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Calcium responses of chicken trigeminal ganglion neurons to methyl anthranilate and capsaicin

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Summary

Using digital fluorescence imaging, we determined the effects of methyl anthranilate (MA), an avian irritant, and capsaicin (CAP), a mammalian irritant, on intracellular calcium ($[Ca^{2+}]_i$) in chicken trigeminal neurons. Concentration–response functions indicated that the threshold for inducing increases in $[Ca^{2+}]_i$ was higher for CAP (30 μmol l^{-1}) than for MA (10 μmol l^{-1}). The maximum magnitudes of $[Ca^{2+}]_i$ in response to MA and CAP were compared after normalization to 40 mmol l^{-1} KCl. At equal concentrations (300 μmol l^{-1}), trigeminal neurons responded with a greater change in $[Ca^{2+}]_i$ to MA (78% of KCl) than to CAP (43% of KCl). Furthermore, at 300 μmol l^{-1}, 48% of neurons responded to MA whereas only 16% responded to CAP. The increases in $[Ca^{2+}]_i$ induced by both MA and CAP were dependent upon extracellular calcium. While the calcium responses to MA were also dependent on extracellular sodium, responses to CAP were not. There were separate but overlapping populations of neurons sensitive to MA and CAP. Taken together, the higher threshold concentration of CAP, the higher response magnitude to MA than CAP and the greater number of neurons sensitive to MA than CAP provide a rationale for the observed behavioral differences of birds to these two compounds. Finally, the findings that the calcium responses to MA and CAP have different ion dependencies and that there are separate populations sensitive to these compounds suggest different transduction mechanisms mediating chicken trigeminal responses to MA and CAP.

Key words: calcium imaging, capsaicin, chick, digital fluorescence imaging, Fura Red, irritation, methyl anthranilate, pain, primary cell culture, trigeminal neuron, repellent.

Introduction

Although the general morphological organization of the peripheral trigeminal system in birds is similar to that of mammals (Dubbeldam and Karten, 1978; Dubbeldam and Veenman, 1978), there are functional behavioral differences in how these taxa respond to exogenous algesic compounds (cf. Clark, 1998a; Clark and Shah, 1991; Clark et al., 1991; Mason et al., 1991; Nolte et al., 1993). Two compounds exemplify the taxonomic differences in behavioral nociceptive responses: methyl anthranilate (MA) and capsaicin (CAP; Clark, 1998b). For example, birds demonstrate a congenital aversion to MA, a chemical compound found in the skin of grapes (*Vitus* spp.), at concentrations that do not evoke behavioral responses from mammals (cf. Clark et al., 1991; Nolte et al., 1993). By contrast, birds are behaviorally insensitive to CAP, a potent mammalian irritant found in chili peppers (*Capsicum* spp.; Mason and Maruniak, 1983; Szolcsanyi et al., 1986). Humans and other mammals demonstrate aversive responses to oral exposures of CAP at 3 p.p.m. (10 μmol l^{-1}; Green, 1989; Simons et al., 2002), yet birds are indifferent to >1000–20 000 p.p.m. (3.3–66 mmol l^{-1}) CAP (Szolcsanyi et al., 1986; Mason et al., 1991; Norman et al., 1992). The reflexive avoidance of MA in birds and of CAP in mammals is mediated through the trigeminal pathway (Mason et al., 1989; Caterina et al., 2000).

Despite the lack of a behavioral response to CAP in birds, the avian nervous system is not completely insensitive to this compound. Sann et al. (1987) showed that injections of 1% CAP into the sciatic nerve of pigeons increased the concentration of substance P in dorsal horn neurons. Harti et al. (1989) demonstrated that a topical application of 1% CAP to the cornea of pigeons caused a decrease in substance P in 50% of the innervating neurons. In both cases, the physiological response to >30 μmol l^{-1} CAP did not appear to be associated with any overt behavioral response indicative of irritation. These observations are consistent with behavioral studies on starlings (*Sturnus vulgaris*). Mason and Clark (1995) showed that although starlings did not demonstrate a congenital behavioral avoidance towards CAP, they could be trained to avoid food treated with CAP in conditioned avoidance paradigms, and avoidance was contingent upon an intact ophthalmic branch of the trigeminal nerve. Together,

these studies indicate that birds can perceive and physiologically respond to CAP *via* pathways and mechanisms typically associated with nociception. However, birds do not perceive CAP as irritating.

The interest in elucidating the mechanisms underlying the mediation and perception of avian irritants is twofold. First, taxonomic differences in the ability to perceive plant chemical defenses such as irritants has profound implications in understanding the evolution of plant–animal interactions that focus on the foraging ecology of animals and seed survivorship/dispersal in plants (Norman et al., 1992; Clark, 1998b; Tewksbury and Nabhan, 2001). Second, understanding the neural coding of irritants will aid in the identification and development of environmentally safe repellents. This is important in resolving conflicts between wildlife and humans, e.g. crop depredations and property damage, while minimizing human impact on wildlife resources (Clark, 1998a,b).

There is considerable information on the effects of exogenous compounds on avian avoidance behavior, both empirically and from a structure–activity perspective (Clark and Shah, 1991, 1994; Clark, 1998a). Yet, we know very little about how these compounds are coded by the nervous system. In the present paper, we characterize the concentration–response relationships for MA and CAP in populations of cultured chick trigeminal neurons using digital fluorescence imaging of intracellular calcium ($[Ca^{2+}]_i$). In addition, we compare mechanisms of neural activation by MA and CAP by determining the relative dependence on extracellular Na^+ and Ca^{2+}.

Materials and methods

Primary culture of trigeminal ganglion neurons

Chicken (*Gallus gallus* Linn., White Leghorn) eggs (Truslow Farms, Chestertown, MD, USA) were incubated at 38°C in a circulating air incubator. Trigeminal ganglia were isolated from 6–10 embryos (day 18 or 19) for each experiment under sterile conditions. The ganglia were collected and minced in iced Hank's Balanced Salt Solution (HBSS; Sigma, St Louis, MO, USA) with 0.5% penicillin–streptomycin (PS; Sigma). The cells were dissociated with 0.1 mg ml^{-1} DNase and 1 mg ml^{-1} collagenase and gentle agitation at 37°C for 45 min. The cells were spun down at 1550 r.p.m. for 3 min and suspended in 2.5 ml of 10% Percoll in HBSS. The cells were carefully triturated 10 times with a silanized fire-polished pipette and 10 times with a small-bore pipette (or until mixture was homogenous). The cell suspension was then layered on top of a 30%/60% Percoll step gradient. Next, the cells were centrifuged at 800 ***g*** for 20 min at 20°C. The 10% Percoll layer impeded the formation of a myelin mat and improved the yield of neurons. Erythrocytes and satellite cells were found in the pelleted layer and the neurons were harvested at the interface between the 30% and 60% layers. The collected neurons were then suspended in 35 ml HBSS+PS. The neurons were centrifuged at 800 ***g*** for 5 min and suspended in 0.6 ml L-15 medium with supplement, and nerve growth factor (NGF; 100 ng ml^{-1}) was added (Baumann, 1993). 50–100 µl of neurons in growth medium were added to cloning cylinders (3 mm i.d.) set with silicone grease on poly-L-lysine/laminin-coated coverslips. The cells were stored in a 38°C, air atmosphere incubator and allowed to settle overnight. The following day, the medium was replaced in each well with 1 ml of fresh medium.

Calcium imaging

Changes in intracellular calcium levels ($[Ca^{2+}]_i$) were measured using ratiometric digital fluorescence calcium imaging of chick trigeminal neurons (Grynkiewicz et al., 1985; Restrepo et al., 1995). Cultured neurons were loaded with Fura Red (Molecular Probes, Eugene, OR, USA) by incubation in 10 µmol l^{-1} Fura Red/AM and 60 µg ml^{-1} Pluronic-127 in Ringer's solution at 24°C for 1 h. FuraRed was used because the excitation wavelengths, 485 nm and 440 nm, do not cause interfering fluorescence from MA as do the wavelengths used to excite Fura 2, the more commonly used Ca^{2+} indicator. Images of fluorescing neurons were acquired with a cooled charge-coupled device (CCD) camera. Autofluorescence was negligible and, with illumination times of 100–200 ms, we did not find appreciable photo bleaching.

Responses of neurons to chemical stimulation

Coverslips with attached neurons were placed in a flow chamber through which Ringer's solution flowed. Chemical stimuli in Ringer's solution were applied to the flow, and pairs of ratio images were acquired every 10 s. Chemical stimuli were applied to the neurons for 15 s and then the chambers were rinsed for 2–3 min between each stimulus. The average fluorescence ratio, F_{440}/F_{485}, an index of $[Ca^{2+}]_i$, was calculated for each neuron using 'regions of interest' drawn automatically for each neuron using Metafluor (Universal Imaging Corp., Downingtown, PA, USA). Responses, expressed as the change in fluorescence ratio, were normalized to the response elicited by the positive control, 40 mmol l^{-1} KCl, which depolarizes neurons, causing a concomitant influx of Ca^{2+} *via* voltage-gated calcium channels.

Ringer's solution (pH 7.4) used for these experiments contained 138.3 mmol l^{-1} NaCl, 5.8 mmol l^{-1} KCl, 1.0 mmol l^{-1} $CaCl_2$, 1.0 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes and 10.0 mmol l^{-1} glucose. Calcium-free Ringer's solution contained 138.3 mmol l^{-1} NaCl, 5.8 mmol l^{-1} KCl, 6.0 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes, 10.0 mmol l^{-1} glucose and 1 mmol l^{-1} EGTA. Sodium-free Ringer's solution contained 138.3 mmol l^{-1} *N*-methyl-D-glucamine, 5.8 mmol l^{-1} KCl, 1.0 mmol l^{-1} $CaCl_2$, 1.0 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes and 10.0 mmol l^{-1} glucose.

Experiment 1. Neuronal population response to MA and CAP

The object of this experiment was to quantify the neuronal population response to MA and CAP stimulation. Two measures of responsiveness were used as an index of cellular

activity: (1) the magnitude of MA- and CAP-induced changes in $[Ca^{2+}]_i$ normalized to that of a positive control, 40 mmol l^{-1} KCl, and (2) the proportion of cells in an experiment with a response greater than 5% of the response to KCl. To determine the concentration–response function of chick trigeminal ganglion (TG) neurons to MA and CAP, neurons were exposed to an ascending concentration series of stimulus, followed by exposure to 40 mmol l^{-1} KCl. In seven experiments, responses to MA were obtained from 12 neurons, and in four experiments, responses to CAP were obtained from 26 neurons.

Experiment 2. Distribution of neuronal sensitivity to MA and CAP

In a second series of experiments, we determined the colocalization of sensitivity to MA and CAP. Fields of neurons were exposed to 100 μmol l^{-1} MA, rinsed for 2 min and then the neurons were exposed to 100 μmol l^{-1} CAP. An equal number of tests had the reverse order of presentation of MA and CAP. After a final rinse, neurons were exposed to 40 mmol l^{-1} KCl. We classified neurons as responsive if the increase in $[Ca^{2+}]_i$ was greater than 10% of the KCl response. This criterion level was used because it represented the mean baseline $[Ca^{2+}]_i$ ± 2.5 S.D. recorded during the interstimulus interval. Responses of 467 neurons stimulated with both MA and CAP were measured in six experiments.

Experiment 3. Ion dependence of neuronal sensitivity

In a third series of experiments, we determined the ion dependence of neuronal sensitivity to MA and CAP. Following an initial stimulation with 100 μmol l^{-1} MA or 100 μmol l^{-1} CAP, the background flow of Ringer's solution was changed to either Na^+-free or Ca^{2+}-free Ringer's solution, and the response to a second stimulation of the appropriate compound was measured. Following replacement of the ion-free solution with normal Ringer's solution and a period of rinsing, the degree of recovery of sensitivity to MA or CAP was determined with a third stimulation. In four experiments for each ion, 12 MA-sensitive neurons were tested for calcium dependence and 11 MA-sensitive neurons were tested for sodium dependence. In three experiments, 17 CAP-sensitive neurons were tested for calcium dependence. In two experiments, 13 CAP-sensitive neurons were tested for sodium dependence.

Results

Growth of trigeminal ganglion (TG) neurons

After 18 h in culture, chick TG neurons were firmly attached to the substrate. Neurons exhibited development of neuritic processes and had phase-bright, round somatic morphology (Banker and Goslin, 1991). Neurons continued to grow and were used in tests 1–6 days after initiation of the cell culture. Once cells began to migrate towards one another and form clusters they were not used in tests.

Response and recovery of TG neurons

Cultured chick TG neurons responded to MA and CAP stimulation with reversible increases in $[Ca^{2+}]_i$. Initial neuronal responses occurred within 5–10 s of exposure to the stimulus, which was the limit of time resolution of the bath application. For both stimuli, responses peaked within 10–15 s of the stimulus flow being initiated. Recovery to baseline $[Ca^{2+}]_i$ typically occurred within 100 s after MA was rinsed from the chamber. Recovery from CAP stimulation took slightly longer, approximately 120 s. Based upon these observations, we set the minimum interstimulus interval to be 120 s for all subsequent experiments.

Experiment 1. Neuronal population response to MA and CAP

Neurons exposed to MA and CAP exhibited positive criterion responses of increasing magnitude as a function of

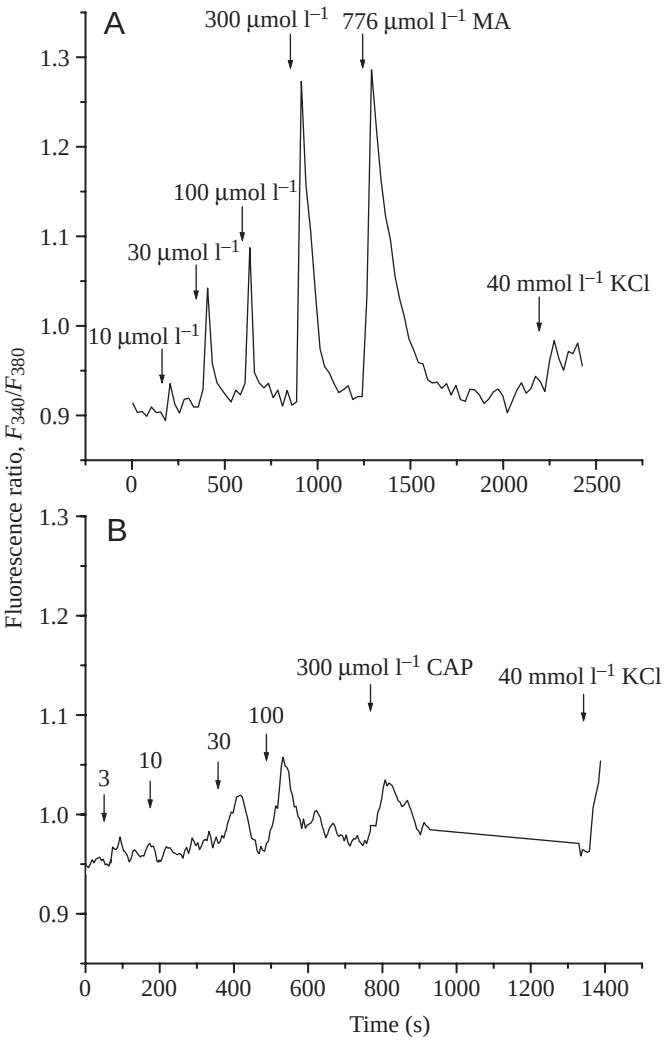

Fig. 1. Typical response profile for a cell indicating changes in fluorescence ratio as a function of time and extracellular concentration of stimulus: (A) methyl anthranilate (MA) and (B) capsaicin (CAP). The latency for peak response was 5–10 s after stimulation.

increasing stimulus concentration (Fig. 1). The proportion of chick TG neurons that responded to stimuli (r) was defined by the relationship $r=a(1-e^{-bx})^c$, where x is the concentration of stimulus, a relates to the maximum number of neurons recruited by increasing concentration of stimulus, b relates to the slope of this recruitment function, and c relates to both the threshold and the slope of the function. In general, the fit to the mean population response values for MA and CAP was good (MA – r^2=0.987, d.f.=2, 4, F=157.946, P<0.001; CAP – r^2=0.999, d.f.=2, 3, F=2799.9, P<0.001; Table 1). At the concentration that produced a saturated response level (300 μmol l^{-1}), the number of neurons responding to MA relative to CAP was 2.8 times greater (47.9% *vs* 16.6%, respectively; Fig. 2). MA-sensitive cells were recruited to the criterion response levels more gradually as MA concentrations increased. By contrast, CAP-sensitive cells were recruited to the criterion response levels over a narrower range of concentration (c.f. parameter c for MA and CAP; Table 1). The concentration of stimulus at which half the population of responsive neurons was activated was higher for MA (92.3 μmol l^{-1}) than for CAP (31.3 μmol l^{-1}).

Differential responsiveness of neurons was apparent when the normalized response amplitude was modeled as well. The asymptotic response amplitude was almost twofold higher for MA-sensitive neurons relative to CAP-sensitive cells (Fig. 3; c.f. parameter a, Table 1). Moreover, the first suprathreshold response concentration was lower for MA (10 μmol l^{-1}) than for CAP (30 μmol l^{-1}). By these two measures, chick neurons were more sensitive to MA stimulation. The recruitment of neural response was more graded over the concentration range for MA-sensitive cells relative to CAP-sensitive cells, with CAP-sensitive cells expressing near asymptotic amplitude responses at the suprathreshold stimulus concentration (~30 μmol l^{-1}). By contrast, the amplitude of responsive neurons at near threshold stimulus concentration (~10 μmol l^{-1}) was 17% of the asymptotic response level for MA-sensitive neurons (Fig. 3). The concentration of stimulus at which the mean response was half maximal was 83.0 μmol l^{-1} for MA and 24.0 μmol l^{-1} for CAP.

Table 1. *Mean parameter values for the concentration–response relationship between stimulus (MA or CAP) and $[Ca^{2+}]_i$ for KCl-sensitive chick TG neurons*

	Methyl anthranilate (MA) Mean ± S.E.M.	Capsaicin (CAP) Mean ± S.E.M.
Population response[1]		
Parameter *a*	45.831±2.640	16.283±0.173
Parameter *b*	0.007±0.002	0.085±0.031
Parameter *c*	0.937±0.231	9.539±9.291
Magnitude response[2]		
Parameter *a*	83.355±3.576	44.750±1.588
Parameter *b*	0.006±0.001	0.181±3.441
Parameter *c*	0.741±0.105	53.122±5420.225

[1]Parameter estimates for the concentration–population response relationship are based upon the proportion of KCl-sensitive neurons responding at or above the criterion value.

[2]Parameter estimates for the concentration–magnitude response relationship are based upon the response of a given cell relative to its response to stimulation with KCl.

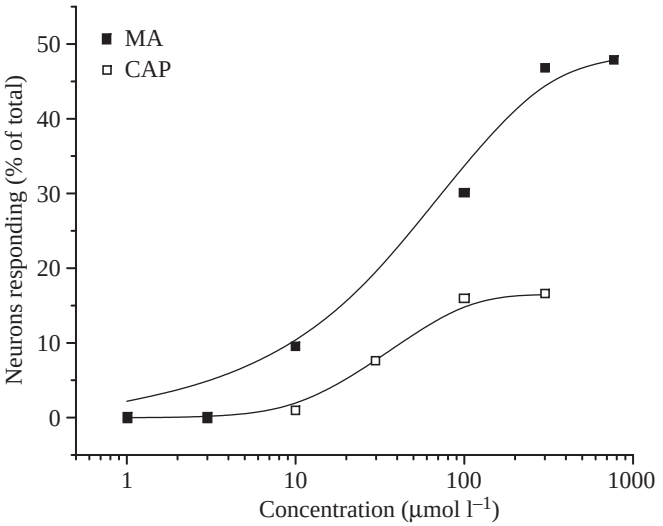

Fig. 2. The proportion of cells responding to criterion condition as a function of stimulus concentration: methyl anthranilate (MA; N=43); capsaicin (CAP; N=55). Positive responses were scored when intracellular calcium concentrations were at least 10% of the level of the terminal stimulation with KCl.

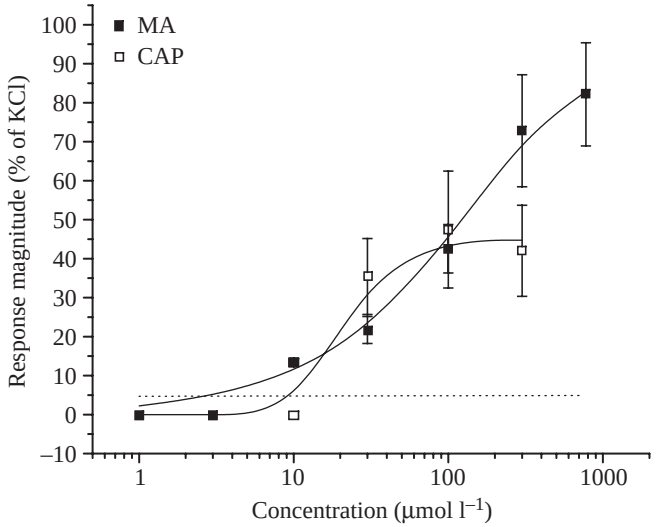

Fig. 3. The mean percent of peak calcium influx (normalized to the response of each neuron to KCl) as a function of the concentration of methyl anthranilate (MA; closed squares) or capsaicin (CAP; open squares). Potassium chloride (40 mmol l^{-1}) was used as a standard depolarizing stimulus. A 5% threshold is indicated by the broken line.

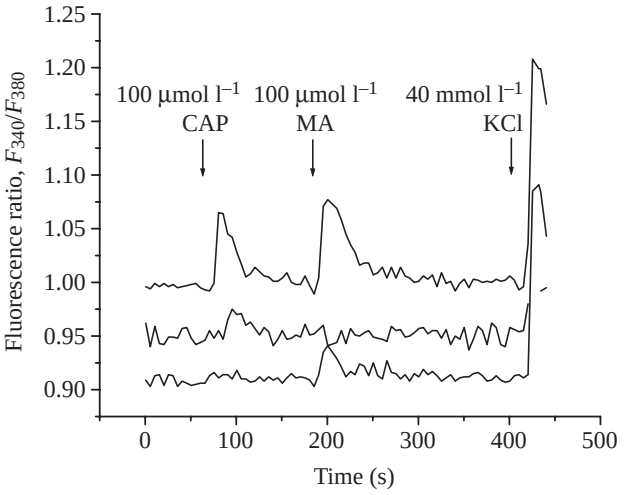

Fig. 4. Trigeminal neurons respond differentially to methyl anthranilate (MA) and capsaicin (CAP). Some neurons responded only to CAP (middle trace), others to MA only (bottom trace) and others to both (top trace). The top trace has been displaced upward 0.05 units for clarity.

Experiment 2. Distribution of neuronal sensitivity to MA and CAP

Most (67.9%) of the KCl-sensitive chick TG neurons did not respond to either stimulus. Slightly more chick TG neurons responded to only MA (16.9%) relative to those responding to only CAP (10.3%). A smaller proportion of KCl-sensitive chick TG neurons was sensitive to both MA and CAP (4.9%). Within this group of neurons, there appears to be no relationship between the magnitude of responses to MA and CAP. As noted above, KCl-sensitive chick TG neurons had a lower threshold for response and higher amplitude of response for MA than for CAP. Fig. 4 is an example of the differential responsiveness to MA and CAP in three neurons that were imaged simultaneously.

Experiment 3. Ion dependence of neuronal sensitivity

The responses of chick TG neurons to both MA and CAP were dependent upon the presence of extracellular calcium. In the presence of 1 mmol l^{-1} extracellular calcium, MA (100 µmol l^{-1}) induced $[Ca^{2+}]_i$ increases to 53.0±40.4% (mean ± S.E.M., N=13) of the KCl standard (Fig. 5A). When the extracellular calcium was removed from the medium, cells failed to respond to 100 µmol l^{-1} MA. When extracellular calcium was re-introduced, the response to 100 µmol l^{-1} MA exposure returned to pretreatment levels (48.1±35.8% of the KCl standard; P>0.05, Wilcoxon test).

A similar dependence on extracellular calcium was observed for CAP stimulation. In the presence of 1 mmol l^{-1} extracellular calcium, $[Ca^{2+}]_i$ increased to 46.1±30.9% of the KCl standard (N=17) after stimulation with 100 µmol l^{-1} CAP (Fig. 5B). When extracellular calcium was removed from the medium, cells failed to respond to 100 µmol l^{-1} CAP. When extracellular calcium was reintroduced the response to 100 µmol l^{-1} CAP returned to pretreatment levels (43.8±27.4% of the KCl standard; P>0.05, Wilcoxon test).

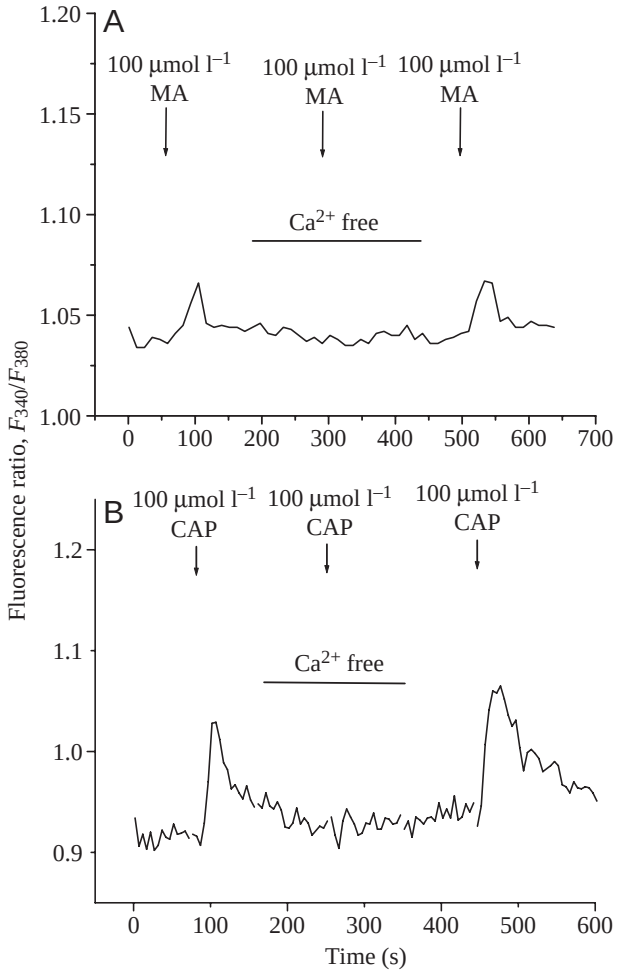

Fig. 5. The response of intracellular calcium to methyl anthranilate (MA) in chick trigeminal ganglion neurons is dependent on extracellular calcium. This figure shows typical chick trigeminal neurons responding to (A) 100 mmol l^{-1} MA and (B) 100 mmol l^{-1} capsaicin (CAP). When the extracellular calcium is removed, the responses are null. When extracellular calcium is re-introduced the responses return.

The responses of chick TG neurons to MA were dependent upon extracellular sodium. For example, in the presence of 138.3 mmol l^{-1} extracellular sodium, $[Ca^{2+}]_i$ increased to 41.6±20.0% of the KCl standard (N=11) in response to 100 µmol l^{-1} MA exposure. Following removal of extracellular sodium, the level of $[Ca^{2+}]_i$ failed to increase upon exposure to 100 µmol l^{-1} MA. Reintroduction of extracellular sodium into the medium resulted in complete recovery of responsiveness to 100 µmol l^{-1} MA. The response level (43.8±27.4%) was similar to that seen in the pretreatment period (pre- *vs* post-sodium removal, P>0.05, Wilcoxon Test; Fig. 6A). By contrast, chick TG neuronal responses to CAP were not dependent upon the presence of extracellular sodium. In the presence of 138.3 mmol l^{-1} extracellular sodium, the

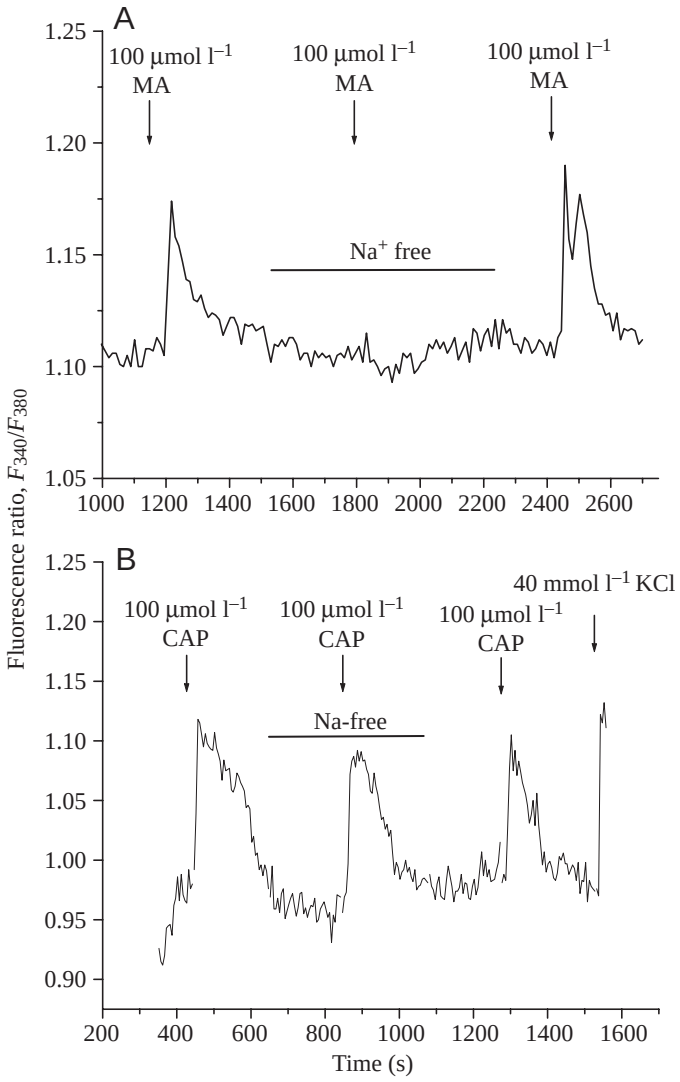

Fig. 6. The physiological response of chick trigeminal ganglion neurons to methyl anthranilate (MA) was also dependent on extracellular sodium, but responses to capsaicin (CAP) were not. (A) The response of a neuron that did not respond to MA when extracellular sodium was removed. The response returned when sodium was re-introduced. (B) A capsaicin-induced increase in intracellular Ca^{2+} even in the absence of extracellular Na^{+}.

$[Ca^{2+}]_i$ level in TG neurons increased to 45.6±29.4% of the KCl standard (N=15) in response to 100 µmol l^{-1} CAP exposure. Following removal of extracellular sodium, calcium responses to 100 µmol l^{-1} CAP exposure were unaffected (51.1±32.1% of KCl response; Fig. 6B). However, responsiveness to CAP was diminished following the replacement of sodium. The response level was 28.4±28.3% of the KCl response, suggesting cellular fatigue.

Discussion

Previous behavioral studies showed that MA is aversive to birds and that CAP is not (Kare, 1961; Szolcsanyi et al., 1986; Mason et al., 1989, 1991; Norman et al., 1992). Because birds were insensitive to concentrations of CAP up to 20 000 p.p.m., investigators believed that birds could not detect CAP. However, subsequent studies showed that starlings detected MA and CAP *via* the trigeminal nerve but that only MA was congenitally aversive (Mason et al., 1989; Mason and Clark, 1995). This study provides physiological data supporting the interpretation that birds are sensitive to both MA and CAP through the trigeminal nerve and that chick TG neurons respond to both MA and CAP with increases in $[Ca^{2+}]_i$. Moreover, the neuronal evidence supports the notion that birds are more sensitive to MA relative to CAP. Chick trigeminal neurons have a lower response threshold for MA than for CAP. At equal exposure concentrations, a greater percentage of chick trigeminal neurons responded to MA than to CAP. Moreover, stimulation with MA induced larger increases in $[Ca^{2+}]_i$ in chick trigeminal neurons than did CAP. We also showed that the neuronal response to MA and CAP is mediated by different cellular mechanisms. The sensitivity of chick TG neurons to MA was dependent on extracellular calcium and sodium. By contrast, the sensitivity of chick TG neurons to CAP was dependent only upon extracellular calcium. Finally, and importantly, we found that different populations of chick TG neurons responded to MA and CAP, with some neurons responding to both compounds.

The somatosensory systems of birds and mammals are superficially similar but differ in their response to some chemical irritants. First, many of the same endogenous compounds (e.g. bradykinin, histamine and serotonin) elicit pain and/or excite nociceptors in birds (Koltzenburg and Lewin, 1997; Gentle and Hill, 1987) and mammals (Kessler et al., 1992). Pain and irritation are modulated similarly in both taxa. Aspirin-like analgesics reduce oral irritation in starlings to the chemical *o*-acetophenone (OAP), a bird irritant similar in structure to MA. Moreover, starlings become sensitized to chemical irritants when treated with prostaglandin E1 (Clark, 1995). Second, thermonociceptors of mammals and birds respond to noxious heat in the same temperature range (Sann et al., 1987; Necker and Reiner, 1980; Nagy and Rang, 2000). Moreover, the responses of thermonociceptive neurons of both taxa were antagonized by capsazepine, an antagonist of the vanilloid receptor, VR1 (Marin-Burgin et al., 2000; Liu and Simon, 2000). Finally, polymodal nociceptors, an important class of neurons that respond to both noxious thermal and mechanical stimuli, are found in both taxa (Necker and Reiner, 1980; Gallar et al., 1993). However, although mammalian polymodal nociceptors are responsive to sub-micromolar concentrations of CAP (Liu et al., 1996), neural sensitivity to similar concentrations of CAP has not been observed in birds (Wood et al., 1988).

Despite these similarities of the somatosensory systems of birds and mammals, major differences also exist. First, birds lack the tetrodotoxin (TTX)-insensitive sodium channels of mammals (Petersen et al., 1987). Second, at concentrations that induce depolarizing currents in rat dorsal root ganglion or trigeminal neurons, neither CAP nor resiniferatoxin, a

capsaicin receptor agonist, induce inward currents or ^{45}Ca uptake in chick dorsal root ganglion (DRG) neurons (Winter et al., 1990). With one exception (Petersen et al., 1987), all studies to date that have examined the sensitivity of avian neurons to capsaicin have used concentrations of CAP below 30 μmol l^{-1}, the threshold of CAP response in our study. Petersen reported that 30 μmol l^{-1} CAP reduced TTX-sensitive sodium currents in chick DRG. Thus, while it is true that chick neurons are much less sensitive to CAP than are mammalian neurons, chick neurons are not totally insensitive. Third, neither corneal (1% CAP=3300 μmol l^{-1}) nor prenatal intraperitoneal (600 mg kg^{-1}) application of CAP in birds caused depletion of substance P- or calcitonin gene-related peptide (CGRP)-containing neurons (Harti et al., 1989) or desensitization to algesic compounds such as histamine, serotonin or bradykinin (Szolcsanyi et al., 1986), which it does in mammals (Holzer, 1991). Fourth, Mason and Clark (1995) showed that starlings could detect CAP *via* the trigeminal nerve but that responses were not nocifensive.

Consistent with the behavioral studies that show lack of nociceptive behavior towards CAP and the mechanistic studies that demonstrate the failure of CAP to deplete pain neurons of neuropeptides in birds, it is likely that the neurons responding to CAP in our study are not nociceptors. Lacking other functional characterization of these neurons, it is difficult to assign them specific physiological significance. Conversely, because MA is aversive to birds, at least some of the population of neurons that we found in chicken to be responsive solely to MA and not to CAP must be nociceptors. Whether they are sensitive to chemical stimulation only or are polymodal, analogous to mammalian polymodal nociceptors, remains to be determined.

From the experimental data, we infer that the sensitivity of chick TG neurons to MA results from an activation of a ligand-activated transduction mechanism. An alternative explanation is that MA and CAP cause membrane perturbation (Feigin et al., 1995). We do not believe this to be the mechanism for several reasons. First, if an increase in intracellular calcium response was due to membrane perturbation, we would expect the calcium response to be independent of sodium. This was not the case for MA, the responses to which were dependent on extracellular sodium. Second, if membrane perturbation was the mechanism driving neuronal response, we would expect an increasing number of neurons to respond as the concentration of irritant increased until at some point all neurons responded. Rather, we observed an asymptote in the numbers of neurons responding to increasing concentrations of both MA and CAP. A fraction of the population of neurons remains insensitive to MA and CAP. Third, we established that different populations of neurons responded to either MA, CAP or both compounds. If membrane perturbation was driving the response, we would not expect differential responses among the neurons. This segregation of the sensitivity to MA and CAP to different subpopulations of neurons suggests that there are at least two different transduction processes, and these are likely to be mediated by ligand–receptor interactions. One possible mechanism mediating the neuronal response to MA that is consistent with the observed dependence on extracellular sodium is the opening of voltage gated calcium channels secondary to a ligand-mediated sodium-dependent membrane depolarization (Kostyuk et al., 1981). This mechanism would explain the complete loss of neuronal sensitivity to MA when sodium is removed from the extracellular medium. It is unlikely that the initial depolarization of sensory neurons by MA is due to one of the transient receptor potential (TRP) ion channel family, of which mammalian and chicken VR1 are members, because these channels are non-specific cation channels.

Responsiveness to CAP may be similar to that described for mammals, albeit with decreased sensitivity. In mammals, CAP activates a non-specific ion channel (VR1), which depolarizes peripheral sensory neurons (Caterina et al., 1997). This channel allows the influx of calcium without dependence on extracellular sodium. Recently, an avian ortholog to the mammalian vanilloid receptor, VR1, has been cloned from chicken dorsal root ganglion (Jordt and Julius, 2002) and characterized. While similar to mammalian VR1 in terms of sensitivity to noxious heat (Petersen et al., 1987; Marin-Burgin et al., 2000) and antagonism of responses to protons by capsazepine (McIntyre et al., 2001), the chicken vanilloid receptor, cVR1, is insensitive to 100 μmol l^{-1} CAP when expressed in *Xenopus* oocytes. Because we observed robust responses to 100 μmol l^{-1} CAP, it is likely that either (1) a different ion channel mediates the neuronal responses we observed to CAP or (2) heterologously expressed channels are less sensitive than the native form. Jordt and Julius also reported that the heterologously expressed channels were insensitive to MA. However, they tested MA at a concentration below the threshold we observed.

A question arising from this research is whether the lack of sensitivity to CAP in birds is evolutionarily primitive or derived? Few data are available to comprehensively address this question. However, there is a suggestion that avian insensitivity is an evolutionarily conserved trait. Facial nerves (N VII) of catfish (*Ictalurus punctatus*) are insensitive to 0.1% CAP (B. Bryant, unpublished data). Similarly, dorsal root nerve fibers in toads (*Bufo bufo*) were unresponsive to CAP up to concentrations of 430 μmol l^{-1}, which is a concentration highly stimulatory to mammals (Hawkins et al., 1991). Brown treesnakes (*Boiga irregularis*) were behaviorally insensitive to ocular application of 1% oleoresin of *Capsicum* (estimated 100 p.p.m. CAP; Clark and Shivik, 2002). All of these taxa, including birds, share a common trait of insensitivity to CAP. The common ancestor of reptiles and birds post-dates the phylogenetic divergence of mammals while the phylogenetic origin of amphibia and teleosts pre-dates that of mammals. The implication of this pattern is that avian insensitivity to CAP is the primitive trait and that mammalian sensitivity is a derived trait.

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