comparable data for nicotinic AChRs (particularly those of neuronal type) are currently lacking we decided to adopt the same combined method (Khiroug *et al*., 1997) to study $[Ca^{2+}]_i$ during development of and recovery from desensitization of nicotinic AChRs. These experiments were supplemented by tests in which the level of $[Ca^{2+}]_i$ was varied by intracellular application of Ca^{2+} buffers.

As an experimental preparation we used rat chromaffin cells in culture, since it is known that in the adrenal medulla acetylcholine (ACh) is the transmitter released from splanchnic nerve fibres (Feldberg *et al*., 1934) to depolarize chromaffin cells, predominantly via nicotinic AChR activation (Yoshizaki, 1975; Kidokoro *et al*., 1982; Gu *et al*., 1996), to secrete catecholamines into the bloodstream (Douglas *et al*., 1967). Activated neuronal nicotinic AChRs are relatively permeable to Ca^{2+} which carries between 2.5 (Zhou & Neher, 1993) and 5% (Vernino *et al*., 1994) of the total current through the open channel. Since the stimulant action of a sustained application of ACh on catecholamine secretion rapidly fades (Douglas & Rubin, 1961) mainly because of desensitization of nicotinic AChRs (Marley, 1988), chromaffin cells appear to be a suitable model in which to test any involvement of Ca^{2+} in desensitization.

Methods

Cell preparation

Rat chromaffin medullar cells were cultured according to the method of Brandt *et al*. (1976). Ether-anaesthetized female rats (200–250 g body weight) were killed by decapitation and their adrenal glands were removed, dissected free of the cortex, and rinsed in a medium (pH 7.2) containing (mM): NaCl 137, KCl 3, Na_2HPO_4 0.7, HEPES 25, glucose 10 and 350 units ml^{-1} of penicillin and streptomycin. Cells were dissociated by treating adrenal tissue fragments at 37°C with collagenase A and DNase

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I (0.5 units ml^{-1} and 10 $\mu g\ ml^{-1}$, respectively) and drawing them gently up and down in a Pasteur pipette every 15–20 min. The cell containing suspension was centrifuged at 500 *g* for 4 min, and rinsed twice with the HEPES-buffered medium. Finally, cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, plated on poly-lysine (1.25 mg ml^{-1})-coated Petri dishes and cultured for 1–2 days under an atmosphere containing 5% CO_2.

Patch-clamp recording

Cell containing dishes (mounted on the stage of an inverted Nikon Diaphot microscope) were superfused at a rate of 5 ml min^{-1} with control saline solution containing (mM): NaCl 132, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, HEPES 10 (pH adjusted to 7.4 with NaOH). Patch pipettes pulled from thin glass (1.5 mm o.d.) had resistance of 1.5–3 MΩ when filled with (mM) CsCl 120, HEPES 20, $MgCl_2$ 1 and Mg_2ATP_3 3. The use of Ca^+ as the main intracellular cation effectively blocked K^+ channels which otherwise might have been activated when $[Ca^{2+}]_i$ was experimentally raised: this notion was confirmed by the lack of any measurable outward current (or leak conductance increase) even when $[Ca^{2+}]_i$ was increased shortly after the whole cell configuration had been established. Unless explicitly stated otherwise, whole-cell patch clamp recordings were combined with confocal $[Ca^{2+}]_i$ imaging: for this purpose 25 μM of the Ca^{2+}-sensitive dye fluo-3 was added to this pipette solution. pH was always adjusted to 7.2 with CsOH. After GΩ seals had been obtained in the whole-cell configuration, membrane potential was normally clamped at −70 mV (unless otherwise indicated). After the whole-cell conditioning a 10 min period of stabilization normally elapsed before the membrane currents were recorded with a List L/M-PC amplifier (List, Darmstadt, Germany). These responses were filtered at 1 kHz and acquired on the hard disk of a PC by means of pCLAMP software (5.5 version; Axon Instruments Inc., Foster City, California). Membrane currents were measured in terms of time to peak, amplitude and exponential decay while the values of charge transfer were obtained by measuring the current area. In some experiments (in which only current measurements but not imaging were performed) the Ca^{2+} buffers BAPTA or EGTA were added to the internal solution or any exogenous buffers omitted all together. In pilot tests, when the patch pipette contained the Ca^{2+} chelator BAPTA (10 mM) as well as the Ca^{2+} sensitive fluorescent dye fluo-3 (see below), nicotine-induced membrane currents were not accompanied by any measurable increase in fluorescence, suggesting that this concentration of BAPTA provided adequate buffering of $[Ca^{2+}]_i$ transients.

Confocal microscopy imaging of $[Ca^{2+}]_i$

For confocal microscopy imaging in the visible light range we used the Ca^{2+}-sensitive dye fluo-3 which emits a fluorescent signal linearly related to the Ca^{2+} concentration within the 10–1000 nM range with additional advantages of very rapid binding-unbinding of free Ca^{2+} and relative insensitivity to pH (Minta *et al*., 1989). Fluo-3 (cell impermeant form, pentapotassium salt; Molecular Probes Inc., Eugene, Oregon, U.S.A.) was routinely applied (25 μM) via the patch pipette. Fluorescent signals were digitized over the whole pericaryon central optical section as 64×32 pixel images in the 32-line rapid scan mode (temporal resolution 320 ms per scan; pixel size 0.6 μm; confocal aperture 200 μm) and analysed with the ImageSpace 3.10 software (Molecular Dynamics, Sunnyvale, California, U.S.A.). $[Ca^{2+}]_i$ transients were measured in terms of fractional amplitude ($\Delta F/F_0$; where F_0 is the baseline fluorescence level, and ΔF is the rise over the baseline) and rise time (10–90% of peak amplitude). Since fluo-3 measurements of $[Ca^{2+}]_i$ are non-ratiometric and thus unable to provide direct measurements of $[Ca^{2+}]_i$ concentrations, baseline fluorescence based on previous calibration experiments with 5 μM ionomycin was estimated to be 41 ± 5 nM (Andjus *et al*., 1996).

A small number of imaging experiments was carried out on intact (non-patched) cells from which only fluorescent signals were recorded: in this case the dye was loaded by preincubation with fluo-3 AM (5 μM) for 45 min at 37°C in the dark followed by extensive washing. High temporal and spatial resolution (10 ms per line; 0.16 μm pixel size) single line scans to examine the perimembrane distribution of $[Ca^{2+}]_i$ were obtained only from such intact cells (in the presence of 0.1 mM Cd^{2+} to block voltage activated Ca^{2+} channels; Kim *et al*., 1995) since the rapid movement of the fast scanning mirror of the confocal microscope introduced recording instability incompatible with patch clamping due to mechanical resonance of the micropipette.

In all imaging experiments (with or without patch clamping) emission of fluo-3 was induced by the 488 nm light band of the Ar-Kr laser and detected by a photomultiplier tube with a combination of 510 nm high-pass and 530 ± 30 nm band-pass filters (MultiProbe 2001 confocal laser scanning microscope; Molecular Dynamics). No detectable dye bleaching was observed under these conditions.

Data analysis

All data are presented as mean ± s.e.mean (n = number of cells) with statistical significance assessed with paired t test (for normally distributed data) or one-way ANOVA test (for non parametric data). A value of $P \leqslant 0.05$ was accepted as indicative of a statistically significant difference.

Drug application and experimental protocols

(−)-Nicotine (hydrogen tartrate salt; purchased from Sigma) was diluted in control saline solution at a final concentration of 1 mM and delivered by pressure application (10–20 p.s.i. via a Picospritzer II; General Valve Co., Fairfields, New Jersey, U.S.A.) from glass micropipettes positioned about 15 μm away from the recorded cell. Initial tests were performed to select a pressure value that did not produce any visible distortion of cell shape which could disturb $[Ca^{2+}]_i$ imaging. Fast and slow desensitization was defined as fading of inward current with a biexponential time course (expressed as τ_1 and τ_2 values) during the continuous pressure application of nicotine (1 mM solution in the pipette) for 2 s. Preliminary tests were also done with 0.1 or 0.01 mM nicotine solution in the pressure pipette: in these cases inward current showed modest fading with monoexponential decay, indicative of partial desensitization only. A standard protocol was employed to induce receptor desensitization (see for instance Chestnut, 1983; MacOnochie & Knight, 1992; Nooney & Feltz, 1995; Khiroug *et al*., 1997). In particular, we investigated onset and steady state of desensitization by measuring the decline in current response to 2 s nicotine (conditioning application), and monitored recovery from desensitization by applying 10–20 ms test pulses at 15–30 s interval. Such slow rates of test pulses avoided contamination by any residual nicotine current after the conditioning application (see MacOnochie & Knight, 1992) and minimized any interaction between test pulses (Chestnut, 1983). Although recovery from desensitization may posses a bi-exponential time course (Feltz & Trautmann, 1982; Boyd, 1987), the present data mainly describe the process of slow recovery from desensitization, which presumably corresponds to the one with a time constant of tens of seconds (Boyd, 1987). Substances other than nicotine were bath-applied or added to the patch pipette.

Results

Nicotine-induced membrane currents and $[Ca^{2+}]_i$ rises

In agreement with previous studies on chromaffin cells (Vernino *et al*., 1994) we also observed that nicotine evoked inward currents and associated rises in $[Ca^{2+}]_i$ (see Figure 1A; 20 ms

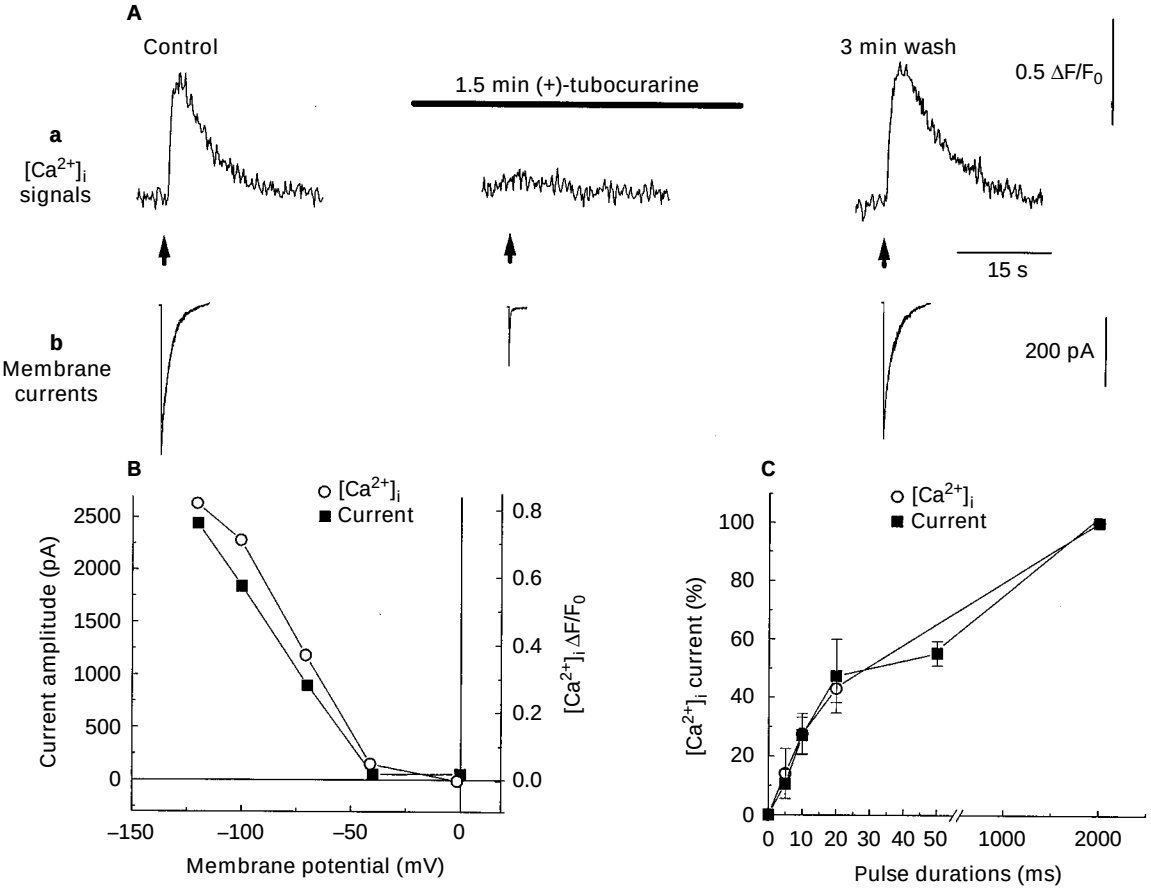


Figure 1 Effects of (+)-tubocurarine, membrane potential or pulse duration on simultaneously recorded $[Ca^{2+}]_i$ rises and membrane currents elicited by nicotine from voltage clamped cells. (A) $[Ca^{2+}]_i$ transients (a) and inward currents (b) elicited by nicotine (1 mM; 20 ms; arrows) in control solution, 1.5 min after bath-applying (+)-tubocurarine (10 μM) and after 3 min washout. (B) Relation between holding membrane potential (abscissa scale) and peak amplitude of 20 ms nicotine-evoked current (left ordinate scale, absolute values) or size of associated $[Ca^{2+}]_i$ rise (right ordinate scale). Data are from the same cell depicted in (A) and are representative of results from 5 cells. Note that to aid comparison of the Ca^{2+} and current data, the latter have been plotted as absolute (positive) values. (C) Plot of duration of nicotine pressure application (indicative of the amount of applied nicotine; abscissa scale) versus $[Ca^{2+}]_i$ and current responses (expressed as % of the maximum response). Data points are from 4–13 cells.

pulse application of this agonist). The current response (−610 pA) peaked in 82 ms while $[Ca^{2+}]_i$ increased in parallel with the inward current and reached a peak of 0.96 $\Delta F/F_0$ at 3 s. Figure 1A also shows that bath-application of the nicotinic antagonist (+)-tubocurarine (10 μM) strongly and reversibly depressed both responses (on average (+)-tubocurarine depressed the charge transfer through activated receptors and the amplitude of $[Ca^{2+}]_i$ rises by 94±1 and 99±0.4%, respectively; $n=3$), indicating that elevation of $[Ca^{2+}]_i$ required nicotinic AChR activation. α-Bungarotoxin (1 μM) was ineffective on responses induced by nicotine (see also Vernino *et al*., 1994; Nooney & Feltz, 1995).

Figure 1B shows that when the holding potential was changed between −120 and 0 mV (moving slowly in a stepwise fashion to various holding levels which were maintained for at least 1 min), the voltage-dependence of inward current and $[Ca^{2+}]_i$ rise was very similar with a comparable degree of outward rectification (same cell as Figure 1A). Sustained membrane depolarization presumably inactivated voltage-sensitive Ca^{2+} currents, thus preventing detectable changes in baseline $[Ca^{2+}]_i$. Peak inward currents and $[Ca^{2+}]_i$ rises had a similar hyperbolic relation to the nicotine doses expressed as pulse duration (Figure 1C; $n=4-13$). Since 10–20 ms pulses elicited approximately half maximal (Figure 1C) and stable responses, subsequent experiments normally employed this length of pressure application for further tests of nicotinic receptor sensitivity: on average the inward current evoked under these conditions was −884±90 pA ($n=12$).

When external Ca^{2+} was omitted while adding 5 mM BAPTA and 3 mM Mg^{2+}, baseline $[Ca^{2+}]_i$ levels decreased by 29±6% and were unchanged (1±0.7%) following application of nicotine ($n=4$). The amplitude of nicotine currents in the same solution was not significantly different (80±13%) from control. Furthermore, in separate experiments, application of the Ca^{2+} channel antagonist Cd^{2+} (0.1 mM) did not significantly depress test currents (89±4% of the ones in control solution; $n=6$) or the $[Ca^{2+}]_i$ transients (96±8%) induced by nicotine. However, on the same cells, Cd^{2+} (0.1 mM) was able to reduce the $[Ca^{2+}]_i$ rise induced by a depolarizing voltage step (from −70 to 0 mV for 2 s) to 23±6% of control ($P<0.01$). Since on cells voltage clamped at −70 mV, voltage-sensitive Ca^{2+} channels could not operate and the nicotine-induced responses were not antagonized by the Ca^{2+} channel blocker Cd^{2+}, it appears that voltage-sensitive Ca^{2+} channels were not involved in the action of nicotine. As also demonstrated by the experiments in Ca^{2+}-free media, the main source of Ca^{2+} for the rise in $[Ca^{2+}]_i$ was therefore from the extracellular compartment via activated nicotinic AChRs.

Desensitization and recovery of nicotinic AChRs

Figure 2A shows an example of the protocol used to test nicotinic AChR desensitization and recovery: after repetitive 20 ms test pulses of nicotine (applied every 30 s to ensure reproducibility; last one shown at the start of the traces) a 2 s

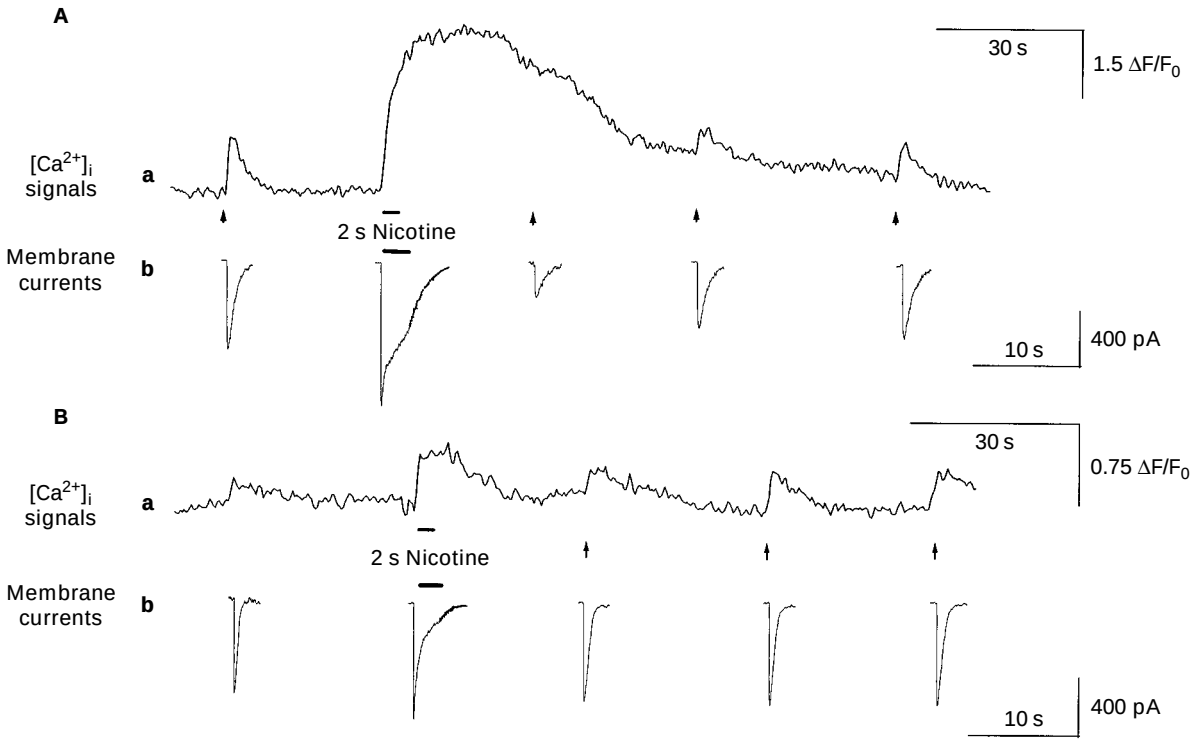


Figure 2 Simultaneous records of $[Ca^{2+}]_i$ transients and inward currents during nicotine-induced desensitization of two voltage clamped cells with different degrees of $[Ca^{2+}]_i$ response. (A) $[Ca^{2+}]_i$ changes (a) and inward currents (b) following 20 ms (arrows) or 2 s (horizontal bar) applications of nicotine (note that timescale for current is much faster than for $[Ca^{2+}]_i$ changes). Note that recovery of test currents proceeded in parallel with decay of $[Ca^{2+}]_i$ transient induced by 2 s nicotine. (B) Comparable records from another cell in which the conditioning pulse (2 s) of nicotine induced a fading inward current (b) but only a modest increase in $[Ca^{2+}]_i$ (a) associated with rapid recovery from desensitization.

administration of the same agonist (horizontal bars) induced a rapidly-waning inward current (−1150 pA peak amplitude; for an example of a fast time base trace of current fade see Figure 6a) which was associated with an increase in $[Ca^{2+}]_i$ (note much slower time calibration) peaking 10 s after the end of the nicotine application. Recovery of baseline current was complete within 10 s but the effectiveness of subsequent test pulses in eliciting current responses was reduced, with recovery attained approximately 90 s from the 2 s nicotine application (Figure 2Ab): 30 s after the conditioning pulse of nicotine the test pulse evoked an inward current which was 41% of control one. As depicted in Figure 2Aa the peak $[Ca^{2+}]_i$ rise following 2 s nicotine application was larger than the ones elicited by 20 ms application (this difference is also quantified in Figure 1C; open circles). Even more striking was the duration of the $[Ca^{2+}]_i$ elevation after 2 s nicotine compared with the one after 20 ms application: such a large difference was made up by the sustained nature of the $[Ca^{2+}]_i$ increase after 2 s nicotine. This is indicated in Figure 2Aa by the slow process (28.4 s half-time decay) of recovery of $[Ca^{2+}]_i$ to baseline level. During this period $[Ca^{2+}]_i$ transients to subsequent nicotine applications were reversibly attenuated (Figure 2Aa).

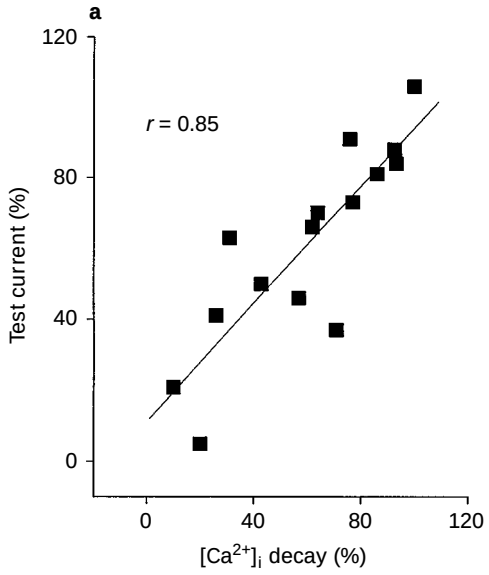
Figure 2B shows a different type of response, recorded from another cell, to the conditioning pulse of nicotine: in this case the peak inward current (−805 pA) largely faded (reaching a level of −180 pA; Figure 2Bb) but was associated with a modest rise in $[Ca^{2+}]_i$ (Figure 2Ba) which dissipated with a 5.3 s half-time. Recovery from desensitization both in terms of current and $[Ca^{2+}]_i$ peaks was already observed at 30 s.

The rather variable time course of $[Ca^{2+}]_i$ transients after 2 s nicotine was exploited to test any correlation between sustained rise in $[Ca^{2+}]_i$ and depression of the nicotine-induced test current and $[Ca^{2+}]_i$ responses 30 s after the conditioning pulse. Figure 3a shows pooled data from 15 cells taken 30 s after the conditioning pulse: on the ordinate scale the residual current responses (% of control) are plotted against the % fall of $[Ca^{2+}]_i$ from its peak (abscissa scale). The regression line fitted to the data had a 0.85 coefficient of correlation (r) indicating that the two parameters were linearly related. Nevertheless, for the same time point (30 s after conditioning) no correlation was found between depression of test currents (or $[Ca^{2+}]_i$ transients) and elevation of $[Ca^{2+}]_i$ measured as % rise over baseline (not shown). Figure 3b shows for the same group of cells the temporal profile of changes in $[Ca^{2+}]_i$ and in nicotine test currents following the conditioning application of nicotine (taken as time zero): 90 s later there was substantial recovery in current amplitude and return of $[Ca^{2+}]_i$ to baseline level.

The large fading of the nicotine current during 2 s application might have been caused by a shift in the current reversal potential rather than receptor desensitization. In order to explore this issue, voltage ramps (400 mV s^{-1}) were applied to the cell once the current decline reached quasi steady-state conditions (just before the end of nicotine delivery). The resulting current/voltage plot was thus compared with the one at the peak of the currents elicited by 20 ms test nicotine pulses while applying the same ramp protocol. In five cells null potentials obtained with control and conditioning pulses were -15 ± 3 and -18 ± 4 mV, respectively ($P>0.05$), ruling out the possibility that current fading was due to a shift in reversal potential.

Recovery of desensitization is affected by manipulating $[Ca^{2+}]_o$ or $[Ca^{2+}]_i$

Although the data collected so far indicated a strong association between recovery from desensitization and $[Ca^{2+}]_i$, even stronger evidence would be obtained by observing corresponding changes when $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ are varied. Figure



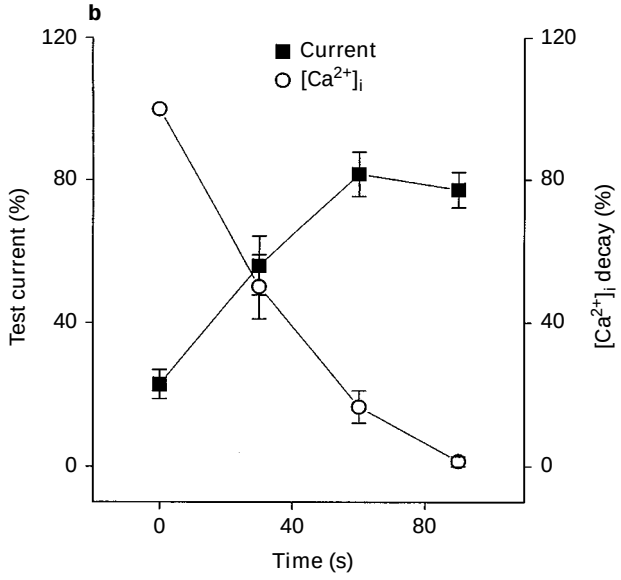


Figure 3 Relation between $[Ca^{2+}]_i$ changes and recovery from desensitization simultaneously measured on voltage clamped cells. (a) Plot of test current amplitude at 30 s from conditioning pulse (expressed as % of control test pulses; ordinate scale) versus decay of $[Ca^{2+}]_i$ transient (as % of peak response; abscissa scale) at the same time point; r = correlation coefficient, $n = 15$. (b) Amplitude of test currents after conditioning pulse (left ordinate scale) and decay of $[Ca^{2+}]_i$ (right ordinate scale) were plotted as function of time from conditioning application of nicotine. Note reciprocal time profile of changes.

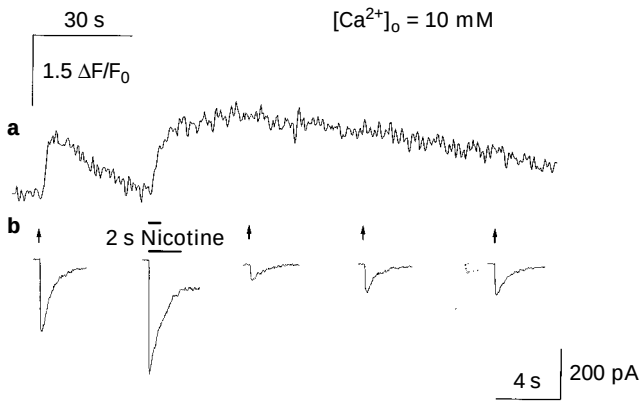


Figure 4 High external Ca^{2+} solution prolonged recovery from desensitization of voltage clamped cell. Changes in $[Ca^{2+}]_i$ (a) and inward currents (b) evoked by test or conditioning pulses of nicotine were simultaneously recorded in the presence of 10 mM Ca^{2+} solution. Following 2 s nicotine application note sustained elevation in $[Ca^{2+}]_i$ associated with slow recovery from desensitization.

4 shows an example of a cell bathed in a solution containing 10 mM Ca^{2+}. By use of the standard protocol described above, it appears that $[Ca^{2+}]_i$ transients (Figure 4a) evoked by 20 ms nicotine had slower decay than in control solution (cf Figure 2A and B); the inward current induced by a 2 s application of nicotine decayed biexponentially in a manner similar to that in control solution, but it was associated with a very long lasting increase in $[Ca^{2+}]_i$ which declined in a complex fashion (decay half-time = 98 s). During the persistent rise in $[Ca^{2+}]_i$, inward currents induced by test pulses were depressed with slow recovery (Figure 4b). On average the currents (30 s after the conditioning pulse) were $28 \pm 4\%$ of preconditioned responses ($n = 4$), a value significantly different from that found in control solution ($56 \pm 8\%$; $n = 15$; see data in Figure 3b). High $[Ca^{2+}]_o$ solution did not significantly change the peak amplitude of the current induced by 2 s nicotine (-1072 ± 388 pA, $n = 4$, vs -1253 ± 126 pA in 2 mM $[Ca^{2+}]_o$, $n = 15$). Similarly, τ_1 and τ_2 values for the fast and slow fade of the nicotine inward current in 10 mM $[Ca^{2+}]_o$ (86 ± 15 and 1108 ± 347 ms, respectively) did not significantly differ from corresponding values in standard external solution (127 ± 16 and 1091 ± 143 ms, respectively).

Even simple inspection of the $[Ca^{2+}]_i$ decay in control bathing solution indicated the relatively slow nature of this process (see Figure 2); nevertheless, in the same cell the $[Ca^{2+}]_i$ decay could vary substantially in relation to the time elapsed from establishing the whole-cell condition. Figure 5a shows an example in which a 2 s application of nicotine elicited a 2.7 $\Delta F/F_0$ rise in $[Ca^{2+}]_i$ 1 min after patching and a 2.8 $\Delta F/F_0$ 10 min later (corresponding values for current peak were -1016 and -1200 pA, respectively; data not shown). While baseline $[Ca^{2+}]_i$ was also similar, the decay half-time of the two $[Ca^{2+}]_i$ signals differed considerably (18 and 38 s, respectively). Also different was the extent of recovery of test currents 30 s after the conditioning pulse (81 and 41% for 1 and 11 min recording, respectively). This observation suggested that continuous dialysis of the cell cytoplasm by the patch pipette interfered with the process of Ca^{2+} removal and with recovery from desensitization. This issue was explored in more detail by recording nicotine currents from cells patched with pipettes containing neither buffers nor fluo-3 (imaging was thus not carried out). Figure 5b shows an example in which after 1 min of whole cell conditioning, test currents (applied at 15 s intervals) largely recovered (to 76% of control) 30 s after a conditioning dose. Ten min later the recovery process after a new conditioning dose was substantially less (33% 30 s after the conditioning application). Tests run at intermediate (6 min) or longer (16 min) times with similar protocols indicate that this process developed gradually and was completed by about 10 min (Figure 5b). The amplitude of test current remained stable throughout the experiment once each recovery phase was completed (see control values before conditioning pulse in Figure 5b). The peak inward current induced by 2 s nicotine was -2400 pA ($\tau_1 = 75$ ms; $\tau_2 = 558$ ms) after 1 min patch clamping and -2000 pA ($\tau_1 = 102$ ms; $\tau_2 = 530$ ms) 15 min later, indicating that the development of desensitization was not dependent on the time from whole-cell establishment. These data, while ruling out the possibility that slow recovery from desensitization was artefactually produced by fluo-3, show that recovery was influenced by the persistence of high $[Ca^{2+}]_i$ which, in turn, presumably depended on the degree of internal Ca^{2+} buffering.

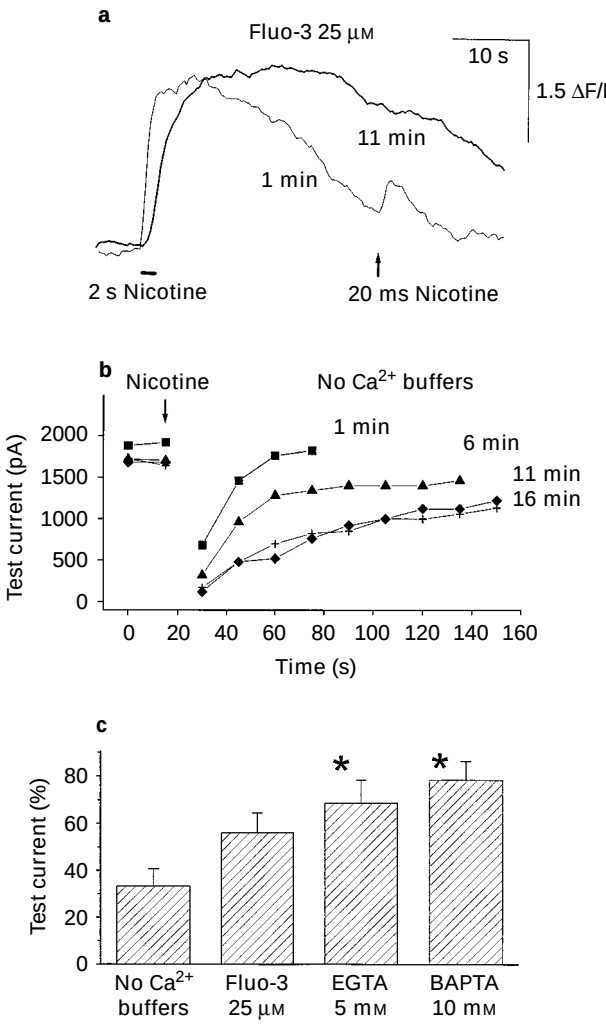


Figure 5 Recovery from desensitization and persistence of $[Ca^{2+}]_i$ rise depended on degree of intracellular Ca^{2+} buffering or duration of whole cell recording. (a) Time course of $[Ca^{2+}]_i$ rise induced by 2 s nicotine (horizontal bar) either after 1 min from obtaining whole cell configuration (thin trace) or 11 min later (thick trace). Note that baseline and peak $[Ca^{2+}]_i$ level were unchanged while decay was prolonged. Both Ca^{2+} responses were recorded from the same voltage clamped cell (concomitant current responses are not shown). Arrow points to response evoked by 20 ms test pulse of nicotine. (b) Time course of recovery of test currents after 2 s conditioning pulse of nicotine (arrow) recorded at 1, 6, 11 or 16 min after whole cell configuration had been obtained. All data were from the same cell patched with an electrode containing no exogenous Ca^{2+} chelators (such as fluo-3, EGTA or BAPTA) and are representative of results obtained from 5 cells. In this type of experiment no imaging was performed. Current amplitude (ordinate scale) is in absolute values. (c) Histograms of depression of test current amplitude 30 s after conditioning dose of nicotine (expressed as % of test currents before conditioning dose; ordinate scale) in cells after 10 min recording with patch pipettes containing no Ca^{2+} buffers ($n=10$), fluo-3 ($n=15$), EGTA ($n=8$) or BAPTA ($n=4$). Asterisks indicate that EGTA and BAPTA data are significantly different from no Ca^{2+} buffers data ($P<0.05$; ANOVA test). Note that, because of the intracellular solution used, simultaneous imaging (data not shown) was carried out only when fluo-3 was applied.

Figure 5c illustrates a summary of data (obtained at about 10 min in whole cell conditions) with various (or no) internal Ca^{2+} chelators: 30 s after a conditioning dose the recovery of nicotinic AChRs from desensitization was limited with no exogenous buffers or fluo-3, while it was significantly larger with EGTA or BAPTA. Since our results were collected 10 min after establishing whole-cell configuration, we routinely measured the recovery from desensitization under steady state conditions.

Role of $[Ca^{2+}]_i$ in onset and steady state desensitization

The trace of Figure 6a exemplifies (on a fast time base) the fade of the inward current evoked by 2 s nicotine. The current declined from a peak amplitude (A_{peak}) of -640 pA to a quasi-steady state level (A_{end}) of -69 pA just at the end of the pulse administration. During nicotine application the current decay from its peak was well fitted with two time constants ($\tau_1=83$ and $\tau_2=536$ ms, respectively). In about 40% of cells after the application of nicotine had ceased, the current response transiently bounced back ('rebound'; see MacOnochie & Knight, 1992) to 29 ± 5% of the peak value (data not shown). On a random sample of 5 cells baseline current was always reattained with a monoexponential time course of 4.6 ± 1.4 s after the rebound peak. Due to its limited occurrence, the rebound phenomenon was not further investigated. Since imaging of $[Ca^{2+}]_i$ had slower sampling rate than current recording, we used different degrees of $[Ca^{2+}]_i$ buffering to ascertain if the onset and steady-state level of desensitization were related to $[Ca^{2+}]_i$. Figure 6b–e shows that peak current amplitude, A_{end}/A_{peak} ratio, τ_1 and τ_2 values did not significantly change in the four experimental conditions tested, namely no exogenous buffers, fluo-3, EGTA or BAPTA. The only value which appeared significantly different was the increase in the peak amplitude of the inward current in the presence of BAPTA. These results suggest that the rise in $[Ca^{2+}]_i$ is not the main factor modulating nicotinic AChR desensitization over this time scale.

Subcellular distribution of $[Ca^{2+}]_i$ rise by nicotine

Averaging Ca^{2+} signals over the whole cell section did not allow the examination of the discrete subcellular distribution of this divalent cation during responses to nicotine application. In order to assess whether whole cell scans were providing adequate information about $[Ca^{2+}]_i$ changes, we performed rapid single line scans (10 ms sampling) on intact, non-patched cells (see Methods) in the presence of 0.1 mM Cd^{2+} which has been previously demonstrated to block $[Ca^{2+}]_i$ rises mediated by voltage-activated Ca^{2+} channels. Furthermore, this approach enabled us to address an additional question, namely if the characteristics of decay of the large $[Ca^{2+}]_i$ transients previously observed under voltage-clamped conditions were comparable to those physiologically exhibited by intact cells. Figure 7a shows that, in an intact cell, $[Ca^{2+}]_i$ grew faster in a 1.6 μm perimembrane segment of the scanning line than in the diameter section of the cell (rise time = 1 and 5 s, respectively), although in both cases the decay proceeded at comparable rates. These findings indicate that the kinetics of Ca^{2+} decay were not lost with whole cell section imaging and that they were similar to the ones at the level of the cell membrane. The same type of analysis performed on five cells provided similar results. The present data thus accord with the observation, based on fura-2 $[Ca^{2+}]_i$ imaging of bovine chromaffin cells, that nicotine increases $[Ca^{2+}]_i$ predominantly at the level of the subplasmalemmal area of the cell (Cheek *et al.*, 1989).

The next question to be addressed was whether peak and decay values of $[Ca^{2+}]_i$ differed significantly between voltage clamped and intact cells. Figure 7b shows that in intact cells the Ca^{2+} channel blocker Cd^{2+} (0.1 mM) significantly decreased the peak $[Ca^{2+}]_i$ response induced by a 2 s nicotine application to a value not different from the average one found in voltage-clamped cells. However, the decay of the $[Ca^{2+}]_i$ signal remained approximately the same regardless of whether cells were intact, treated with Cd^{2+} or voltage-clamped (Figure 7c).

Discussion

The principal finding of the present study is the novel observation on rat chromaffin cells that the speed of recovery of nicotinic AChRs from desensitization was related to the time

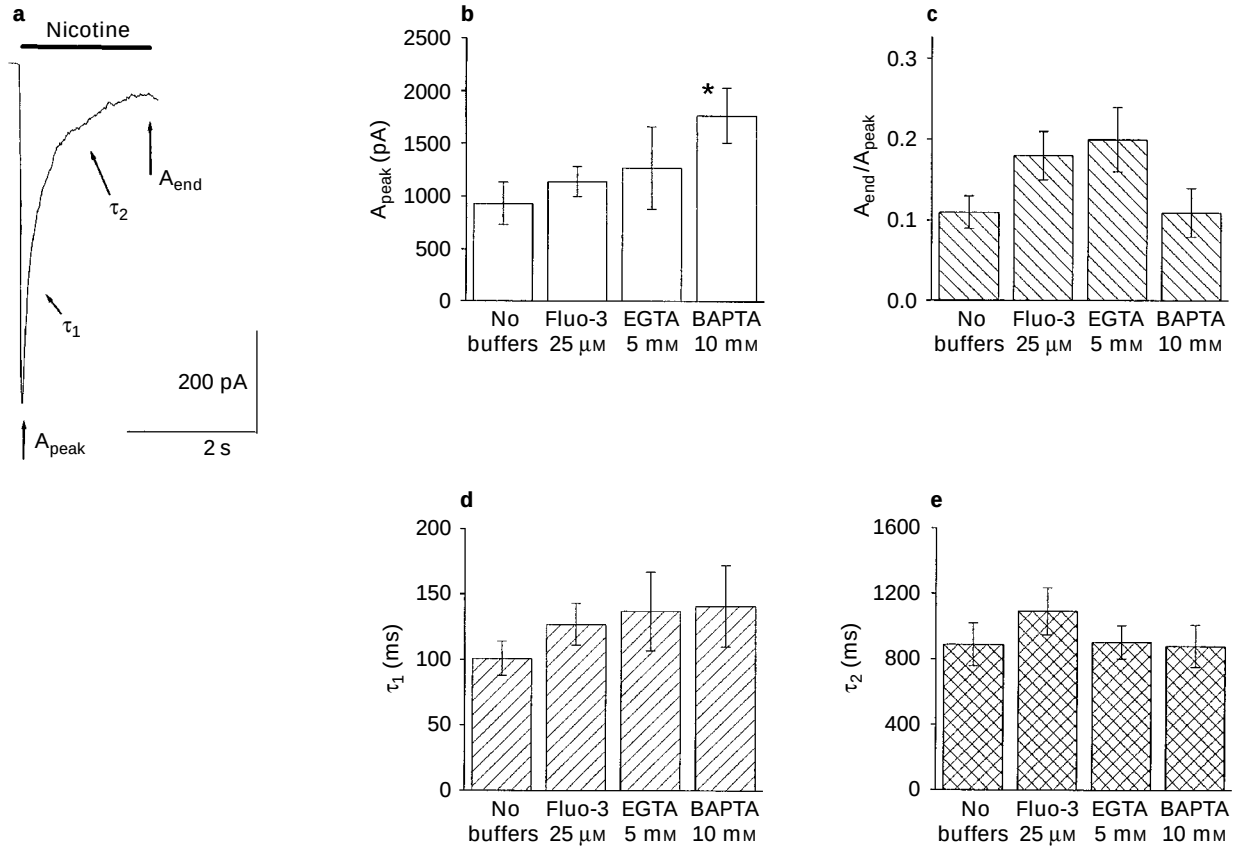


Figure 6 Onset and steady state level of desensitization during different degree of $[Ca^{2+}]_i$. Trace in (a) shows inward current induced by 2 s pulse of nicotine (horizontal bar). A_{peak} = peak current amplitude; τ_1 = first decay time constant; τ_2 = second decay time constant; A_{end} = current level at the end of nicotine application. (b–e) Histograms of these parameters in cells recorded in the absence of Ca^{2+} buffers, or after addition of fluo-3, EGTA or BAPTA intracellularly. Asterisk in (b) indicates that the BAPTA data are significantly different from no buffer data ($P<0.05$; Student's t test). For further details see legend to Figure 5c.

course of $[Ca^{2+}]_i$ changes. Onset and steady state level of desensitization were apparently independent of $[Ca^{2+}]_i$ transients.

Nicotine-induced responses of chromaffin cells

The responses observed in the present study were mediated by neuronal nicotinic AChRs since they were elicited by nicotine and blocked by (+)-tubocurarine (for review see Volle, 1980). The average reversal potential of the nicotine-induced current was about −15 mV, a value close to that (−7 mV) obtained by Nooney *et al.* (1992) for bovine chromaffin cells under similar conditions. These observations thus imply that activated nicotinic AChRs are permeable to mono and divalent cations. Inward currents evoked by 10–20 ms applications of nicotine were associated with $[Ca^{2+}]_i$ transients which grew with a slower time course (Vernino *et al.*, 1994). The $[Ca^{2+}]_i$ signals were mainly due to influx of Ca^{2+} through nicotinic AChRs since they were blocked by (+)-tubocurarine, prevented in Ca^{2+} free media, unaffected by the Ca^{2+} channel blocker Cd^{2+} and shared the same sensitivity to membrane potential and pressure application of nicotine as the inward currents. A previous investigation carried out on rat parasympathetic ganglia found that Cd^{2+} blocked currents through nicotinic receptor channels with an IC_{50} value of 1 mM (Nutter & Adams, 1995) which is 10 times larger than the concentration used in the present study. It is therefore not unexpected that in our study a low concentration of Cd^{2+} spared nicotine-evoked responses, while it strongly decreased $[Ca^{2+}]_i$ rises through Ca^{2+} channels. A previous investigation has also shown that, on chromaffin cells, the nicotine-induced $[Ca^{2+}]_i$ rise is indeed due to influx rather than intracellular release of this cation because a variety of experimental approaches to manipulate internal Ca^{2+} stores, including the application of thapsigargin, an inhibitor of $[Ca^{2+}]_i$ sequestration, failed to affect the Ca^{2+} signal (Vernino *et al.*, 1994). On rat chromaffin cells 2.5–5% of the nicotinic AChR current is carried by Ca^{2+} (Zhou & Neher, 1993; Vernino *et al.*, 1994): this observation explains why in Ca^{2+} free solution there was insignificant depression of membrane currents.

Molecular biology studies have indicated that chromaffin cells can express various subtypes of nicotinic AChR made up by heterologous assembly of α_3, α_5 and β_4 subunits (Criado *et al.*, 1992; Campos-Caro *et al.*, 1997) plus a distinct α_7 homomeric receptor blocked by α-bungarotoxin (Garcia-Guzman *et al.*, 1995). Since nicotinic responses observed in the present study as well as in former investigations (Fenwick *et al*, 1982; Vernino *et al.*, 1994; Nooney & Feltz, 1995) were insensitive to α-bungarotoxin, it appears that the α_7 receptor did not contribute to the current or $[Ca^{2+}]_i$ responses.

Rapid confocal imaging of $[Ca^{2+}]_i$

Since $[Ca^{2+}]_i$ imaging of the whole optical section of a single chromaffin cell provides only an average of $[Ca^{2+}]_i$ changes, it is possible that $[Ca^{2+}]_i$ signal measured smooths out subtle and rapid variations at different subcellular compartments. In order to explore this possibility single line scans were performed on intact cells superfused with Cd^{2+} containing solution: at the perimembrane segment the $[Ca^{2+}]_i$ signal induced by nicotine had faster rise time confirming its transmembrane origin. However, the rate of decay of $[Ca^{2+}]_i$ was essentially the same at the perimembrane area or through the entire cell section.

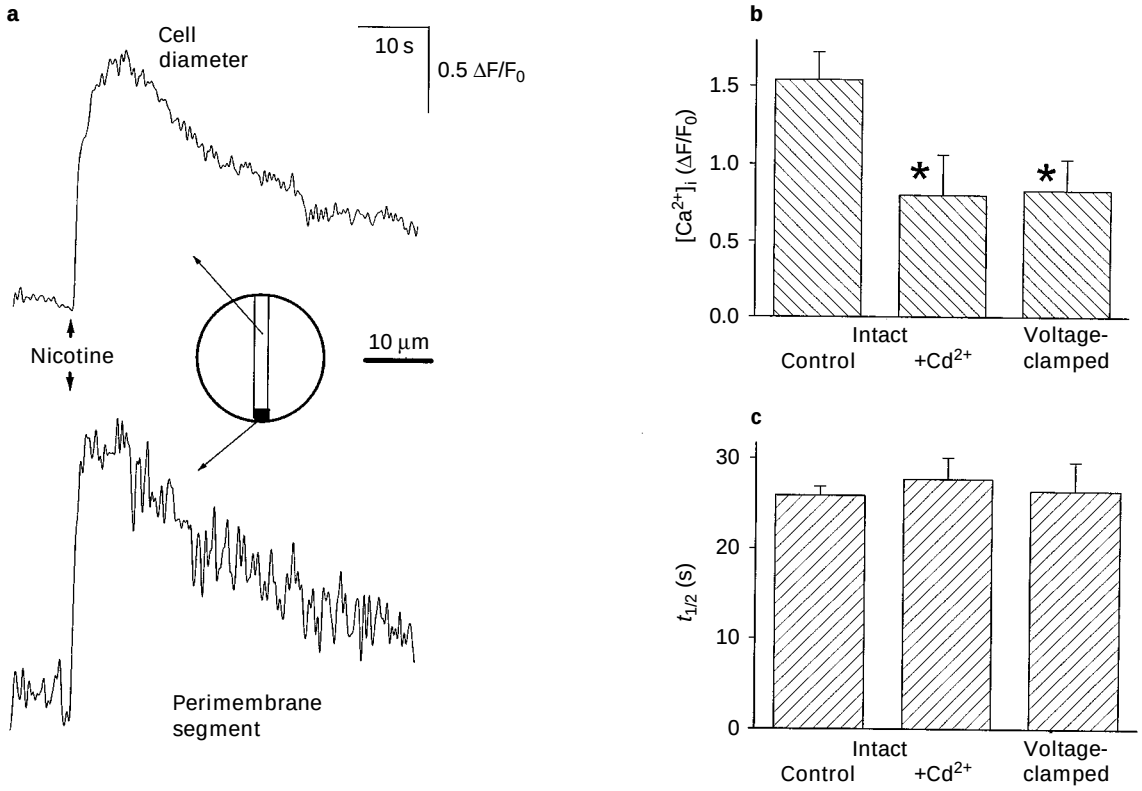


Figure 7 Characteristics of nicotine-induced $[Ca^{2+}]_i$ changes in intact (non-patched) versus voltage clamped patched cells. (a) Comparison of time course of $[Ca^{2+}]_i$ changes recorded with a single line scan (top; see scheme in inset) or within a 1.6 μm section of the perimembrane area (bottom) from an intact cell exposed to 2 s nicotine (arrows). (b) Histograms of fluorescence signal (ordinate scale) recorded from intact cells (either in control or in 0.1 mM Cd^{2+} containing solution) or from voltage clamped cells (whole cell patch configuration) following pressure application of 2 s nicotine. Intact cells were loaded with bath-applied fluo-3 AM while voltage clamped cells received fluo-3 through the patch pipette. Asterisks in (b) indicate that responses in Cd^{2+} solution or from voltage clamped cells are significantly different from those of intact cells in control solution ($P<0.05$; ANOVA test). Note that there was no difference in signal amplitude between intact cells (in Cd^{2+} solution) cells and voltage clamped cells. (c) Histograms of half decay time of $[Ca^{2+}]_i$ rise induced by 2 s nicotine in conditions as detailed in (b). Note that regardless of the experimental conditions the decay of $[Ca^{2+}]_i$ was similar.

The process controlling the slow decay of $[Ca^{2+}]_i$ transients was not examined in the present study and will require a separate investigation. However, it is worth noting that $[Ca^{2+}]_i$ decayed from its peak at a similar rate in intact or voltage clamped cells, suggesting that voltage clamping under whole cell patch conditions did not apparently introduce an artefactually slow decline of $[Ca^{2+}]_i$ which might have distorted the operation of nicotinic AChRs.

Role of Ca^{2+} in desensitization of nicotinic AChRs

The most direct approach to assess the role of Ca^{2+} in nicotinic AChR desensitization is by imaging $[Ca^{2+}]_i$ changes while measuring currents induced by nicotine. A complementary, albeit indirect, approach was to observe the onset of desensitization during controlled alterations in $[Ca^{2+}]_i$ brought about by experimental manipulations with various chelating agents (Miledi, 1980; Chestnut, 1983). We dissected out various phases of desensitization which comprised biexponential onset (observed as τ_1 and τ_2 values of current fade during 2 s application of nicotine; MacOnochie & Knight, 1992), apparent steady state level (measured as fractional current decrease at the end of the same application) and recovery from desensitization (measured as % amplitude of test currents versus preconditioned controls). Amongst the various phases of desensitization investigated for their dependence on $[Ca^{2+}]_i$ only the time course of recovery was strongly correlated with the decay rate of $[Ca^{2+}]_i$ from its peak (induced by 2 s nicotine). Thus, a persistently high $[Ca^{2+}]_i$ was always associated with slower desensitization recovery.

Such a conclusion was based on four lines of evidence: (1) although cells displayed considerable individual variability in the duration and extent of changes in $[Ca^{2+}]_i$, in each case desensitization recovery was related to how persistent the $[Ca^{2+}]_i$ rise was; (2) raising $[Ca^{2+}]_o$ from 2 to 10 mM was associated with much slower recovery from desensitization and very sustained increase in $[Ca^{2+}]_i$. A comparable observation has been made on nicotinic AChRs of the frog neuromuscular junction after application of ruthenium red which is supposed to increase $[Ca^{2+}]_i$ (Chestnut, 1983). Even if an elevation in $[Ca^{2+}]_o$ can modulate the opening of nicotinic AChRs (Amador & Dani 1995), this phenomenon was not expected to influence the way in which desensitization was measured in the present study, since test currents were always expressed as % of the ones observed in the same solution. Furthermore, neither the amplitude of the conditioning current nor its fading (which reflects desensitization onset) was altered in high $[Ca^{2+}]_o$. (3) Intracellularly applied EGTA or BAPTA ensured a rapid recovery from desensitization while omission of exogenous Ca^{2+} buffer slowed it down. (4) As in the same cell the duration of the $[Ca^{2+}]_i$ rise evoked by 2 s nicotine became longer with time elapsed after the establishment of whole-cell conditions, there was a concomitant slowing of recovery from desensitization. Presumably ongoing cell dialysis by the patch pipette solution largely removed physiological buffers and intracellular factors required to terminate the rise in $[Ca^{2+}]_i$. This phenomenon was first noted with fluo-3 loaded cells and subsequently confirmed by analysing recovery from desensitization in cells patched without any Ca^{2+} chelator in the pipette solution.

The present results may have some wider implications: for example, the duration of the whole-cell recording, the time (during the recording session) selected for the desensitization tests, as well as the presence of exogenous chelators in the patch pipette might largely determine the outcome of experiments investigating the role of Ca^{2+} in receptor desensitization. Our data also indicate that voltage clamping the cell did not lead to underestimation of the role of $[Ca^{2+}]_i$ in desensitization recovery. In fact, despite the different $[Ca^{2+}]_i$ amplitude between intact and patched cells the decay of $[Ca^{2+}]_i$ from its peak (which is the crucial element to control recovery from desensitization) proceeded at a similar rate.

Is the onset of desensitization dependent on $[Ca^{2+}]_i$?

Early work on nicotinic AChRs of skeletal muscle fibres has suggested that $[Ca^{2+}]_o$ is important in the control of onset and extent of desensitization (Manthey, 1966; Magazanik & Vyskocil, 1970), although it has subsequently been shown that desensitization still takes place in the absence of $[Ca^{2+}]_o$ and is reduced by an injection of EGTA into the postsynaptic fibre (Miledi, 1980). Unlike muscle nicotinic AChRs, neuronal-type receptors of chromaffin cells are highly permeable to Ca^{2+} (Vernino *et al*., 1994) which is thus potentially capable of modulating the process of desensitization. Nevertheless, desensitization of the population response of chromaffin cells to carbachol has been shown to be independent of $[Ca^{2+}]_i$ (Sasakawa *et al*., 1985). The present investigation based on a higher resolution approach at single cell level confirmed a lack of effect of $[Ca^{2+}]_i$ on the onset or degree of desensitization, as shown by the use of different levels of $[Ca^{2+}]_i$ buffering, and argues against a direct role of this cation in promoting desensitization. Since steady state desensitization appears to be a process distinct from recovery of desensitization in terms of sensitivity to $[Ca^{2+}]_i$, it is possible that each process is a potential target for independent modulation by intracellular second messengers.

Molecular mechanisms underlying modulation by $[Ca^{2+}]_i$ of nicotinic AChR recovery

The present data can be interpreted as due to either a direct modulation by lingering $[Ca^{2+}]_i$ of nicotinic AChRs or a $[Ca^{2+}]_i$-dependent metabolic process controlling receptor function (see for example Boyd, 1987). Although the long lasting nature of $[Ca^{2+}]_i$ increase by nicotine is compatible with the first hypothesis, initially put forward by Manthey (1966), the second possibility accords with recent biochemical and electrophysiological observations. In fact, molecular biology studies have shown that desensitization is an intrinsic property of ligand-gated ionotropic receptors (see review by Lena & Changeux, 1993) and that is can be modulated by intracellular second messengers via phosphorylation of certain receptor subunits (Levitan, 1994). Several studies have focussed on protein kinase C (PKC) as the $[Ca^{2+}]_i$ activated effector which modulates desensitization of nicotinic AChRs. However, the situation remains unclear, since on chromaffin cells PKC activity has been found to promote (Downing & Role, 1987), to decrease (Lin *et al*., 1993) or to have no effect on nicotinic AChR desensitization (Loneragan *et al*., 1996). Recent work on nicotinic AChRs of muscle fibres (Hardwick & Parsons, 1996) has suggested that recovery from desensitization is regulated by the fine balance between PKC and phosphatase activity (which are both $[Ca^{2+}]_i$ dependent): further experiments are necessary to establish whether this process also takes place on nicotinic AChRs of chromaffin cells.

Regardless of the molecular substrate responsible for controlling recovery from desensitization, a mechanistic interpretation of the present data might be provided by assuming that on chromaffin cells the operation of nicotinic AChRs can be described with a kinetic scheme similar to the one used for muscle AChRs (Cachelin & Colquhoun, 1989) and which is a modification of the classical cyclic model of receptor desensitization (Katz & Thesleff, 1957). In this case the transition of the agonist-bound activated receptor to each one of the two sequential, desensitized states (corresponding to fast and slow phases of desensitization) would not be influenced by Ca^{2+} (or a Ca^{2+} dependent messenger) because the τ_1 and τ_2 values of current fading and the steady state desensitization level were unchanged by manipulations of $[Ca^{2+}]_i$. It therefore seems that the action of $[Ca^{2+}]_i$ is downstream of the fast desensitized state and may consist of a stabilizing effect on the nicotinic AChR in a slow desensitized conformation either by discrete binding to certain sites of the receptor (Lean & Changeux, 1993) or via Ca^{2+} dependent phosphorylation/dephosphorylation of the receptor complex.

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