mACHRs in the Grasshopper Brain Mediate Excitation by Activation of the AC/PKA and the PLC Second-Messenger Pathways

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mAChRs in the grasshopper brain mediate excitation by activation of the AC/PKA and the PLC second-messenger pathways. J Neurophysiol 87: 876–888, 2002; 10.1152/jn.00312.2001. The species-specific sound production of acoustically communicating grasshoppers can be stimulated by pressure injection of both nicotinic and muscarinic agonists into the central body complex and a small neuropil situated posterior and dorsal to it. To determine the role of muscarinic acetylcholine receptors (mAChRs) in the control of acoustic communication behavior and to identify the second-messenger pathways affected by mAChR activation, muscarinic agonists and membrane-permeable drugs known to interfere with specific mechanisms of intracellular signaling pathways were pressure injected to identical sites in male grasshopper brains. Repeated injections of small volumes of muscarine elicited stridulation of increasing duration associated with decreased latencies. This suggested an accumulation of excitation over time that is consistent with the suggested role of mAChRs in controlling courtship behavior: to provide increasing arousal leading to higher intensity of stridulation and finally initiating a mating attempt. At sites in the brain where muscarine stimulation was effective, stridulation could be evoked by forskolin, an activator of adenylate cyclase (AC); 8-Br-cAMP-activating protein kinase A (PKA); and 3-isobuty-1-methylxanthine, leading to the accumulation of endogenously generated cAMP through inhibition of phosphodiesterases. This suggested that mAChRs mediate excitation by stimulating the AC/cAMP/PKA pathway. In addition, muscarine-stimulated stridulation was inhibited by 2′,3′-dideoxyadenosine and SQ 22536, two inhibitors of AC; H-89 and Rp-cAMPS, two inhibitors of PKA; and by U-73122 and neomycin, two agents that inhibit phospholipase C (PLC) by independent mechanisms. Because the inhibition of AC, PKA, or PLC by various individually applied substances entirely suppressed muscarine-evoked stridulation in a number of experiments, activation of both pathways, AC/cAMP/PKA and PLC/IP3/diacylglycerine, appeared to be necessary to mediate the excitatory effects of mAChRs. With these studies on an intact “behaving” grasshopper preparation, we present physiological relevance for mAChR-evoked excitation mediated by sequential activation of the AC- and PLC-initiated signaling pathways that has been reported in earlier in vitro studies.

INTRODUCTION

Acetylcholine (ACh) is a major and widespread transmitter in sensory neurons and interneurons of the insect brain (Gerschenfeld 1973; Sattelle 1985). As in vertebrates, nicotinic and muscarinic acetylcholine receptors (n- and mAChRs) can be distinguished (Breer 1981; Breer and Sattelle 1987; Pitman 1971); but in contrast to vertebrates, mAChRs generally occur in much lower concentration in insect nervous tissue than nAChRs (Knipper and Breer 1988).

mAChRs belong to a family of proteins with seven transmembrane domains that, on binding of ACh, activate heterotrimeric G proteins, thereby influencing the levels of intracellular second-messenger molecules. Five different subtypes (m1–m5) have been cloned from vertebrates that differ in their selectivity for agonists/antagonists, in their localization within the CNS, in their coupling to different G proteins, and thus in their effects on second messengers (reviewed by Bonner 1989; Felder 1995; Jones 1993). Typical effects evoked by muscarinic receptors are the inhibition of adenylate cyclase (AC), resulting in decreased intracellular levels of cAMP mediated by subtypes m2 and m4, and the activation of phospholipase C (PLC) mediated by the subtypes m1, m3, and m5, leading to the generation of the second-messenger inositol-1,4,5-triphosphate (IP3) and diacylglycerine (DAG) (reviewed by Bonner 1989; Felder 1995; Jones 1993). Although less frequently, activation of the adenylate cyclase pathway by muscarinic agonists has also been documented in a few vertebrate preparations (Brown and Rietow 1981; Enyedi et al. 1982; Olianas and Onali 1992). Substantial cross-talk leading to the sequential activation of one of these pathways after the other has been confirmed in heterologous expression systems (Felder et al. 1989; Jones et al. 1991).

mAChRs have been found in various insect nervous systems (reviewed by Hannan and Hall 1993; Trimmer 1995), but only one insect mAChR, from the Drosophila brain, has been cloned to date (Onai et al. 1989; Shapiro et al. 1989). Its sequence displays a high degree of homology to vertebrate mAChRs and its heterologous expression in different vertebrate (Blake et al. 1993; Shapiro et al. 1989), and a Drosophila cell line (Millar et al. 1995) demonstrated an increase in cytosolic IP3 and Ca2+ concentrations following activation by muscarinic agonists and a selectivity for antagonists that is similar to vertebrate m1 and m3 subtypes but distinct from m2 AChRs. Although detailed pharmacological classification of invertebrate mAChRs generally does not correlate well with that of vertebrates (reviewed by Hannan and Hall 1993), binding studies with muscarinic agonists and antagonists, evidence for coupling to different second-messenger pathways and heterogeneous physiological responses to muscarinic stimulation...
mediated by modulation of ligand- and voltage-gated ion channels (David and Pitman 1993, 1996a; Parker and Newland 1995) indicate that a variety of mAChR subtypes is also present in insects (Locusta migratoria synaptosomes: Knipper and Breer 1988; Periplaneta americana DUM-neurons: Lapied et al. 1992; honeycock, cockroach and housefly heads: Abdallah et al. 1991; Manduca sexta: Trimmer and Weeks 1993).

Evidence for the coupling of insect mAChRs to second-messenger pathways derives from studies on various species (reviewed by Trimmer 1995; Trimmer and Qazi 1996). In agreement with the results of the expression studies of the cloned Drosophila mAChR (Millar et al. 1995), an increase in phosphatidylinositol turnover was found in insect nervous systems (David and Pitman 1994; Duggan and Lunt 1988; Trimmer and Qazi 1996) in response to the application of muscarinic agonists. Membrane depolarization and/or increased excitability of the neurons following muscarinic stimulation seen in the same preparations (David and Pitman 1996a,b; Lapied et al. 1992; LeCorronc and Hue 1993; Trimmer 1994) could be mediated by activation of PLC, but the downstream mechanisms have not been described in detail and their potential link to the observed currents is difficult to prove. Hyperpolarizing currents that have been attributed to activation of presynaptically located mAChRs inhibit transmitter release from locust synaptosomes (Knipper and Breer 1988) acting via a reduction in cAMP-level. Similar effects on the synaptic release of ACh were also seen in cockroach and locust sensory afferents (LeCorronc and Hue 1993; Le Corronc et al. 1991; Parker and Newland 1995), presynaptic terminals of locust wing stretch receptor neurons (Leitich and Pitman 1995) and larvae of tobacco hawkmoths (Trimmer and Weeks 1993). The second-messenger cGMP participates in various behaviors of insects (chemosensory processing: Bicker 1998; eclosion behavior: Ewer and Truman 1996; modulation of photoreceptors: Schmachtenberg and Bicker 1999), but to date no direct coupling of this pathway to mAChRs has been confirmed (Trimmer and Qazi 1996).

Insect mAChRs have been attributed with two main functions: the inhibition of transmitter release from sensory terminals and the regulation of the excitability of motoneurons or interneurons (e.g., LeCorronc and Hue 1993; Trimmer and Weeks 1989, 1993). Studies in Manduca sexta larvae suggested that mAChRs mediating increased excitability of proleg retractor motorneurons following strong sensory stimuli provide a form of “motor arousal” to increase the sensitivity for small sensory stimuli (Trimmer and Weeks 1993). In addition, central rhythm-generating circuits underlying various insect behaviors can be activated by global application of muscarinic agonists (locomotion: Büschges et al. 1995; Ryckebusch and Laurent 1993; pharyngeal movements: Gorczyca et al. 1991; chewing: Trimmer 1995), but the neurons directly affected by muscarinic stimulation remained unidentified.

Recently, our pharmacological studies in the protocerebrum of gomphocerine grasshoppers revealed a new functional role for mAChRs, showing them to be the basis for specific arousal underlying the selection and control of singing behavior (stridulation) appropriate for a certain behavioral situation (Heinrich et al. 2001a,b). Male grasshoppers perform a variety of different species- and context-specific songs that are used for intraspecific communication (reviewed by Elsner 1994). The muscles responsible for the hindleg movements underlying sound production are activated by thoracic pattern generating networks (Hedwig 1992; Ronacher 1989). These networks are controlled by the brain via descending command neurons with their dendritic arborizations in the protocerebrum dorsal and posterior to the central body complex (Hedwig 1994, 1995; Hedwig and Heinrich 1997). Microinjection of neuroactive substances into the dendritic area of these command neurons and into the upper or lower division of the central body complex revealed a central role of cholinergic activation in the control of stridulatory behavior (Heinrich et al. 1997, 2001a).

Stimulation of both nAChRs and mAChRs by specific agonists induced stridulatory sequences similar to the natural behavior. However, the time courses of induced activity were clearly different, with activation of mAChRs leading to stridulation of slower developing intensity and longer duration when compared with the nicotinic effects. Following a single experimental application of Ach imitating the presynaptic release of the presumed natural transmitter, the muscarinic system is only weakly activated and stridulatory sequences are mainly triggered by activation of nAChRs (Heinrich et al. 1997, 2001b). The slower but longer-lasting activation of the muscarinic system accumulates excitation during long episodes of courtship stridulation. The level of activity of the second-messenger pathways initiated by mAChR activation determines the behavioral threshold to engage in singing behavior and the selection of stridulatory patterns according to the progress of courtship, culminating in an attempt to mate with the female (Heinrich et al. 2001a,b).

In this study, we extended the pharmacological investigations toward an analysis of the intracellular processes that translate the stimulation of mAChRs into excitation of brain neurons that control the activity of the command neurons. For this purpose, we used membrane permeable agents known to activate or inhibit the enzymes of the three second-messenger pathways cAMP, IP3/DAG, and cGMP. The pharmacological agents were applied at sites where muscarine reliably elicited stridulatory behavior. Their ability to induce stridulation and their effect on muscarine-induced excitation were investigated. In this report, we present strong evidence that mAChRs in the cephalic control system for stridulatory behavior of grasshoppers mediate long-lasting excitation by activation of both AC leading to the formation of cAMP, and PLC generating the second messengers IP3 and DAG. Because inhibition of each of these pathways can completely suppress muscarine-stimulated stridulation, they seem to be sequentially activated.

METHODS

Animals

Adult specimen of the gomphocerine grasshoppers Omocestus viridulus (L., 1758) (O.v.) and Chorhippus biguttulus (L., 1758) (Ch.b.) were caught in the vicinity of Göttingen, Germany, and kept separately in the laboratory for up several weeks. Additional Ch.b. were reared from eggs that were collected in the previous summer and kept at 4°C for ≥4 mo. The nymphs hatched after ~1 wk at 26°C and were raised on wheat and supplemental food for crickets (Nekton, Pforzheim) at a 16/8 h light-dark-cycle. All experiments were conducted with male adults at temperatures of 20–25°C.
The front cuticle of the head capsule was opened with a razor blade to expose the dorsal surface of the brain. The rest of the animal was left intact and capable of moving all its appendages freely, particularly its hindlegs used in stridulation. To record the stridulatory movements with two optoelectronic devices (Helversen and Elsner 1977), a piece of reflecting foil (Scotchlite 3 M, type 7610; 2 mm diam) was glued to the femur of each hindleg. The up and down movements of each hindleg were thus transformed into voltage signals proportional to the amplitude of movement. Additionally, the sounds produced during stridulation were recorded by a custom-made microphone.

Injection of drugs

The neuroactive substances [ACh, muscarine, 8-Br-cAMP, 8-Br-cGMP, 3-isobutyl-1-methylxanthine (IBMX) and forskolin obtained from Sigma-Aldrich; phorbol-12-myristate-13-acetate (PMA), SQ 22536, 2',5'-dideoxyadenosine (ddAdo), Rp-isomer of cAMPs (Rp-cAMPS), d-erythro-sphingosine, thapsigargin and zaprinast obtained from Calbiochem and H-89 and U-73122 obtained from Biomol] were dissolved in grasshopper saline (Clements and May 1974) to give concentrations of 10−5 M. Forskolin, ddAdo, H-89, PMA, d-erythro-sphingosine, thapsigargin, U-73122, and zaprinast were first dissolved in dimethyl sulfoxide (DMSO) before saline was added to give a final concentration of 5% DMSO. Solutions of DMSO in saline had no effect, neither stimulatory when injected alone nor inhibitory when co-injected with muscarine, on the performance of stridulatory behavior. The agents were injected into a specific region of the brain using a pressure injection device (WPI, model PV 820). Double-barrel microcapillaries connected to a three-way stop-cock allowed application of approximately the same amount of two substances at the same site in the protocerebrum. Before the experiment, the tips of the capillaries were broken under visual control to produce tip diameters of ∼10−15 µM. The pressure and pulse duration were then adjusted so that ∼1−3 nl of a given substance was applied per injection. This had previously been confirmed by measuring the volumes of droplets injected into petroleum jelly (Hedwig and Heinrich 1997).

Data processing

The recorded signals were digitized on-line by means of a A/d-converter card (Real Time Devices AD3300) with the software TurboLab 4.3 (Bressner Technology, Germany) and stored as data files. The sampling rate for recording the stridulatory movements, the sound and the injection pulses was 3 kHz. The software NEUROLAB (Hedwig and Knepper 1992; Knepper and Hedwig 1996) was used for visual examination and filtering of the original data. Using the calculating program Excel (Microsoft), diagrams were generated and subsequently imported into the graphics program CorelDraw 7 (Microsoft), where they were assembled into figures.

Evaluation and statistical analysis of experimental results

When testing a substance for its ability to induce stridulation at a given site within the brain, experiments were defined as successful if stridulation was released in at least three successive trials. One injection of a stimulating drug usually released several sound sequences that were separated by short pauses. The sum of the durations of all individual sound sequences released by one stimulation was taken as the total duration of stridulation. When investigating putative inhibitory effects on muscarine-stimulated stridulation, the average duration of muscarine-induced songs was calculated from at least three trials, executed at intervals of 5 min. After application of the substance under investigation, experiments were defined as successful if the first pulse of muscarine following injection of that substance did not elicit stridulation or if the duration was below the range of 2 SDs of the average duration of muscarine-induced songs performed before inhibition. The inhibition was classified as irreversible if the duration of stridulation never recovered into that range again.

From the recorded data, the latencies between injection pulses and onsets of stridulation, and the total duration of the released stridulatory activity (see preceding text) were determined. Analyses were performed using relative values calculated by setting the longest latency and longest duration of all songs stimulated during a given experiment as 100%, except for the experiments shown in Fig. 2C, middle, where the duration resulting from the first injections of each series was set to 100%. We used the Wilcoxon-Mann-Whitney test to determine significant differences in the latency and duration of stridulation released by two different substances (Figs. 1A and 2, left and middle). Potential changes in the duration of muscarine-induced stridulation following the injection of another substance (Figs. 2, right, 3−5) were evaluated by a nonparametric Kruskal-Wallis test followed by Dunn’s multiple comparison test. This nonparametric test was chosen because we generally included all experiments, whether successful interference with muscarine stimulation occurred or not, into the analysis.

![Figure 1](http://jn.physiology.org/)

**FIG. 1.** Stimulation of grasshopper stridulation by cholinergic agonists. A: injections of acetylcholine (ACh; •) and muscarine (■) into the protocerebrum elicited species-specific stridulatory hindleg movements after distinct latencies and of distinct durations. B: increased volumes of muscarine used for stimulation led to prolonged stridulatory behavior (■) that started after progressively shorter latencies (□). C: repeated application of identical muscarine injections at regular intervals of 5 min elicited stridulation of gradually increasing durations (■) and decreasing latencies (□). Left: 1 experiment. Middle and right: averaged of 27 experiments. RHL/LHL: movements of the right/left hindleg; error bars: SD; *P < 0.05; **P < 0.001; ***P < 0.0001.
the statistical analysis, and therefore a normal distribution of the stimulated durations of stridulation could not be assumed. An unbiased inclusion of all experimental data into the analysis results in comparatively conservative statements about significances, allowing only strong and/or robust effects to alter the control parameters significantly. Potential changes in the latency and the duration of songs released by a series of pulses of muscarine were analyzed by the ANOVA test for repeated measures (Fig. 1, B and C) in combination with Tukey’s multiple comparison test. To provide an estimate of the variance of the measured parameters, SDs of the means were calculated and included into the histograms. All statistical analysis was performed using the program Prism 2.01 (GraphPad Software). As we found no differences in the reaction of the two species, *O.* viridulus and *Ch.* biguttulus, used in the experiments (see Fig. 2, A–C), the results were pooled for certain analyses.

**RESULTS**

**Stimulation of mAChRs**

**MUSCARINE-STIMULATION RELEASES STRIDULATION SIMILAR TO THE NATURAL BEHAVIOR.** Microinjection of small amounts of muscarine into the central protocerebrum of *O. viridulus* and *Ch. biguttulus* lead to the release of long-lasting stridulation, divided into separate song sequences, that is indistinguishable from natural stridulation. In *O. viridulus*, calling and courtship songs can be elicited. Pharmacologically stimulated courtship songs follow a gradual increase in intensity and duration of individual song sequences that is similar to spontaneous courtship (Hedwig and Heinrich 1997; Heinrich et al. 1997). In *Ch. biguttulus*, calling and courtship songs differ only slightly.
in their movement and sound patterns (Elsner 1974; Helversen 1972) and the species-specific movements and activity/pause patterns can reliably be reproduced by pharmacological stimulation (Heinrich et al. 1998).

When ACh and muscarine were injected into the same site within the protocerebrum, muscarine-stimulation evoked significantly prolonged stridulation [63.8 ± 16.9% (mean ± SD) for muscarine compared with 10.5 ± 5.5% for ACh, n = 7; \( P = 0.01 \)] with a slower onset (54.8 ± 20.4% for muscarine compared with 12.3 ± 14.8% for ACh; \( P = 0.01 \)) and a gradual increase of intensity (Fig. 1A). This confirmed earlier data that showed a fast nicotinic and a slower but longer-lasting muscarinic response following stimulation with the presumed natural transmitter ACh (Heinrich et al. 1997).

**AMOUNT OF MUSCARINE INJECTED DETERMINES THE TIME COURSE OF STIMULATED STRIDULATION.** In a series of preparations, the amount of muscarine injected to a fixed site in the protocerebrum was altered by varying the duration of the pressure pulse used for its ejection from the capillary. The average relative duration of the released stridulatory behavior increased with the duration of pressure pulses from 34.2 ± 16.9% (n = 8) after pulses of 50 ms to 75.8 ± 12.0% (n = 8) following muscarine pulses of 300 ms (Fig. 1B). Further increasing the pulse duration evoked no additional increase in the duration of the induced stridulation. In contrast, the latency between injection pulse and onset of stridulation was inversely related to the amounts of muscarine used for stimulation (Fig. 1B). The mean relative latency decreased gradually from 93.9 ± 10.0% (n = 8) after pulses of 50 ms to 56.1 ± 17.9% (n = 9) after muscarine pulses of 200 ms and remained unchanged with larger volumes of muscarine.

**REPETITIVE MUSCARINE STIMULATION ALTERS THE TIME COURSE OF RELEASED SONGS.** Repeated injections of muscarine, applied to the same site at regular intervals of 5 min, induced stridulation of gradually increasing duration. As illustrated by one typical experiment with Ch.b. (Fig. 1C), this increase in duration saturated after the third or fourth stimulation and was associated with a decrease in the latency to the onset of singing. The statistical analysis of 27 experiments revealed a significant increase of the duration from 100% following the first muscarinic stimulation to 191 ± 106.2% (\( P = 0.001 \)) after the fourth pulse of muscarine (Fig. 1C). In the same experiments, the latency decreased significantly from 100 to 49.5% ± 26.2% (\( P = 0.001; \) Fig. 1C) The intervals between individual muscarine stimuli could be extended to ≤10 min and still produce the alterations of durations and latencies, although they were weaker with longer intervals. This demonstrated that the experimentally evoked muscarinic excitation not only outlasts the period of muscarine-induced stridulation (usually 1–2 min) but persists for ≤10 min.

For studies of potential excitatory or inhibitory effects of pharmacological agents known to interfere with intracellular signaling pathways, it was crucial to maintain a constant level of basal excitation resulting from previous injections of muscarine. This was achieved by periodically injecting standard volumes of muscarine in fixed intervals (usually 5 min) throughout the entire experiment. Drugs that potentially inhibited singing behavior were applied within the interval between two muscarine pulses, whereas potential activators were applied instead of muscarine pulses.

To identify the second-messenger pathways that mediate excitation following activation of mAChRs, we employed substances known to permeate cell membranes and to alter the activity of certain second-messenger pathways. References for the successful application in an invertebrate preparation are provided for each substance used in this study.

**Activation of the cAMP pathway**

**ACTIVATION OF AC OR PKA STIMULATES STRIDULATION.** At those sites in the grasshopper brain where muscarine successfully elicited stridulation, activation of AC by injection of forskolin (Arrese et al. 1999) and activation of PKA by microinjection of 8-Br-cAMP (Lundquist and Nässel 1997; Smith et al. 1984) reliably released long-lasting stridulatory behavior. With respect to the movement patterns and the coordination of the hindlegs, no differences were seen between muscarine-induced songs and songs stimulated with forskolin or 8-Br-cAMP (data not shown). Similar amounts of forskolin and muscarine induced stridulatory activity of about the same duration (Figs. 2A, left). In contrast, the duration of stridulation released by 8-Br-cAMP was significantly shorter when compared with muscarinic stimulation (Fig. 2B, left). For O.v., the average relative duration of muscarine-induced songs was 70.8 ± 25.7% (n = 14), whereas injection of 8-Br-cAMP was followed by 21.6 ± 18.9% (n = 19; \( P = 0.001 \)) relative duration of stridulation. For Ch.b., the mean relative duration of stridulation after injection of muscarine (72.5 ± 22.7%, n = 15) was also significantly (\( P = 0.05 \)) longer than stridulation induced by 8-Br-cAMP (47.2 ± 28.5%, n = 12). Because we found no differences in the results obtained with the two different grasshopper species, O.v. and Ch.b., in these or any other experiments (see also Figs. 2, A and C), the results, after calculating the relative values, were pooled for all further analysis. Comparison of the latencies of muscarine-induced stridulation with latencies after injection of forskolin (Fig. 2A, middle) or 8-Br-cAMP (Fig. 2B, middle) revealed no significant differences.

To investigate the effects of forskolin and 8-Br-cAMP on muscarine-induced stridulatory activity, we compared the time courses of songs released by injection of muscarine before and after injection of one of these substances to the same sites in the protocerebrum. Neither of the pharmacological agents changed the latency (data not shown) nor the duration of muscarine-induced stridulation significantly (Fig. 2, A and B, right), suggesting that they did not cause any persistent excitation in addition to the excitation resulting from the preceding muscarine stimulation.

**INHIBITION OF PHOSPHODIESTERASE (PDE) RELEASES STRIDULATION.** Inhibition of cyclic nucleotide-dependent phosphodiesterases, e.g., by IBMX, has been shown to cause accumulation of the cyclic nucleotides cAMP and cGMP in insect neurons (Timmer and Qazi 1996). Injection of IBMX into the grasshopper brain elicited coordinated stridulatory movements that were indistinguishable from muscarine-stimulated stridulation induced at the same site within the brain (data not shown). However, the time course of the released stridulation differed in that the average relative duration of stridulatory behavior after injection of IBMX was significantly shorter than following injection of muscarine (Fig. 2C, left). In O.v. it decreased from 46.6 ± 31.6% (n = 23) for muscarine to 17.4 ± 9.5%
Inhibition of the cAMP-pathway

INHIBITION OF AC SUPPRESSES MUSCARINE-STIMULATED STRIDULATION. We used ddAdo (Fryer and Zucker 1993) and SQ 22536 (Zhang et al. 1999) to inhibit AC. Injection of ddAdo led to a reversible decrease in the duration of muscarine-stimulated stridulatory activity in 7 experiments, whereas in another 14 experiments, the subsequent muscarine-stimulated song duration remained within the range of two times the SD of the average of the control stimulations (see METHODS, section for criteria that define successful experiments). In the representation of all the experiments performed with ddAdo, the average relative duration of muscarine-induced songs of 69.2 ± 14.2% (n = 21) was significantly (P = 0.05) reduced to 38.8 ± 35.8% (n = 10) during a period of ≤5 min after the injection of ddAdo (Fig. 3A). The durations then gradually increased to reach control levels between 10 and 15 min after the injection of ddAdo (72.1 ± 22.6%, n = 14).

When injecting SQ 22536 to inhibit adenylyl cyclase, muscarine-induced stridulation was irreversibly inhibited in three experiments and reversibly inhibited in 12 different animals. The average relative duration of stridulatory activity elicited by muscarine before injection of SQ 22536 of all 32 trials was significantly (P = 0.001) reduced from 65.3 ± 18.9% (n = 32) to 23.6 ± 35.5% (n = 14) during the first 5-min interval after application of SQ 22536 (Fig. 3B). The average effect reversed quickly and the durations nearly reached control level within the following 5-min period (54.4 ± 37.2%, n = 29).

INHIBITION OF PKA SUPPRESSES MUSCARINE-STIMULATED STRIDULATION. To inhibit PKA activity, we injected either H-89 (Nagano et al. 1998; Smith et al. 1996) or Rp-cAMPS (Lundquist and Nässel 1997; Smith et al. 1996) to the same sites in the protocerebrum where muscarine stimulated stridulation. In six experiments, injections of H-89 led to a reversible inhibition of muscarine-induced stridulation, and in two other trials, the inhibition was irreversible. The average relative duration of muscarine-induced songs of all 16 experiments conducted was significantly reduced after injection of H-89 (Fig. 3C). The duration decreased from 67.5 ± 16.0% (n = 16) before to 20.9 ± 35.7% (n = 7; P = 0.01) within 5 min and 33.5 ± 26.4% (n = 12; P = 0.05) between 5 and 10 min after application of H-89. Beyond 10 min after H-89 injections, no inhibitory effects on muscarine-induced stridulation remained.

Rp-cAMPS, a competitive antagonist for cAMP-mediated activation of PKA, profoundly inhibited the ability of muscarine to elicit long-lasting stridulation in 21 of 40 grasshoppers tested. It evoked a fully reversible inhibition in eight trials and an irreversible inhibition of muscarine-induced stridulatory activity in another 15 experiments. An analysis of all experiments conducted with Rp-cAMPS revealed that the average relative duration of muscarine-induced stridulation after its injection decreased gradually (Fig. 3D). Compared with other inhibitors used in this study, its effect was slower in onset and persisted for longer periods (compare Figs. 3, A–C, and 4A). Immediately after Rp-cAMPS application, the duration of stridulation was not altered (68.0 ± 16.6%, n = 40) before compared with 64.5 ± 38.4%, n = 10, 0–5 min after injection of Rp-cAMPS). During the following 40 min, it decreased dramatically to 20.7 ± 26.7% (n = 24; P = 0.001) in the interval between 35 and 40 min after Rp-cAMPS injection. In those cases where the inhibition was reversible, the recovery occurred gradually, starting between 20 and 140 min after the injection of Rp-cAMPS. Therefore decreasing vitality during these long-lasting experiments might have been the reason why some grasshoppers apparently did not recover from Rp-cAMPS-mediated inhibition of stridulation.

All inhibitors used to reduce the activities of either AC or PKA evoked a complete and reversible suppression of muscarine-stimulated stridulation in some experiments (H-89: 5 experiments; SQ 22536: 10; ddAdo: 3; Rp-cAMPS: 14). This
suggests that mAChR-mediated excitation may depend on the activation of AC- and cAMP-mediated stimulation of PKA. Activation of this pathway appears to be obligatory for mediating the excitatory effects of mAChR stimulation in the protocerebral circuits that control stridulation.

**Activation/inhibition of other second-messenger pathways**

**IP3/DAG-MEDIATED PATHWAY IS NECESSARY TO MEDIATE THE EXCITATORY EFFECTS OF mAChRs.** To investigate a possible involvement of IP3 or DAG in mediating the muscarinic excitation, we injected U-73122 (Vroemen et al. 1997) to inhibit PLC, the initial enzyme of this pathway. In 8 of 12 experiments, a reversible reduction in the duration of muscarine-induced stridulation was found. The average relative duration of songs elicited by muscarine in all experiments decreased significantly (P = 0.01) from 73.5 ± 15.1% (n = 12) to 26.7 ± 27.3% (n = 10) in the period of ≤5 min after injection of U-73122 (Fig. 4A). This effect dropped below significance level in the following 5-min interval and appeared to be fully reversed in the time period between 10 and 15 min after the application of the PLC inhibitor (62.3 ± 32.3%, n = 12). Employment of neomycin (Shibanaka et al. 1993), a substance that reduces PLC activity by binding to the enzyme’s substrate PIP2, produced a gradually reversing reduction of muscarine-stimulated stridulation from 69.9 ± 15.4% (n = 32) before to 47.3 ± 23.9% (n = 11; P = 0.01) after neomycin application (Fig. 4B), very similar to the effects of U-73122. Thus two substances known to diminish the activity of PLC by different mechanisms affected mAChR-mediated excitation in a similar way.

Because in some of these experiments muscarine-stimulated stridulation was completely suppressed by both inhibitors of PLC activity, U-73122 (n = 6) and neomycin (n = 4), activation of PLC (as activation of AC and PKA; see preceding text) also appears to be an obligate step for generating mAChR-mediated excitation in the brain region studied.

To further explore the mechanism of excitation initiated by activation of PLC, we employed the Ca2+-transport-ATPase inhibitor thapsigargin (Zimmermann and Walz 1997, 1999) to prevent refilling of calcium stores. However, thapsigargin had no effect on the duration of muscarine-induced stridulation (Fig. 4C) nor did it elicit stridulatory activity when applied alone (data not shown). Assuming the effectiveness of thapsigargin, excitation seemed not to be strongly dependent on the release of Ca2+ from IP3-dependent internal stores. To study a potential effect mediated by DAG, the other product of PLC, we tested the phorbol ester PMA (Baines and Downer 1992; Gu and Singh 1997), which is known to activate protein kinase C (PKC) and δ-erythro-sphingosine (Crow and Forrester 1993), an inhibitor of PKC. Neither of the two substances supposed to interfere with the activity of PKC had a modulatory effect on the latency or the duration of stridulation elicited by muscarine when injected at identical sites in the protocerebrum. Although in 4 of 18 experiments δ-erythro-sphingosine inhibited muscarine-induced stridulation according to the criteria defined, these effects were too weak to allow conclusions to be drawn (Fig. 4D). Neither was it possible to elicit stridulatory behavior by injection of PMA at sites where muscarine before and afterward reliably stimulated stridulation. Taken together, these results indicate that the PLC pathway is necessary for mediating the muscarinic excitation in the control system for stridulation in grasshopper brains. However, further studies need to delineate the pathway downstream from the generation of IP3 and DAG by PLC and the mechanism that couples the PLC pathway to the AC pathway or vice versa (see DISCUSSION).

cGMP-MEDIATED PATHWAY IS NOT INVOLVED IN MEDIATING THE EXCITATORY EFFECTS OF mAChRs. In analogy to the experiments carried out with the rather unspecific PDE inhibitor IBMX (compare Fig. 2C), we also tested for the involvement of cGMP-specific PDE by injection of the specific inhibitor zaprinast (Paupardin-Tritsch et al. 1986). At sites where muscarine reliably elicited stridulation, injection of zaprinast never induced stridulatory activity according to the criteria for a successful elicitation (see METHODS) (Fig. 5A, left). In addition, latencies (not shown) and the durations of muscarine-induced stridulation were not altered by preceding injections of zaprinast (Fig. 5A, right). This suggested that the increased excitation following injections of IBMX was based on the reduced hydrolysis of cAMP rather than of cGMP. This was supported by the result that direct increase of cGMP levels through injections of 8-Br-cGMP (Schmachtenberg and Bicker 1999), at sites in the grasshoppers brain where muscarine reliably

**FIG. 4.** Interference with the inositol-1,4,5-triphosphate (IP3)/diacylglycerol (DAG) second-messenger pathway. Average relative duration of muscarine-induced stridulation before (■) and after (□) application of U-73122 (A), neomycin (B), thapsigargin (C), and δ-erythro-sphingosine (D). All substances were prepared at a concentration of 10-5 M and were injected with identical pulse parameters to the same site within the protocerebrum where stridulation was stimulated with muscarine at regular intervals of 5 min. Error bars: SD; *P < 0.05; **P < 0.001; ***P < 0.0001.
discharge systems, the physiological significance of any cellular or subcellular mechanism observed can be directly determined. Our recent studies (Heinrich et al. 2001b) suggested that mAChR-mediated excitation lowers the behavioral threshold to perform stridulation in response to relevant sensory stimuli and determines the selection of song patterns associated with a particular behavioral situation. The results presented in this publication support this view and reveal that mAChRs in the central protocerebrum of grasshoppers mediate long-lasting and temporally accumulating excitation by sequential activation of the AC/cAMP/PKA and the PLC/IP3/DAG second-messenger pathways.

CONTRIBUTION OF mAChRs IN THE CENTRAL PROTOCEREBRUM TO THE CONTROL OF STRIDULATION IN GRASSHOPPERS. Various studies (reviewed by Elsner 1994) suggested that the species- and behavioral context-specific stridulation patterns of grasshoppers are generated by metathoracic rhythm-generating circuits. The performance of a particular pattern is initiated and maintained by tonic activating input from cephalothoracic command neurons, three types of which have been identified in the species O. viridulus (Hedwig 1994, 1995; Hedwig and Heinrich 1997). All command neurons identified so far in various species extend most of their dendrites within a small brain neuropil close to the posterior and dorsal border of the central body complex without extending any arborizations into this prominent structure implicated with sensory integration and motor control (Homberg 1993; Huber 1960a,b; Strauss and Heisenberg 1993). However, these command neurons may only relay the excitation that is initially generated by yet unidentified presynaptic neuromuscular involved in the initiation and selection of stridulation. Since in various species injections of cholinergic agonists into the two brain regions mentioned elicited stridulation that was composed of different patterns performed in the correct sequence (Heinrich et al. 1997, 2001a), the circuits concerned with the decision when and which pattern to sing may be, at least in part, located within these areas.

Previous pharmacological studies on this preparation have revealed an important role of cholinergic excitation mediated by both nicotinic and muscarinic receptors (Heinrich et al. 1997). ACh also seems to be the natural transmitter in the cephalic control system of cricket stridulation (Wenzel and Hedwig 1999; Wenzel et al. 1998). Whereas presynaptically released ACh induces rapid depolarization via nicotinic receptors, thereby triggering an individual sequence of stridulation, muscarinic receptors may only weakly be activated by the transient presence of ACh. Stimulation of stridulation with ACh led to rapidly initiated stridulation that in most cases was ceased before stridulation stimulated by muscarine at the same site within the brain had even started (see Fig. 1A). However, if activated by muscarine or repeated injections of ACh, mAChR-mediated excitation outlasted the period of evoked stridulation and progressively accumulated with repeated stimulations (Fig. 1C) (Heinrich et al. 2001b). In response to this elevation of the basal state of excitation, the effects of subsequent stimulations increased as was reflected by shorter latencies and longer durations of stridulation following identical stimuli. Muscarinic receptors have been suggested to contribute to the control of stridulation by initiating mechanisms for specific arousal, according to integrated sensory information relevant to singing behavior, internal physiological states, and previous activity in the respective circuits (Heinrich et al. 2001a,b). This functional role played by mAChRs in the grasshopper brain appears to be similar to muscarinic effects on.

**DISCUSSION**

The aim of this study was to determine the sign transduction pathway activated by the mAChRs in the cephalic control circuits for stridulation in grasshoppers. Experiments were performed with restrained but functionally intact grasshoppers. The singing behavior that is controlled by the brain regions under study was used as a monitor for the level of excitation induced by small volumes of injected neuroactive agents. Although pharmacological studies on fully intact and behaving preparations face certain limitations compared with studies on isolated cells, on tissue preparations or on heterologous expression systems, the physiological significance of any cellular or subcellular mechanism observed can be directly determined. Our recent studies (Heinrich et al. 2001b) suggested that mAChR-mediated excitation lowers the behavioral threshold to

![Diagram](http://jn.physiology.org/DownloadedFrom/10.220.33.1 HTTP://JN.PHYSIOLOGY.ORG/DownloadedFrom/10.220.33.1 on May 7, 2017)
sensory afferent-to-interneuron and sensory afferent-to-motorneuron synapses in cockroaches and larvae of tobacco hornworns (LeCorronc and Hue 1993; LeCorronc et al. 1991; Trimmer 1994; Trimmer and Weeks 1989, 1993). In both preparations, muscarinic agonists increased the sensitivity to subsequent synaptic release of ACh by decreasing the spike initiation threshold of particular postsynaptic neurons. This mechanism is thought to provide specific arousal that may prioritize certain sensory input and prime a particular motor output.

**COUPLING OF mAChRs IN THE BRAIN OF GRASSHOPPERS TO INTRACELLULAR SIGNALING PATHWAYS.** To determine the second-messenger pathways that mediate the excitatory effects of mAChR activation in the protocerebrum of grasshoppers, membrane-permeable substances that have been demonstrated to interfere with intracellular signaling cascades in insects were injected to sites within the brain, where muscarine elicited a certain duration of stridulation. Because there was no way of measuring the accumulation of these substances inside the neurons that are relevant for the control of stridulation, each experiment was analyzed with respect to the effectiveness of a certain volume of muscarine to stimulate stridulation at the same injection site. The results therefore are based on the assumption that both muscarine and the test substance acted on the same neurons. However, only relatively strong effects obtained in a relatively high number of experiments reached significance level in the evaluation of all experiments with a given substance. Interpretation was difficult when drugs had little or no effect because stridulation is known to be influenced by various specific and unspecific stimuli and sufficient accumulation of a substance within neurons and its actions on the specific set of enzymes expressed in these cells are not certain. To minimize unspecific effects on the signaling pathways in question, usually two or more drugs that activated or inhibited a certain enzyme or mechanism were used to confirm the respective results.

Direct activation of the enzymes AC and PKA by forskolin and 8-Bromo-cAMP and elevation of cAMP through inhibition of PDE by IBMX reliably induced stridulatory behavior at sites where muscarine was also effective (Fig. 2). Although forskolin has been demonstrated to modulate voltage- and ligand-gated ion channels independently of cAMP production in various preparations (reviewed by Laurenza et al. 1989), the stimulating effects in the protocerebrum of grasshoppers appear to be mediated by activation of adenylate cyclase because stridulation could also be induced by 8-Bromo-cAMP and IBMX. In addition to stimulating the cAMP-signaling pathway from within, muscarine-stimulated stridulation could be completely suppressed by ddAdo and SQ 22536, two inhibitors of AC; H-89, a direct inhibitor of PKA, and Rp-cAMPS preventing the activation of PKA by endogenously generated cAMP (Fig. 3). These results indicate that mAChRs in the cephalic control circuits for stridulation mediate excitation by activating the AC/cAMP/PKA signaling pathway. Whereas the GC/cGMP pathway appeared not be involved in this context (see Fig. 5), inhibition of PLC activity by U-73122 and neomycin, which act by different mechanisms, also completely suppressed muscarine-stimulated stridulation (Fig. 4, A and B). Stimulation of PLC by mAChRs, leading to the generation of the two second messengers IP3 and DAG, has been demonstrated for various vertebrate (Nathanson 1987; Peralta et al. 1988; Shapiro et al. 1988) and invertebrate preparations (Hwang et al. 1999; Millar et al. 1995; Trimmer and Berridge 1985; Trimmer and Qazi 1996) and may be regarded as a common mechanism that mediates excitatory responses to neurons. Three of the five subtypes of mAChRs cloned from vertebrates (Bonner 1989; Felder 1995; Jones 1993) and one subtype cloned from Dro sophila (Millar et al. 1995) have been shown to increase cytosolic IP3 concentrations following activation by muscarinic agonists. Depolarization and/or increased excitability of postsynaptic inter- and motoneurons seen in functional insect preparations (LeCorronc and Hue 1993; Trimmer and Weeks 1989, 1993), are suggested to depend on this type of mAChR coupling to the PLC-signaling pathway (David and Pitman 1994, 1996a; Trimmer 1994; Trimmer and Qazi 1996), although the mechanisms initiated by IP3 (and possibly also by DAG) and the ion channels that finally mediate the physiological effects have only been partially identified.

Coupling of mAChRs to AC/cAMP-signaling pathways has been demonstrated for various vertebrate and invertebrate preparations. In vertebrates, the m2 and m4 AChR subtypes usually reduce AC activity through an inhibitory G protein (Bonner 1989; Felder 1995; Jones 1993). In insects, muscarinic agonists may also typically reduce cAMP levels by activation of certain mAChR subtypes. Studies on synaptosomes from locusts (Knipper and Breer 1988, 1989) and cockroaches (LeCorronc et al. 1991) revealed hyperpolarizing currents and inhibition of subsequent transmitter release associated with decreased levels of cAMP following mAChR activation. Similar effects that modulated the synaptic release of ACh according to previous synaptic activity were also seen in sensory afferent terminals (LeCorronc and Hue 1993; Leitch and Pitman 1995; Trimmer and Weeks 1989). However, analysis of tissue extracts from Manduca sexta nerve cords revealed no changes of cAMP concentrations following exposure to muscarinic agonists (Trimmer and Qazi 1996). This suggested that the potential changes of cAMP levels in presynaptic terminals were too small or too short to be detected against the background of cAMP from other cells or that mAChRs may only be capable of reducing elevated cAMP levels.

In contrast to the results of most previous studies, mAChRs in the brain of the grasshopper mediate excitation by activation of AC, increased levels of cAMP and stimulation of PKA. A stimulation of stridulation by mAChRs evoked by inhibition of “inhibitory neurons” that contribute to the control of stridulation could be excluded because uncoordinated mixtures of different song patterns as seen after disinhibition with picrotoxin (Heinrich et al. 1998) never occurred on stimulation with muscarinic agonists (Trimmer and Qazi 1996). This suggested that the potential changes of cAMP levels in presynaptic terminals were too small or too short to be detected against the background of cAMP from other cells or that mAChRs may only be capable of reducing elevated cAMP levels.

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usually been performed in the presence of inhibitors of PDEs and effects on cAMP levels required high concentrations of muscarinic agonists (Felder et al. 1989; Jones et al. 1991), a physiological significance for this positive coupling of mAChRs to AC activity could not be established. In the cephalic circuits for the control of stridulation in grasshoppers, both the presumed natural transmitter ACh and specific muscarinic receptor agonists induced long-lasting excitation with the potential to accumulate with repeated stimulation (Heinrich et al. 2001b; this study). These effects were completely suppressed by muscarinic antagonists and inhibitors of both AC and PKA, suggesting that activation of the AC/cAMP/PKA pathway (as activation of PLC; see following text) may be a major and necessary step for mediating mAChR-initiated excitation instead of being merely a byproduct of another pathway or an artifact generated by overstimulation of the system.

The functional contribution of mAChRs to the control of stridulation has been demonstrated to lie in the accumulation of excitation provided by sensory input relevant to singing behavior (Heinrich et al. 2001b). This basic level of excitation or arousal determines the behavioral threshold to initiate stridulation in response to sensory stimuli and the selection of song patterns employed, according to increasing arousal during the progress of courtship (Heinrich et al. 2001a,b). These functions may rely entirely on the characteristics of the second-messenger pathways that are coupled to the respective subtypes of mAChRs. These characteristics include a rather weak activation on transient presynaptic release of ACh, a prolonged activity of key enzymes and/or persistent changes of structures mediating the physiological responses (e.g., ion channels), and the potential to progressively increase the activity level of the signaling pathway, thereby adjusting the basal state of excitation to the previous synaptic stimulation.

EVIDENCE FOR SEQUENTIAL ACTIVATION BY mAChRs OF THE AC- AND THE PLC- INITIATED SECOND-MESSERGER PATHWAYS. Complete suppression of muscarine-stimulated stridulation by inhibitors of AC and PKA (Fig. 3) and by two inhibitors of PLC with different mechanisms of action (Fig. 4, A and B) suggested that both pathways are present in the same neurons and necessary to generate the excitatory effects of muscarine. Stridulation could be stimulated by sole activation of AC or PKA or by inhibition of PDE activity. Also, forskolin-stimulation of AC, the key enzyme that initiates this signaling pathway, evoked stridulation with similar latencies and durations as those induced by muscarine when injected to the same sites in the brain (Fig. 2A). Taken together, these results suggest that both signaling pathways, AC/cAMP/PKA and PLC/IP₃/DAG, are most likely sequentially activated instead of being initiated independently by different subtypes of mAChRs. A parallel effect on cAMP and IP₃ second-messenger pathways has been demonstrated in studies on cultured neuronal cell bodies (Lapied et al. 1992), on cell lines transfected with muscarinic receptor subtypes (Peralta et al. 1988), and on isolated cilia of rat olfactory neurons (Vogl et al. 2000). However, muscarinic agonists evoked opposing effects in these preparations, e.g., stimulation of phosphoinositide hydrolysis and inhibition of AC or opposing hyper- and depolarizing currents, indicating a differential regulation or even a mutual inhibition (Vogl et al. 2000) of both signaling pathways. Sequential activation of AC and PLC has also been reported from various studies, e.g., on human neuroblastoma cells (Baumgold and Fishman 1988) and various transfected cells (Felder et al. 1989; Jones et al. 1991; Peralta et al. 1988). A physiological relevance for neuronal function remained unclear because relatively high agonist concentrations were needed to substantially stimulate both pathways in a given preparation (Felder et al. 1989; Jones et al. 1991) and high levels of expression of a given receptor can lead to an increase in amplitude of the signal transduction response and a loss of selectivity (Schwarz et al. 1993).

Our studies in the grasshopper brain indicate that AC, cAMP, and PKA are involved in one part of the combined signaling pathway, and PLC, generating the potential second messengers IP₃ and DAG, is a component of the other part. However, no components downstream to these intermediate steps have yet been identified. In particular, the substances thapsigargin, PMA and α-erythro-sphingosine failed to demonstrate the involvement of IP₃-sensitive Ca²⁺ liberation or PKC activity in mediating the physiological effects of PLC activation. Further studies will be necessary to elucidate the downstream components of this combined signaling pathway and the sequence and mechanism of coupling between both parts. Possible mechanisms for a coupling between AC- and PLC-initiated pathways have been suggested by earlier studies.

AC/cAMP/PKA PATHWAY ACTIVATES PLC. In principle, PLC could be stimulated by phosphorylation, as it has been demonstrated for PLC subtypes implicated with growth and differentiation processes (reviewed by Rhee and Choi 1992). However, mAChR-mediated phosphorylation of PLC subtypes contributing to neuronal signaling had either no or inhibitory effects on PLC activity (Liu and Simon 1996; Rhee and Choi 1992).

PLC/IP₃/DAG PATHWAY ACTIVATES AC. At least two mechanisms could account for activation of AC by PLC-initiated signaling pathways. DAG, generated by PLC-mediated phosphoinositide hydrolysis could activate PKC, which has been shown to phosphorylate and activate the AC2 subtype (Boi et al. 1997; Zimmermann and Tausig 1996). Other subtypes such as AC1 (Vorherr et al. 1993; Wu et al. 1993) and an AC found in Drosophila (Levin et al. 1992; Livingstone et al. 1984) can be activated by Ca²⁺. This mechanism has already been described in connection with mAChR stimulation, although high agonist concentrations or conditions that inhibited cAMP degradation by PDEs called into question its potential physiological relevance (Baumgold and Fishman 1988; Felder et al. 1989; Jones et al. 1991; Peralta et al. 1988).

Based on the results of the above-mentioned studies, it appears more likely that mAChRs contributing to the control of stridulation in grasshoppers may initially activate the PLC pathway that, subsequently, leads to stimulation of AC and its downstream effects that finally generate neuronal excitation. Experiments that combine direct activation of one signaling pathway (e.g., the cAMP pathway with forskolin) with specific inhibition of the other (e.g., inhibition of PLC with U-73122) are suited to determine the sequence of their activation. In addition, the nature of coupling between both parts of this combined signaling pathway and possible interactions with other neurotransmitter or neuromodulator systems that affect the performance of stridulation will be a focus of our future studies.
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REFERENCES


