The Journal of Neuroscience, December 1, 1999, *19*(23):10280–10288

Actin Filaments and the Opposing Actions of CaM Kinase II and Calcineurin in Regulating α7-Containing Nicotinic Receptors on Chick Ciliary Ganglion Neurons

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Nicotinic acetylcholine receptors containing α7 subunits have a high relative permeability to calcium and influence numerous calcium-dependent cellular events. On chick ciliary ganglion neurons the receptors are concentrated on somatic spines containing actin filaments. Using conventional whole-cell patch-clamp recording from dissociated ciliary ganglion neurons, we show that responses from α7-containing receptors undergo substantial rundown when the receptors are repeatedly challenged with nicotine. Stabilization of actin filaments with phalloidin partially prevents the rundown, whereas collapse of actin filaments with latrunculin A exacerbates it. The rundown depends on calcium influx through the receptors because it requires receptor activation and can be prevented by replacing extracellular calcium with barium or by intracellular dialysis with BAPTA. Thapsigargin and ryanodine each inhibit the rundown, demonstrating further a requirement for calcium release from internal stores. Blockade of calmodulin by calmidazolium or blockade of CaM kinase II with either KN93 or autocamtide-2-related inhibitory peptide each prevents the rundown; blockade of the phosphatase calcineurin with either cyclosporin A or deltamethrin increases the rundown. The results indicate a balance of calcium-dependent kinase and phosphatase activities in regulating the function of α7-containing receptors. Manifestation of the rundown depends in part on the loss of intracellular components via dialysis because little rundown is seen if perforated patch-clamp recording is used to monitor receptor responses even in latrunculin A-treated cells. A membrane-permeable calcineurin inhibitor, however, still decreases the nicotinic response in a calcium-dependent manner, confirming that calcium-dependent phosphoregulation of α7-containing receptors occurs in the intact cell.

Key words: nicotinic; acetylcholine; receptors; rundown; CaM kinase II; calmodulin; calcineurin; spines; α7; bungarotoxin; synaptic; ciliary

Excitation in the nervous system is often mediated by synaptic contacts terminating on dendritic spines (Harris and Kater, 1994; Schikorski and Stevens, 1997). Spine structure is thought to be important for confining the chemical consequences of synaptic signaling to local domains (Koch and Zador, 1993; Yuste and Denk, 1995). This is particularly relevant for calcium entry through NMDA receptors (Muller and Connor, 1991; Mainen et al., 1999) in which confinement serves two purposes, localizing downstream events such as induction of long-term potentiation (Engert and Bonhoeffer, 1997) and reducing the likelihood of calcium-induced cytotoxicity (Choi, 1992).

The spine contains regulatory machinery to limit calcium influx caused by synaptic activity. Calcium entering through NMDA receptor channels activates calmodulin that exerts relatively rapid negative feedback regulation via receptor inactivation. The inactivation involves both a direct interaction of calmodulin with the C terminal of NMDA receptors and a disruption of interactions with α-actinin, linking the receptors to the actin cytoskeleton (Legendre et al., 1993; Wyszynski et al., 1997; Zhang et al., 1998; Krupp et al., 1999). Calcium also causes a slow onset, long-lasting rundown of NMDA receptor function, and the effect depends on the state of actin filaments; collapse of the filaments accelerates the rundown, whereas stabilization of the filaments retards it (Rosenmund and Westbrook, 1993a,b). Calcium-dependent inactivation and rundown may share a final common pathway in the case of NMDA receptors (Westbrook et al., 1997). Interestingly, the calcium/calmodulin-dependent phosphatase calcineurin also diminishes NMDA receptor function (Lieberman and Mody, 1994), and NMDA receptor activation can destabilize actin filaments via a calcineurin-dependent process (Halpain et al., 1998). This may contribute to the rundown of NMDA responses.

Nicotinic acetylcholine receptors containing the α7 gene product (α7-nAChRs) are widely expressed in the nervous system (Couturier et al., 1990; Schoepfer et al., 1990; Anand et al., 1993; Conroy and Berg, 1998). The receptors have a high relative calcium permeability, equivalent to that of NMDA receptors (Bertrand et al., 1993; Seguela et al., 1993). On chick ciliary neurons, α7-nAChRs are concentrated on somatic spines tightly folded into discrete clumps (Shoop et al., 1999) underlying an extended presynaptic calyx (Hess, 1965). Presynaptic stimulation can evoke large, rapidly decaying currents through the receptors (Zhang et al., 1996; Ullian et al., 1997). Physiological concentrations of calcium potentiate the α7-nAChR response by acting at an extracellular site on the receptor (Bonfante-Cabarcas et al., 1996; Galzi et al., 1996; Liu and Berg, 1999).

The present experiments were undertaken to determine

Received July 13, 1999; revised Aug. 31, 1999; accepted Sept. 17, 1999.

This work was supported by the National Institutes of Health Grants NS 12601 and 35469 and Tobacco-Related Disease Research Program Grant RT65-0050. Q.-s.L. is an American Heart Association Postdoctoral Fellow.

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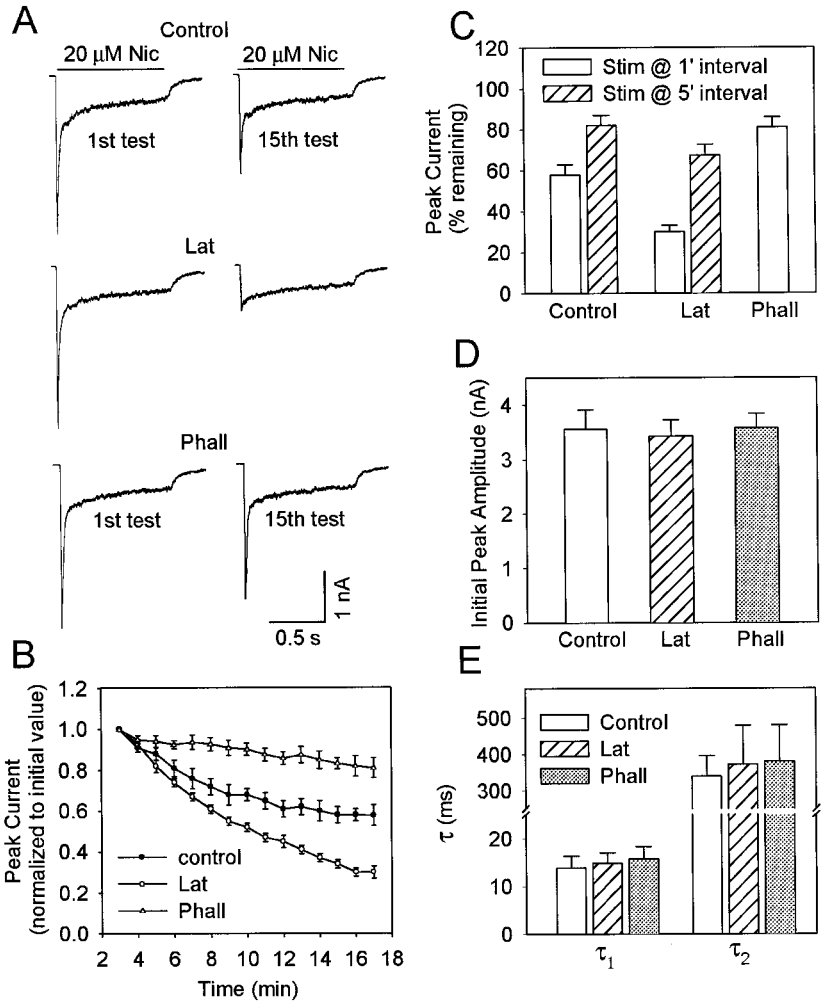

Figure 1. Activity-dependent rundown of the nicotinic response and the effects of the actin cytoskeleton. *A,* Whole-cell conventional patch-clamp recordings of nicotine-induced currents in dissociated chick ciliary ganglion neurons. Nicotine (20 μM; *Nic*) was delivered for a duration of 1 sec from a rapid applicator at 1 min intervals over a 15 min test period. The first (*1st test*) and last (*15th test*) responses are shown. Cells contained either untreated actin filaments (*Control; top*), actin filaments collapsed by treatment with latrunculin A (*Lat; middle*), or actin filaments stabilized with phalloidin (*Phall; bottom*). The holding potential was −60 mV. *B,* Time course of rundown for the nicotinic response. Nicotine-induced peak currents were normalized to the initial amplitude for each individual cell; values represent the mean ± SEM of eight cells per condition. *C,* Proportion of the peak current remaining at the end of the 15 min test period for cells treated as indicated and stimulated (*Stim*) either at 1 min (*open bars*) or 5 min (*hatched bars*) intervals. The rundown was stimulation dependent, augmented by latrunculin A treatment ($p < 0.005$), and diminished by phalloidin ($p < 0.01$). *D,* Peak amplitude of the initial response to nicotine. No significant differences were found among the groups ($p > 0.5$). *E,* Decay time constants for the fast and slowly decaying components. No significant differences were found among the groups ($p > 0.5$).

whether the actin cytoskeleton plays a role in α7-nAChR function. Furthermore, we wanted to determine whether calcium influx provides negative feedback control as it does for NMDA receptors. The results demonstrate that activity-dependent rundown of the α7-nAChR response does occur but that it results from a novel mechanism involving calcium release from internal stores and activation of CaM kinase II. Both the integrity of the actin cytoskeleton and the phosphatase calcineurin act to oppose the rundown. CaM kinase II and calcineurin may provide antagonistic-paired mechanisms for rapid and versatile calcium-dependent regulation of α7-nAChR function.

MATERIALS AND METHODS

Cell preparations. Dissociated ciliary ganglion neurons were prepared from 13- to 14-d-old chick embryos along previous lines (Margiotta and Gurantz, 1989) exactly as described recently (Liu and Berg, 1999). The dissociated cells were allowed to settle in whole culture medium onto glass coverslips or 35 mm plastic tissue culture dishes (Falcon; Fisher Scientific, Houston, TX) coated with 1–3 mg/ml poly-D-lysine for at least 2 hr before analysis. In some cases, cells were treated with 5 μM latrunculin A for 2–3 hr, after an initial 1.5–2 hr period of cell attachment. The cells were taken for recording within 6 hr of dissociation and until that time were maintained in a humidified tissue culture incubator with 95% air/5% CO_2 at 37°C.

Electrophysiology. Whole-cell patch-clamp recordings were obtained from isolated neurons at room temperature as described previously (Hamill et al., 1981; Zhang and Berg, 1995). Both conventional and perforated patch-clamp (Horn and Marty, 1988; Rae et al., 1991) recording methods were used. These procedures, including pipette preparation, patch formation and criteria for acceptance, series resistance measurement and compensation, and recording and analysis of membrane currents for dissociated ciliary ganglion neurons under these conditions, were similar to those used previously (Zhang et al., 1994; Zhang and Berg, 1995) and have been described recently in detail (Liu and Berg, 1999). For conventional (dialyzing) whole-cell experiments the intracellular solution contained (in mM): 140 CsCl, 2 $MgCl_2$, 2 EGTA, and 10 HEPES, pH 7.2 (with CsOH). In some experiments the 2 mM EGTA was replaced with 10 mM Cs_4BAPTA, and the 140 mM CsCl was reduced to 130 mM to maintain osmolarity. The ATP-generating system used in some experiments contained (in mM): 120 CsCl, 2 $MgCl_2$, 5 Mg-ATP, 2 EGTA, 20 phosphocreatine, and 10 HEPES, pH 7.2 (with CsOH); creatine phosphokinase was included at 50 U/ml. In cases in which intracellular dialysis via the patch pipette was used to deliver compounds, a waiting period of 3 min was imposed between the time of membrane rupture and the first test with nicotine. High molecular weight chemicals likely to interfere with formation of the gigaseal were loaded into the pipette by backfilling, and intracellular solution alone was used to fill the pipette tip. In these cases a 3 min delay was used between gigaseal formation and membrane rupture to permit mixing of the pipette contents by diffusion before intracellular dialysis was initiated. The intracellular solution in perforated patch experiments contained (in mM): 145 CsCl, 2 $MgCl_2$, and 10 HEPES, pH 7.2 (with CsOH). The holding potential was −60 mV throughout this study. Cells were individually stimulated with 1 sec pulses of 20 μM nicotine delivered with a rapid applicator (Zhang et al., 1994) usually at 1 min intervals, although some experiments used longer intervals as indicated. Only one cell was tested in each culture dish. Unless otherwise indicated, values are presented as the mean ± SEM and were evaluated for significance using either the paired or unpaired *t* test as appropriate.

Materials. White Leghorn chick embryos were purchased locally and

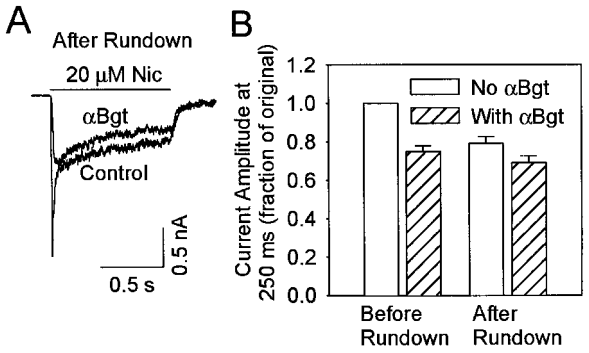

Figure 2. Subtype specificity of nicotinic receptor rundown. *A,* Whole-cell patch-clamp recordings showing the total and α-bungarotoxin (*αBgt*)-resistant portion of the nicotine-induced current in the same neuron at the end of the 15 min rundown period. Nicotine (20 μM; *Nic*) was delivered for 1 sec at 1 min intervals over a 15 min period before testing immediately before (*Control*) and after (*αBgt*) incubating with 100 nM αBgt for 3 min. Most of the slowly desensitizing response was resistant to αBgt and therefore attributable to α3*-nAChRs. *B,* Proportion of the slowly desensitizing response attributable to α3*-nAChRs before and after rundown. The amplitude of the nicotine-induced current (*open bars*) was measured 250 msec after initiating the response in the same neurons before (*Before Rundown*) and after (*After Rundown*) delivering the 15 min stimulation protocol. αBgt was then applied to the same neurons to determine the proportion of the response caused by α3*-nAChRs at the end of the rundown period (*hatched bars*). The proportion of the response attributable to α3*-nAChRs before rundown was determined by measuring the 250 msec values in records obtained previously (Liu and Berg, 1999). The 15 min stimulation protocol produced a decrease in the slowly desensitizing αBgt-sensitive response measured at 250 msec ($p \leq 0.01$) as it did in the rapidly desensitizing peak response attributable to α7-nAChRs (see Fig. 1), but it produced no significant rundown of the α3*-nAChR response ($p > 0.3$). The values represent the mean ± SEM of 6–10 cells and have been normalized for the current detected initially.

maintained at 37°C in a humidified incubator. Chemicals were purchased from the following sources: α-bungarotoxin (αBgt) from Biotoxins (St. Cloud, FL); latrunculin A and Cs_4BAPTA from Molecular Probes (Eugene, OR); calmidazolium from Research Biochemicals (Natick, MA); thapsigargin, ryanodine, KN92, cyclosporin A, deltamethrin, and autocamtide-2-related inhibitory peptide (AIP) from Calbiochem (La Jolla, CA); and KN93 from both Research Biochemicals and Calbiochem, with identical results. All other reagents were purchased from Sigma.

RESULTS

Actin filaments and the activity-dependent rundown of α7-nAChR responses

Rapid application of 20 μM nicotine to a dissociated ciliary ganglion neuron voltage-clamped at −60 mV produces a biphasic inward current as monitored by conventional patch-clamp recording (Fig. 1*A, top*). The large, rapidly desensitizing portion of the current has been attributed to α7-nAChRs (Zhang et al., 1994) and represents >90% of the peak current initially (Liu and Berg, 1999). Repeated stimulation of the receptors at 1 min intervals caused a substantial rundown of the α7-nAChR response over a 15 min period (Fig. 1*B,C*). The 1 min intervals were chosen to allow recovery from agonist-induced desensitization between trials. When the intervals were extended to 5 min, less rundown occurred during the same test period (Fig. 1*C*). The results indicate that the rundown of α7-nAChR responses depends on receptor activation.

The state of the actin cytoskeleton influenced the extent of rundown. Latrunculin A is a membrane-permeant compound that collapses actin filaments and can induce retraction of dendritic

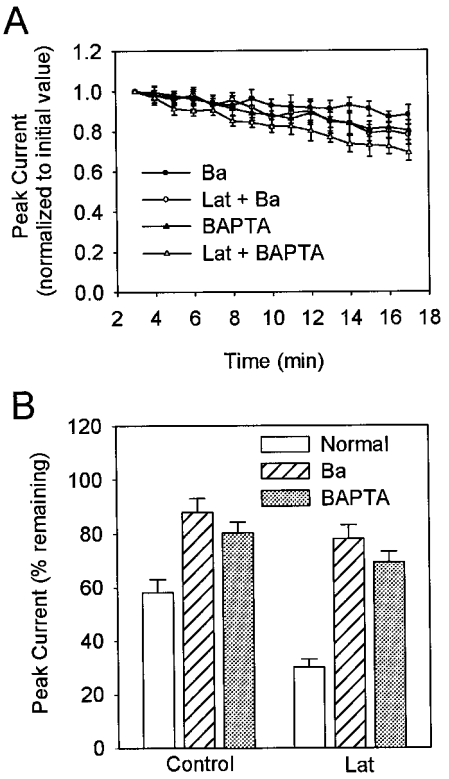

Figure 3. Dependence of α7-nAChR rundown on intracellular calcium accumulation. *A,* Changes in the time course of rundown caused by replacing extracellular calcium with barium (*Ba*) or internally dialyzing with 10 mM BAPTA (*BAPTA*) both in untreated cells and in cells treated with latrunculin A (*Lat*). Stimulation protocols are described in Figure 1. Values represent the mean ± SEM of six to eight cells each and have been normalized for the peak current present at the outset. *B,* Comparison of the peak current amplitude remaining at the end of the test period for untreated (*Control*) and latrunculin A-treated (*Lat*) neurons tested with normal recording solution (*open bars*; taken from Fig. 1*E*), extracellular barium instead of calcium (*hatched bars*), and intracellular BAPTA (*stippled bars*). The calcium replacement and the internal perfusion with BAPTA each protected against rundown in both the control and latrunculin A-treated cells ($p < 0.005$ in all cases).

spines in culture (Allison et al., 1998). Treating ciliary ganglion neurons in culture with 5 μM latrunculin A for 2–3 hr before testing accentuated the rundown; again, the extent of rundown depended on the amount of stimulation (Fig. 1*A, middle, B,C*). In contrast to the latrunculin A results, intracellular dialysis of the neurons with 5 μM phalloidin from the patch pipette to stabilize actin filaments caused the activity-dependent rundown to be much attenuated (Fig. 1*A, bottom, B,C*). Neither the latrunculin A nor the phalloidin treatment significantly affected the initial response of the neurons (Fig. 1*D*), nor did they affect the time constants for decay of either the fast or slowly desensitizing component (Fig. 1*E*). The results show that the rapidly decaying α7-nAChR response runs down during the 15 min test period in an activity-dependent manner and that the rundown is augmented by destabilization of actin filaments. The finding that latrunculin A-treated cells had the same initial peak amplitude response as did control cells indicates that disruption of the actin cytoskeleton did not itself produce rundown.

Selectivity of the rundown for nAChR subtype

Although all of the rapidly decaying nicotinic response from ciliary ganglion neurons is produced by α7-nAChRs, most of the

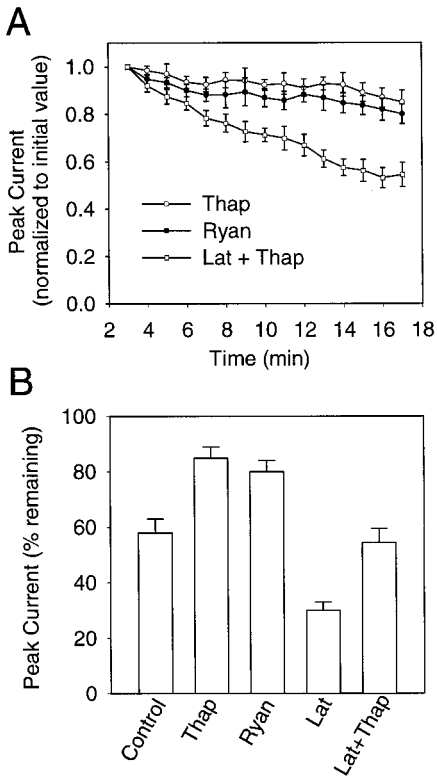

Figure 4. Calcium-induced calcium release from internal stores helps mediate rundown of the α7-nAChR response. *A,* Time course of rundown for nicotinic currents after blockade of calcium release from internal stores in cells with and without latrunculin A treatment. Normal (*Control*) or latrunculin A-treated (*Lat*) cells were internally dialyzed with 10 μM ryanodine (*Ryan*) or 1 μM thapsigargin (*Thap*) before testing. In the case of thapsigargin, the cells were also incubated with the compound 10 min before patch formation. *B,* Peak current remaining at the end of the rundown period, normalized for the size of the initial response in the same cell. Ryanodine and thapsigargin each significantly retarded rundown in control cells ($p < 0.01$ and 0.005, respectively), as did thapsigargin in latrunculin A-treated cells ($p < 0.005$). Values represent the mean ± SEM of six to eight cells each.

slowly decaying response is produced by α3*-nAChRs composed of α3, β4, α5, and sometimes β2 subunits (Vernallis et al., 1993; Zhang et al., 1994, 1996; Conroy and Berg, 1995; Ullian et al., 1997). On ciliary ganglion neurons the α3*-nAChRs are found both at postsynaptic densities and on somatic spines (Jacob et al., 1984; Loring and Zigmond, 1987; Shoop et al., 1999). Responses from α7-nAChRs and α3*-nAChRs can be dissected by incubating the neurons with αBgt that, at nanomolar concentrations, completely blocks the former while having no effect on the latter (Zhang et al., 1994, 1996; Ullian et al., 1997). When this is done, only a small fraction of the slowly decaying response is seen to be αBgt sensitive (Zhang et al., 1994, 1996; Ullian et al., 1997; Liu and Berg, 1999), possibly representing alternative behavior of α7-nAChRs that otherwise desensitize rapidly or resulting from a minor population of novel receptors in the ganglion (Pugh et al., 1995).

Normally the most straightforward way of determining whether α3*-nAChRs undergo activity-dependent rundown would be first to isolate the α3*-nAChR response by incubating neurons in αBgt (to block α7-nAChRs) and then to measure the peak amplitude of the response elicited at 1 min intervals throughout the 15 min test period. The difficulty with this procedure is that blockade of α7-nAChRs would prevent any α3*-nAChR rundown dependent on α7-nAChR activity, e.g., that resulting from calcium influx (see below). Accordingly, we omitted the αBgt blockade and instead focused on the total current amplitude 250 msec after initiation of the nicotinic response. At this late time almost all of the response is produced by α3*-nAChRs, because of the rapid desensitization of α7-nAChRs. Mean values of 0.98 ± 0.05 and 0.78 ± 0.03 nA (mean ± SEM; $n = 6$ cells) were obtained for the current amplitudes at 250 msec before and after the test period, respectively. This represents a rundown of ~20% for the combined αBgt-sensitive and -insensitive portions of the slowly desensitizing response. Applying 100 nM αBgt to the same neurons after the rundown period showed that ~90% of the current response at 250 msec was resistant to toxin and therefore the product of α3*-nAChRs (Fig. 2). This demonstrated not only that α3*-nAChRs undergo relatively little rundown but also that the activity-dependent decline in the α7-nAChR response seen previously did not represent a conversion to a slowly desensitizing species.

A more precise estimate of α3*-nAChR rundown was obtained by determining what fraction of the original response at 250 msec was also αBgt resistant. These measurements were made on current traces obtained previously with perforated patch-clamp recording (Liu and Berg, 1999). The comparisons were justified because those recordings yielded whole-cell peak current amplitudes and time courses that were indistinguishable from the values obtained here with conventional patch-clamp recording before rundown. The analysis showed that before rundown ~75% of the response at 250 msec was resistant to αBgt blockade (Fig. 2*B*). Comparing this with the 90% αBgt resistance seen after rundown indicates that essentially all of the 20% rundown seen in the total current at 250 msec represents loss of the small, slowly decaying toxin-sensitive response. There is almost no rundown of the α3*-nAChR response.

The results demonstrate that the activity-dependent rundown observed here is receptor specific. For the large, rapidly desensitizing α7-nAChR response the rundown can be dramatic, eliminating almost all of it in latrunculin A-treated cells. Because, as noted previously, the fast rise time of the rapidly decaying α7-nAChR response enables it to account for >90% of the whole-cell peak response initially, changes in the whole-cell peak current have been measured in the experiments below to examine the rundown mechanism of α7-nAChR responses. The α3*-nAChR peak response in isolation is nearly one-fourth as large as the α7-nAChR response but contributes little to the combined peak current normally because it has a slower rise time (Liu and Berg, 1999).

Dependence of the rundown on intracellular calcium

Because α7-nAChRs have a high relative permeability to calcium, a likely explanation for the dependence of rundown on receptor activation is that it permits calcium influx. This was tested in two ways. First, extracellular calcium was replaced with barium. Although complete calcium removal can reduce the peak response caused by α7-nAChRs to a third, barium substitution generates a response nearly as large as that seen in calcium (Liu and Berg, 1999). Substitution of 2 mM barium for extracellular calcium in the present experiments almost completely prevented the activity-dependent rundown of α7-nAChR responses (Fig. 3). This was true both for control cells and for cells in which actin filaments had been dispersed by treatment with latrunculin A.

A second test for the importance of intracellular calcium in causing the rundown was performed by dialyzing cells with 10 mM

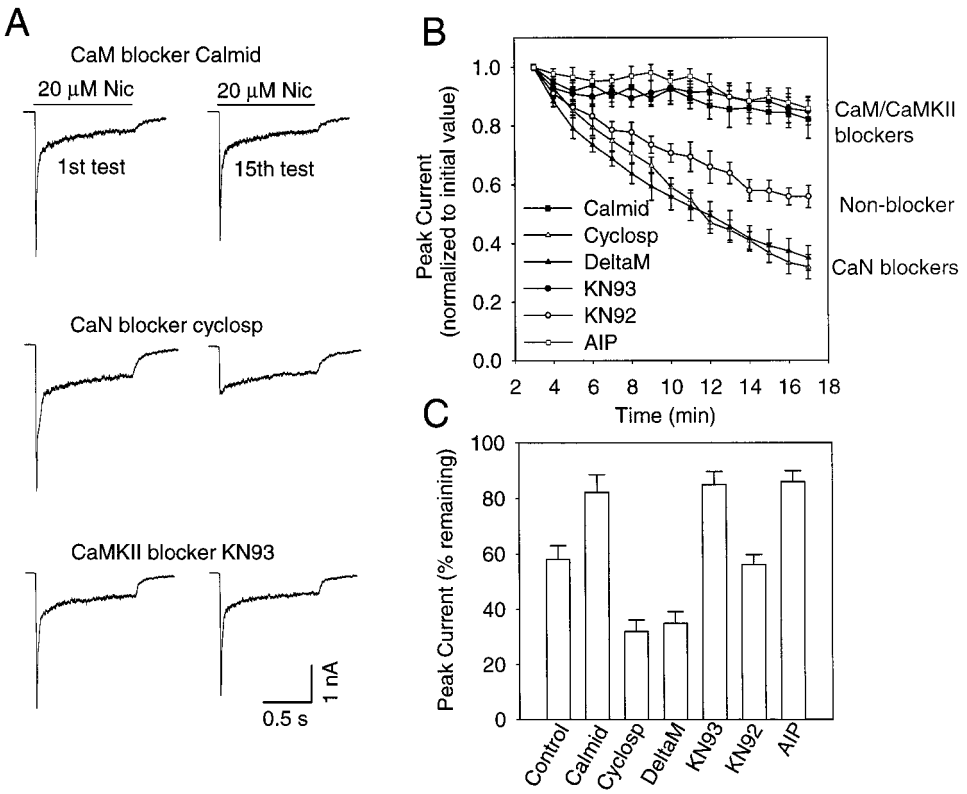

Figure 5. Calcium-dependent phosphoregulation of α7-nAChR responses. *A,* Whole-cell patch-clamp recordings from neurons at the beginning (*1st test*) and end (*15th test*) of the 15 min rundown protocol when the cells were internally dialyzed with the calmodulin (*CaM*) antagonist calmidazolium (*Calmid*; 10 μM; *top*), the calcineurin (*CaN*) inhibitor cyclosporin A (*cyclosp*; 200 nM; *middle*), or the CaM kinase (*CaMKII*) inhibitor KN93 (*KN93*; 50 μM; *bottom*). *B,* Rundown time course for the α7-nAChR response when cells were internally dialyzed with the indicated compounds from the patch pipette during the rundown protocol. Nicotine was first applied 3 min after dialysis was initiated by rupture of the membrane under the patch. Deltamethrin (*DeltaM*; 200 nM; a calcineurin inhibitor), KN92 (*KN92*; 50 μM; an inactive analog of KN93), autocamtide-2-related inhibitory peptide (*AIP*; 5 μM; an inhibitor of CaM kinase II), and other compounds described in *A* were tested. *C,* Peak current remaining at the end of the rundown period for each of the test conditions, normalized to the initial peak response in the same cells. In *Control*, the patch pipette contained normal solution. The calmodulin and CaM kinase blockers each inhibited rundown ($p < 0.01$ and 0.005, respectively), whereas the calcineurin blockers increased it ($p < 0.01$); the inactive KN analog had no effect ($p > 0.5$). Values represent the mean ± SEM of six to eight cells each. *Nic*, Nicotine.

BAPTA from the patch pipette. Again, the rundown observed both in control cells and in latrunculin A-treated cells was much less than that seen with normal recording solution in the pipette (Fig. 3). Both the barium substitution of extracellular calcium and the intracellular perfusion with BAPTA demonstrate that calcium influx is necessary for rundown of the α7-nAChR response.

Because of the requirement for calcium influx, we next tested whether calcium-induced calcium release from internal stores contributed to the rundown. This was done in two ways. One made use of ryanodine that blocks release from internal stores (Coronado et al., 1994). Internally dialyzing the cells with 10 μM ryanodine from the patch pipette produced a significant decrement in the rundown (Fig. 4). The other procedure made use of thapsigargin that blocks a calcium-dependent ATPase in the endoplasmic reticulum responsible for sustaining internal calcium stores; blockade depletes the stores (Thastrup et al., 1990). In this case the cells were incubated with 1 μM thapsigargin for 10 min before internal perfusion via the patch pipette at the same concentration. The compound produced a significant decline in the rundown over the 15 min test period (Fig. 4). Thapsigargin had a smaller but significant effect on latrunculin A-treated cells; either release from internal stores could not be completely blocked with the actin cytoskeleton disrupted, or an additional factor contributed to the rundown under these conditions. Neither ryanodine nor thapsigargin alone altered the initial peak amplitude response; values of 4.15 ± 0.44 nA ($n = 6$) and 3.23 ± 0.17 nA ($n = 8$) were obtained for ryanodine- and thapsigargin-treated cells, respectively. Neither value is significantly different from that of untreated cells ($p > 0.05$).

Phosphoregulation of α7-nAChR rundown

Rundown of membrane currents seen with conventional patch-clamp recording can often be overcome by supplying an ATP-generating system via the pipette (e.g., see references in Rosenmund and Westbrook, 1993b). When this was done in the present experiments, little activity-dependent rundown was observed in the α7-nAChR response. Thus the proportion of peak current remaining at the end of the 15 min test period was 92.8 ± 6.1% ($n = 6$) and 57.8 ± 5.1% ($n = 8$), with and without the ATP-generating system, respectively ($p < 0.001$). This finding, plus the requirement for intracellular calcium build-up, raised the possibility that calcium-dependent phosphoregulation of the receptor was responsible for the rundown. As a first step, we tested for involvement of calmodulin. Intracellular dialysis with the calmodulin antagonist calmidazolium (10 μM) caused a substantial inhibition of the activity-dependent rundown (Fig. 5*A, top, B,C*).

Calmodulin can activate both calcineurin (phosphatase IIB) and CaM kinase II in neurons. Because an ATP-generating system inhibited the rundown, one might have imagined that a calmodulin-dependent phosphatase like calcineurin would promote rundown. Surprisingly, the opposite was found. Inclusion of a nonspecific phosphatase inhibitor, CsF, in the patch pipette exacerbated rundown, paralleling that seen in latrunculin A-treated cells (data not shown). Intracellular dialysis during the 15 min test period with either 200 nM cyclosporin A or 200 nM deltamethrin, both potent and selective calcineurin antagonists, also significantly augmented the rundown (Fig. 5*A, middle, B,C*). In contrast, intracellular dialysis with either 50 μM KN93 or 5 μM AIP significantly inhibited the activity-dependent rundown (Fig. 5*A, bottom, B,C*). Intracellular dialysis with KN92, an inactive analog of KN93, had no effect (Fig. 5*C*). KN93 (Sumi et al., 1991) and AIP (Ishida et al., 1995) are both widely used as specific blockers of CaM kinase II; AIP has been shown to have no effect on CaM kinase IV (Ishida et al., 1995). Accordingly, the results demonstrate an obligatory

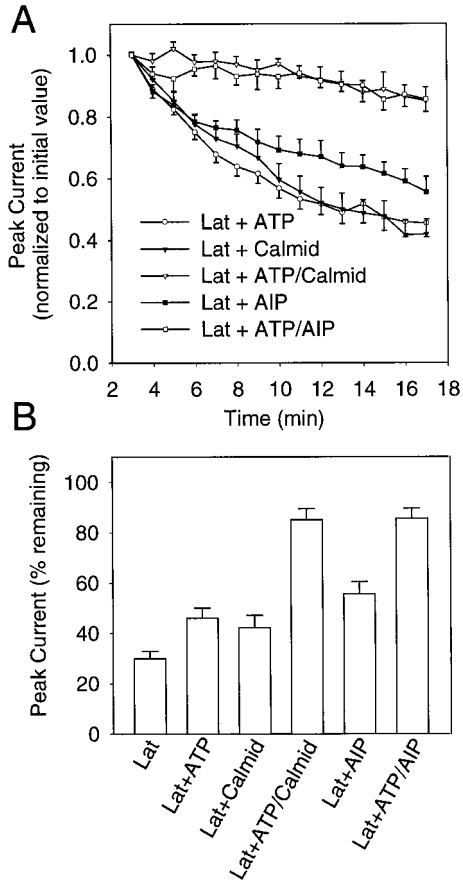

Figure 6. Calcium-dependent phosphoregulation in latrunculin A-treated cells. *A,* Time course of rundown in latrunculin A-treated (*Lat*) cells internally dialyzed with the indicated compounds from the patch pipette. Drug concentrations were the same as in Figure 5, except that an ATP-generating system (*ATP*) was included where indicated. *B,* Peak current remaining at the end of the rundown period for each of the test conditions, normalized to the initial peak response in the same cells. The ATP-generating system, the calmodulin blocker [calmidazolium (*Calmid*)], and the CaM kinase blocker [autocamtide-2-related inhibitory peptide (*AIP*)] each partially inhibited the rundown ($p < 0.01$, 0.05, and 0.001, respectively), although not as much as that seen in control cells lacking exposure to latrunculin A (see Fig. 5 and text). Combining the ATP-generating system with either the calmodulin blocker or the CaM kinase II blocker produced more substantial inhibition ($p < 0.001$), rivaling that obtained in cells without latrunculin A treatment. Values represent the mean ± SEM of six to eight cells each.

role of CaM kinase II in producing the rundown and an opposing protective effect of calcineurin.

Similar experiments were performed with latrunculin A-treated cells because they normally undergo more severe rundown. The ATP-generating system and calmidazolium dialysis each significantly inhibited the rundown, but neither alone achieved as much blockade as seen in control cells (Fig. 6). Together, however, they blocked most of the rundown. The same pattern was observed with AIP that alone only partially prevented rundown. Together with the ATP-generating system, AIP blocked most of the rundown in latrunculin A-treated cells (Fig. 6).

Requirement for a dialyzable component

The rundown experiments described thus far were performed with conventional patch-clamp recording. To determine whether intracellular dialysis was required for the rundown, we performed similar experiments using amphotericin-perforated patch-clamp recording. This procedure avoids disruption of the intracellular contents (Horn and Marty, 1988; Rae et al., 1991). Surprisingly, not only control cells but also latrunculin A-treated cells exhibited little activity-dependent rundown under these conditions (Fig. 7). Nonetheless, even intact cells are subject to phosphoregulation of the α7-nAChR response. This was shown by extracellular perfusion of cells with the membrane-permeant calcineurin inhibitor deltamethrin (200 nM). The perfusion was initiated 3 min after establishing a baseline recording and control response. Deltamethrin produced a significant time-dependent decrease in the α7-nAChR response (Fig. 7), and the decrease was activity dependent (Fig. 7*C*). Moreover, replacing the extracellular calcium with barium prevented the deltamethrin-induced decrease (Fig. 7). Thus, even in cells accessed by perforated patch-clamp recording, calcineurin acts to counterbalance an activity- and calcium-dependent decrease in the α7-nAChR response. In these respects the decrease mimics the rundown mediated by CaM kinase II seen in dialyzed cells. It was not possible to test the effects of specific CaM kinase II blockade directly under these conditions because AIP is not membrane permeant and bath-applied KN93 inhibits α7-nAChRs (perfusion with 10 μM KN93 for even a few seconds dramatically and reversibly reduces both the α3*- and α7-nAChR responses; data not shown). Nonetheless, the results show that a diffusible component, lost during conventional patch-clamp recording, is responsible for retarding activity-dependent rundown and that α7-nAChRs are subject to ongoing calcium-dependent phosphoregulation in intact cells.

DISCUSSION

The principal results reported here are that α7-nAChR responses undergo activity-dependent rundown requiring calcium influx and release from internal stores, activation of calmodulin, and participation of CaM kinase II. The rundown is exacerbated by collapse of the actin cytoskeleton and depends prominently on the loss of a diffusible component that may be needed either to facilitate calcium sequestration normally or to control receptor phosphoregulation. Calcineurin acts to oppose the rundown and, in undialyzed cells, primarily counterbalances the effects of CaM kinase II. Thus the two enzymes provide antagonistic mechanisms for achieving calcium-dependent regulation of α7-nAChRs. The consequence is likely to be a quick-response robust system for controlling calcium influx into the spines.

Calcium-dependent rundown of α7-nAChR responses has been seen previously, but no kinase or phosphatase involvement was identified (Bonfante-Cabarcas et al., 1996). Multiple agents were used in the present studies to demonstrate the roles of specific calcium-dependent enzymes. In addition to CsF as a nonspecific inhibitor of phosphatases, cyclosporin and deltamethrin were used to show that calcineurin protected against the rundown. Similarly, both KN93 and AIP were used to show that CaM kinase II promoted the rundown. Blockade by either KN93 or AIP has widely been considered diagnostic for CaM kinase activity, and AIP is reported to act selectively on CaM kinase II. Chick ciliary ganglion neurons are known to contain CaM kinase II (Lengyel et al., 1996, 1998).

Several features of the phosphoregulation deserve comment. Calmodulin is known to regulate a number of ion channels by direct interaction (Levitan, 1999). The best studied case involving ionotropic receptors is that of NMDA receptors in which one effect of calmodulin is mediated via a direct interaction with the C-terminal portion of the NR1 subunit, causing calcium-

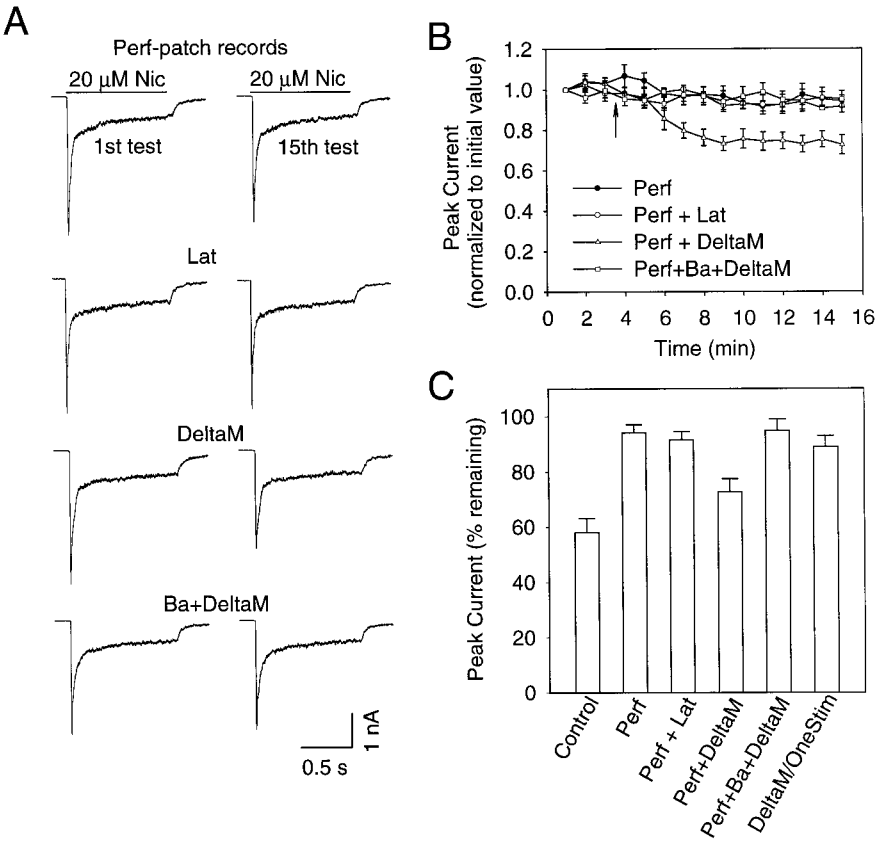

Figure 7. Dependence of rundown on intracellular dialysis. *A,* Perforated patch-clamp recordings (*Perf-patch records*) of responses elicited by 20 μM nicotine (*Nic*) from the same cell at the beginning (*1st test*) and end (*15th test*) of the standard rundown protocol. *Top*, Control cells. *Second*, Latrunculin A-treated cells (*Lat*). *Third*, Deltamethrin-treated cells (*DeltaM*). *Bottom*, Deltamethrin-treated cells tested in barium instead of calcium (*Ba + DeltaM*). *B,* Time course of rundown monitored with perforated patch-clamp recording in control (*Perf*) and latrunculin A-treated cells (*Perf + Lat*) under normal conditions or after bathing with 200 nM deltamethrin (*Perf + DeltaM*, initiated at the *arrow*) or after bathing with deltamethrin after replacing extracellular calcium with barium (*Perf + Ba + DeltaM*). *C,* Peak current remaining at the end of the rundown period for each of the test conditions, normalized to the initial peak response in the same cells. Rundown in the perforated patch-clamp configuration both for normal and latrunculin A-treated cells was significantly less ($p < 0.001$) than that seen with conventional patch-clamp recording (*Control,* from Fig. 1*C*, shown for comparison). The membrane-permeant calcineurin inhibitor deltamethrin reduced the response during the perforated patch recording ($p < 0.05$), and the reduction was blocked by replacing the extracellular calcium with barium ($p > 0.5$ vs control; $p < 0.05$ vs deltamethrin) or by stimulating three times before applying the deltamethrin and then not again until the end (*DeltaM/OneStim*; $p > 0.5$ vs control; $p < 0.05$ vs deltamethrin with regular stimulation). Values represent the mean ± SEM of six to eight cells each.

dependent inactivation of the receptor (Legendre et al., 1993; Wyszynski et al., 1997; Zhang et al., 1998). Calmodulin also acts via calcineurin to inhibit NMDA receptor function (Lieberman and Mody, 1994) and to destabilize actin filaments, promoting collapse of the dendritic spines (Halpain et al., 1998). In contrast, the present results show that calcineurin protects the α7-nAChR response against rundown and that calmodulin activates CaM kinase II to promote α7-nAChR rundown. CaM kinase II actually appears to enhance the responses of several other kinds of ionotropic receptors, including AMPA, kainate, NMDA, and $GABA_A$ receptors (McGlade-McCulloch et al., 1993; Kolaj et al., 1994; Lledo et al., 1995; Wang and Kelly, 1995; Wang et al., 1995). Direct phosphorylation is known to accelerate nAChR desensitization (for review, see Swope et al., 1999), but desensitization did not appear in any normal sense to play a role here because no change was observed in the decay rate of α7-nAChR responses during the rundown.

What role does the actin cytoskeleton play in α7-nAChR regulation? The somatic spines are sustained by actin filaments; collapse of the filaments causes spine retraction and dispersal of the resident α7-nAChRs over the cell body into numerous microclusters of variable size (R. Shoop and D. Berg, personal observations). Interestingly, this dispersal process has no effect by itself on the functionality of α7-nAChRs because latrunculin A-treated cells have the same whole-cell peak nicotine response initially as do control cells. The implication is that the effective balance of regulatory elements controlling α7-nAChR function is not disturbed by disruption of actin links as long as calcium influx is avoided. The relevance of the actin cytoskeleton becomes apparent, however, when examining activity-dependent α7-nAChR rundown by conventional patch-clamp recording. In this case, the rundown is accelerated by collapse of the actin filaments and diminished when they are stabilized. The fact that very little rundown of the α7-nAChR response occurs with perforated patch-clamp recording shows that intracellular dialysis potentiates the rundown. The results suggest that the actin cytoskeleton helps retain a necessary component(s) or stabilizes a regulatory configuration that is lost after dialysis in a calcium-dependent manner.

The state of actin polymerization has been shown previously to influence the rate of rundown for NMDA responses, and in this case the effect was attributed to the ability of the actin cytoskeleton to compartmentalize a regulatory component required for NMDA receptor function (Rosenmund and Westbrook, 1993a). Similarly, the actin cytoskeleton supporting somatic spines may be important for tethering regulatory components in the immediate vicinity of α7-nAChRs. The components may constrain the receptors in a protected conformation or may counterbalance negative regulatory machinery like that of the CaM kinase II pathway. Precedence for specific interactions of this kind comes from the example of actin-associated protein α-actinin supporting NMDA receptor function; calmodulin disrupts the α-actinin–receptor interaction and promotes receptor inactivation (Krupp et al., 1999). In the case of α7-nAChRs, the postulated protective interactions would presumably be destabilized by calcium influx in a way that facilitated removal of the components by intracellular dialysis. Collapse of the actin filaments would accelerate the loss.

A different possibility is that actin filaments may indirectly retard activity-dependent rundown of α7-nAChR responses by supporting calcium sequestration. The rundown clearly depends on calcium influx and calcium release from internal stores. Intra-

cellular dialysis would disrupt compartments responsible for sequestering calcium. As a result, intracellular calcium levels would be more vulnerable to changes imposed by α7-nAChR activation. Collapse of the actin filaments and retraction of the somatic spines could increase the efficiency of the dialysis and thereby might enhance intracellular calcium buildup with attendant rundown of the α7-nAChR response.

The finding that the α7-nAChR response underwent activity-dependent rundown while the α3*-nAChR response did not implies specificity in the effect. The α3*-nAChRs are repeatedly activated by the stimulation protocols used here, and many of the receptors are concentrated on somatic spines as are the α7-nAChRs (Shoop et al., 1999). Either the receptors are subject to entirely different regulatory machinery based on their subunit composition, or the regulatory consequences of calcium influx are confined to receptors in the immediate vicinity. The high relative calcium permeability of α7-nAChRs may expose them transiently to higher local concentrations of intracellular calcium. An unanswered question is whether calcium influx through other channels such as voltage-gated calcium channels could substitute for influx through α7-nAChRs in producing the rundown; calcium currents in ciliary ganglion neurons displayed substantial rundown themselves, preventing an assessment of their contribution to α7-nAChR rundown (Q.-s. Liu and D. Berg, unpublished observations).

Much remains to be learned about the molecular mechanisms responsible for α7-nAChR rundown. For example, the molecular targets of CaM kinase II-mediated phosphorylation and calcineurin-mediated dephosphorylation are not known. *In vitro* analysis of α7 fusion proteins fails to detect CaM kinase II phosphorylation (Moss et al., 1996), raising the possibility that additional components intervene in the pathway. Similarly, the ability of an ATP-generating system to block activity-dependent rundown is not understood. Conceivably the ATP is needed to support calcium sequestration or removal; without it, the calcium buildup may quickly reach threshold for activating the CaM kinase II pathway. Alternatively the ATP may be crucial for maintaining the phosphorylated state of some regulatory component.

The purpose of concentrating α7-nAChRs on somatic spines is unknown but may have to do with calcium influx. By acting to limit the spread of calcium entering through α7-nAChRs, the spines may both protect the cell against cytotoxic effects and facilitate calcium-mediated regulatory events, enabling high local intracellular concentrations of calcium to be achieved. The antagonistic roles of CaM kinase II and calcineurin identified here should be instrumental in the regulation of synaptically driven calcium influx through α7-nAChRs. The balance between these two opposing regulatory effects may be determined by the frequency of synaptic stimulation and accompanying calcium pulses occurring in the spines (De Koninck and Schulman, 1998). It will be important to understand these regulatory pathways, to determine the molecular targets of the kinases and phosphatases, and to assess the roles of such mechanisms *in situ*.

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