Agonist activation of cytosolic Ca\textsuperscript{2+} in subfornical organ cells projecting to the supraoptic nucleus

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The subfornical organ (SFO) is sensitive to both ANG II and ACh, and local application of these agents produces dipsogenic responses and vasopressin release. The present study examined the effects of cholinergic drugs, ANG II, and increased extracellular osmolarity on dissociated, cultured cells of the SFO that were retrogradely labeled from the supraoptic nucleus. The effects were measured as changes in cytosolic calcium in fura 2-loaded cells by using a calcium imaging system. Both ACh and carbachol increased intracellular ionic calcium concentration ([Ca\textsuperscript{2+}]) in a few retrogradely labeled cells. The cholinergic effects on [Ca\textsuperscript{2+}], could be blocked with the muscarinic receptor antagonist atropine and with the more selective muscarinic receptor antagonists pirenzepine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP). In addition, the calcium in the extracellular fluid was required for the cholinergic-induced increase in [Ca\textsuperscript{2+}]. These findings indicate that ACh acts to induce a functional cellular response in SFO neurons through action on a muscarinic receptor, probably of the M1 subtype and that the increase of [Ca\textsuperscript{2+}], at least initially, requires the entry of extracellular Ca\textsuperscript{2+}. Also, consistent with a functional role of M1 receptors in the SFO are the results of immunohistochemical preparations demonstrating M1 muscarinic receptor-like protein present within this forebrain circumventricular organ.

cholinergic; muscarinic angiotensin II; osmosensitive magnocellular neurons; vasopressin; oxytocin

THE SUBFONNICAL ORGAN (SFO) is a forebrain structure that lacks a blood-brain barrier and is generally recognized for its important role in monitoring indexes of body fluid homeostasis (15, 25). The SFO and other structures lying along the lamina terminalis, specifically the median preoptic nucleus and the organum vasculosum, constitute a local neural network that is essential for normal body fluid and cardiovascular regulation (14, 16). Efferent connections from the SFO project to a number of forebrain structures such as the hypothalamic paraventricular nucleus (PVN), the supraoptic nucleus (SON), the amygdala, and perifornical hypothalamic, which are sites of integration and control of effector mechanisms (behavioral, hormonal, and autonomic) that collectively contribute to body fluid and cardiovascular regulation (14, 16).

The SFO is sensitive to osmotic stimuli (37), ANG II (26, 39), and ACh (7, 38, 40). Such extracellular humoral factors rely on pathways between the SFO and the SON for the control of vasopressin and oxytocin release. Magnocellular neurons of the PVN and SON (24, 29, 30) are key components of this forebrain network because they synthesize and control the secretion of vasopressin and oxytocin (10, 20, 32, 35), which affect total body sodium and water balance and distribution.

Relatively little is known of the individual cell types of the SFO that are affected by osmotic stimuli, ANG II, and ACh or of the mechanisms by which their efferent signals to humoral stimuli are generated and how coded neural information projects to forebrain nuclei receiving and integrating information related to body fluid and cardiovascular homeostasis, such as the magnocellular neurons. However, evidence indicates that the SFO is not composed of a homogeneous cell type. In addition to the expected variety of glia, there appears to be a variety of neuronal perikarya based on morphology (7). Electrophysiological studies have also suggested heterogeneity in the structure of the SFO composed of superficial and deep neurons (4, 5).

Our previous work has shown that SFO neurons can be retrogradely labeled by injection of tracer into the SON/perinuclear SON region (17), which contains terminal fields from the SFO (33). Retrograde tracer can be reliably injected into this terminal field of SFO projections without inadvertent diffusion of tracer from the delivery site into the ventricular system. The retrograde label can be visualized both in fixed coronal sections of the SFO, where it appears in an annular

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pattern, and in acutely dissociated cultured neurons of the SFO (17).

Previous studies (see Refs. 12, 17) have demonstrated the utility of $[\text{Ca}^{2+}]_i$ imaging in examining the response of dissociated and cultured cells of circumventricular organs. The present study used retrograde label to identify dissociated neurons of the SFO that had in vitro efferent connections with the SON. $[\text{Ca}^{2+}]_i$ imaging was then used to measure the response of identified neurons to ANG II, increased extracellular osmolarity (sodium concentration), and cholinergic agonists and antagonists.

MATERIALS AND METHODS

All rats, both adults and mothers with preweaning pups, were Sprague-Dawley-derived animals (Harlan; Indianapolis, IN). Rats were housed either individually (adults) or in groups, in the case of mothers and pups in a light-controlled room (12:12-h light-dark cycle) with food and water provided ad libitum for 4–7 days before they were used.

Eleven-day-old rats received pressure injections (under methoxyflurane inhalation anesthesia) of 1,1',dioctadecyl-3,3,3',3'-tetramethindocarbocyanine perchlorate (DiI; 50 mg/ml ethanol; Molecular Probes, Eugene, OR) aimed bilaterally at the SON. The injector was angled 20° lateral from the perpendicular to avoid the lateral ventricles and advanced so that its tip was stereotaxically placed at a target 1.3 mm behind bregma, 4.5 mm either side of midline, and 8.3 mm below the skull. Three days were allowed for retrograde transport, and then the SFO was removed for dissociation. The retrograde pattern of labeling was verified in coronal sections (Fig. 1). As previously reported (17), the pattern observed in coronal sections of the SFO was a concentration of retrogradely labeled cells in an annulus at the perimeter of the organ with a paucity of cells in the core of the structure. In patch-clamp electrophysiological studies conducted in our laboratory (17), individual labeled dissociated cells were easily visualized using rhodamine-labeled microspheres and microscopic epifluorescence. However, in the calcium imaging system used in the present studies, a narrow bandwidth of light was used, and DiI provided more intense fluorescence than rhodamine-labeled microspheres.

Following previously published protocols (18, 19), dissociated cells of the SFO were obtained and cultured for studies over 1–4 days postdissociation. Briefly, for the dissociation, five or six rat pups (14 days old) were decapitated, and each head was placed in ice-cold 70% ethanol. The brain was removed rapidly, placed in ice-cold Hanks’ balanced salt solution (HBSS; $+20$ mM HEPES buffer), and a thick coronal slice was made to include tissue from the rostral level of the optic chiasm caudal to the collicular level. Dissection studies we previously carried out in animals that had been systemically injected with Evans blue (1% solution), which labels brain areas outside the blood-brain barrier (i.e., circumventricular organs, such as the SFO), have shown that the SFO can be isolated and removed from these coronal slices with minimal non-SFO tissue attached (non-SFO tissue is mainly from the hippocampal commissure). Isolated SFOs from five six pups were transferred to ice-cold Earle’s salt solution (ESS; $+20$ mM HEPES buffer) for 1 h and then treated with dispase 1 (3 U/ml; Boehringer Mannheim, Indianapolis, IN) for 1 h at 35°C. After three washes in culture medium, a cell suspension was prepared by trituration of the fragments through a fire-polished Pasteur pipette ($15–20\times$) until the tissue fragments were visibly dissociated, and then, several drops of the medium containing the cells were plated onto previously precoated (0.1 mg poly-l-lysine/ml) round 25-mm coverslips and incubated in a humidified atmosphere (plus 5% CO$_2$) at 37°C. The culture medium was Dulbecco’s modified Eagle’s nutrient mixture F-12 HAM (1:1) with 10% fetal bovine serum (heat inactivated at 56°C for 1 h) and 1% i-glutamine-penicillin-streptomycin solution added (except where noted, all solutions are from Fisher). After 1–2 h postplating to allow the cells to adhere to the coverslips, 1–2 ml of additional medium was added to the culture wells; thereafter the medium was changed every 1–2 days. Within hours of dissociation, the cells began to differentiate: the neurons remain simply spherical and may develop one or two processes, but the glia develop either multiple astrocyte-like processes or become flat large cells with prominent nuclei but no processes.

Changes in cytosolic $[\text{Ca}^{2+}]_i$ were measured with fura 2 ratio fluorescence as previously described (12, 36). Cells were loaded with fura 2 by incubating cells grown on 25-mm coverslips (see above) in DMEM containing 0.1% BSA and 2 mM fura 2-AM (Molecular Probes) for 60 min at 37°C. During the incubation, fura 2-AM is absorbed by the cell and hydrolyzed only within living cells to impermeant fura 2. The coverslip was washed once with DMEM-BSA and once with HEPES-buffered ESS containing 10 mM sodium bicarbonate and 0.1% BSA. Calcium measurements were made using the latter as bath solution. To examine the effects of stimulation in a calcium-free bath, the extracellular medium was custom made without $\text{Ca}^{2+}$, and the $\text{Ca}^{2+}$ chelator, 1 mM EGTA, was added.

For $[\text{Ca}^{2+}]_i$ measurements and pharmacological manipulations, the coverslip was mounted in a brass bath chamber that was attached to the microscope stage. A circulating water jacket maintained a 37°C bath temperature, and all test solutions added were of the same temperature. The bath solutions could be siphoned off and the coverslip washed to permit subsequent testing. $[\text{Ca}^{2+}]_i$, was measured using a video microscopic digital image analysis system (Photon Technology International, South Brunswick, NJ). Excitation wavelengths of 340 and 380 nm were obtained with a 75-W xenon arc lamp, two separate monochromators, and a chopper. The excitation light was delivered from the mono-

Fig. 1. The typical pattern of retrograde label in a coronal section at approximately midrostral caudal level of the subfornical organ (SFO) 3 days after injection of the fluorescent tracer $1,1',\text{dioctadecyl-3,3,3',3'-tetramethindocarbocyanine perchlorate (DiI} into the perinuclear supraoptic nucleus (SON) area. The scale bar represents 100 μM.
chroomatography to a Nikon Diaphot microscope via fiber optics. A 405-nm dichroic mirror separated the excitation and emitted light. Fluorescence was observed via the microscope-computer system after passing through a 510-nm broad band filter. Excitation image pairs (340/380 nm) were acquired every 10 s with a silicon intensified target camera (Hamamatsu, Bridgewater, NJ) and a 512 × 480 frame grabber. The data were recorded on hard disk after several video frames were averaged to reduce the level of noise. An increase in [Ca^{2+}]_{i} produces a shift in fluorescence intensity from 380 to 340 nm and thus an increase in the 340/380 ratio. A positive change in intracellular [Ca^{2+}]_{i} was judged visually as a detectable change in color of the cell as the computer scanned the field every 10 s. Only after the experiment was completed were the accumulated data sets from positive responders analyzed and the [Ca^{2+}]_{i} converted to numerical concentrations. A response to stimulation was easily discerned as a sudden and sharp change in the color of the cell.

The system had been calibrated in a previous study on neurons (36). Briefly, pseudocolor maps of absolute [Ca^{2+}]_{i} were constructed on a pixel by pixel basis, and the values over the entire cell were averaged to obtain whole cell calcium concentration. Calcium concentration was calculated using the equation [Ca^{2+}]_{i} = K_{d}(Sf/Sb)(R - R_{min})/(R_{max} - R), where K_{d} is the dissociation constant of fura 2 (224 nM), R is the 340/380 ratio, R_{max} and R_{min} are the ratios obtained with saturated and zero levels of calcium, respectively, Sf and Sb are the 380-nm excitation fluorescence in the presence of EGTA and at saturation level, respectively. The values of R_{min} (0.45), R_{max} (8.5), and Sf/Sb (8.1) were determined by exposing cells to 5 mM ionomycin, 10 mM nigericin, and 2 mM gramicidin in a 100 mM K+ solution containing either 0 or 10 mM calcium.

The coverslip (200 µl bath solution) was systematically scanned with the computer to identify a microscopic field that contained one or more Dil-labeled cells. Three measures at 10-s intervals were taken as baseline, then the test solution (200 µl) was added via bolus injection and 12 subsequent measures were taken. Because in most cases unlabeled cells were present in the field, responses from these cells were also recorded as a matter of course.

Immunocytochemistry testing for the presence of M_{1} muscarinic receptor protein in the SFO was performed in seven male rats (300–350 g). Rats were anesthetized (pentobarbital sodium, 50–75 mg/kg) and perfused with 0.01 M PBS followed by 4% paraformaldehyde in 0.1 M PBS. The brains were removed and placed in 20% sucrose dissolved in 0.01 M PBS overnight and then coronally sectioned on a freezing microtome (40 µm). Tissue sections from five rats were incubated in 1 mg/ml nickel-diaminobenzidine tetrahydrochloride (Sigma) dissolved in 0.01 M PBS with 0.02% hydrogen peroxide. The intensity of staining was microscopically monitored to the desired ratio of signal to background. The sections were mounted on gelatinized slides, dried overnight, dehydrated in alcohol, and placed under a coverslip with DePeX (Electron Microscopy Sciences, Ft. Washington, PA).

RESULTS

Because of a long-standing interest in the coupling of the classic (renal) renin-angiotensin system with the brain-renin angiotensin systems through the SFO (22–24; see Refs. 14, 16 for review), we initially investigated the effects of increased extracellular ANG II on SFO neurons. In only 1 of 23 retrogradely labeled neurons and 3 of 44 unlabeled cells was there an increase in [Ca^{2+}]_{i} in response to the addition of 200 µl ANG II solution to the 200 µl bath solution (acetate salt, Sigma; final concentration 1 µM). To examine the possibility that the paucity of responding cells was due to inactivation of the ANG II receptors by nonspecific factors in the culture medium, several covenslips were subjected to a “starvation protocol” in which the cells were incubated in BSA-free media for ~18 h. In this case, 0 of 9 retrogradely labeled neurons and 2 of 15 unlabeled cells responded to the ANG II stimulation. In addition to these initial studies, we again tested the [Ca^{2+}]_{i} response of cells to ANG II (10 µM final concentration) after all the cholinergic manipulations were completed (see below). Of the 195 cells tested, 12% of 42 retrogradely labeled neurons and 26% of 153 unlabeled cells increased [Ca^{2+}]_{i}. See Fig. 2 for the pattern of averaged [Ca^{2+}]_{i} response of ANG II stimulation.

In other studies, a total of 103 cells (35 labeled) that had been grown in media of 298 mosM for at least 18 h were held at a basal 276 mosM for 1 h and then osmolality was increased by ~10% (final concentration 304–308 mosM; osmolality was increased via addition of basic solution rendered hyperosmotic via addition of NaCl). No cell showed any alteration in [Ca^{2+}]_{i}.

We then turned our attention toward studying the effects of cholinergic receptor agonists on SFO neurons. In initial cholinergic studies, when the cells were examined for responsiveness to carbachol (carbamylcholine chloride, Sigma; 100 µM), 32% of 22 retrogradely labeled neurons and 45% of 33 unlabeled cells showed...
an increase in \([\text{Ca}^{2+}]_i\). In addition, 10 of 10 cells recovered their basal level of \([\text{Ca}^{2+}]_i\) after three washes of bath solution. The cells clearly recover their basal levels after washing. There is a significant \((F_{14,28} = 15.5, P < 0.05)\) difference in the response of the cells to carbachol between the first and second application.

Similarly, cells that responded to ACh (1 mM) by increasing \([\text{Ca}^{2+}]_i\) could be returned to basal levels by application of atropine (1–10 \(\mu\)M) in 1 of 2 retrogradely labeled neurons and in 9 of 10 unlabeled cells. Overall, retrogradely labeled cells showed a tendency, although not significant, for a smaller increase in \([\text{Ca}^{2+}]_i\) than the unlabeled cells in response to ACh stimulation (Fig. 4). Further experiments were conducted to examine the nature of the muscarinic receptor that mediated the increase in \([\text{Ca}^{2+}]_i\). In 13 cells (2 with retrograde label) that had responded to application of ACh (1 mM) with increased \([\text{Ca}^{2+}]_i\), when the ACh was removed via three washes of bath solution and a combination of ACh and pirenzepine dihydrochloride (RBI, Natick, MA) was added (1 mM each), no increase in \([\text{Ca}^{2+}]_i\) occurred. However, after three more washes and application of ACh alone, a recovered, albeit reduced, increase in \([\text{Ca}^{2+}]_i\) occurred (Fig. 5A).

Fig. 3. The combined average response of cells (3 of 19 labeled) to bath application of carbachol (100 \(\mu\)M) indicated by the arrow. There is a significant \((F_{14,252} = 19.7, P < 0.05)\) rapid and sustained increase in \([\text{Ca}^{2+}]_i\). The inset shows the initial response of 3 cells to carbachol and the repeated response to carbachol of the same cells after 3 washes of bath solution. The cells clearly recover their basal levels after washing. There is a significant \((F_{14,28} = 15.3, P < 0.05)\) difference in the response of the cells to carbachol between the first and second application.

Fig. 4. The average response of retrogradely labeled neurons (\(n = 9\)) and unlabeled cells (\(n = 15\)) to bath application of ACh (1 mM) indicated by the arrow. Both show a significant \((F_{14,308} = 13.3, P < 0.05)\) rapid and sustained increase in \([\text{Ca}^{2+}]_i\). There is no significant \((F_{14,308} = 0.7, P > 0.05)\) difference in the response pattern between the labeled and unlabeled cells.

Fig. 5. A: the combined average response of the cells to the specific muscarinic M1-receptor antagonist pirenzepine (Pip; 2 of 13 cells labeled). In the first situation, ACh (1 mM) was added alone to the bath. A rapid and sustained increase in \([\text{Ca}^{2+}]_i\) is observed. After 3 washes with bath solution, ACh is again added but in combination with pirenzepine. No clear increase in \([\text{Ca}^{2+}]_i\), is observed. Again after 3 washes, ACh is added alone. Now a somewhat recovered response is seen, although clearly lower than the initial response. There is a significant \((F_{28,336} = 8.6, P < 0.05)\) difference in the pattern of response to ACh between the three situations. B: the combined average response of cells (2 of 12 labeled) that had an elevated level of \([\text{Ca}^{2+}]_i\) due to immediately previous application of ACh (1 mM). At the time indicated by the arrow 4-diphenylacetoxy-N-methylpiperdine methiodide (100 \(\mu\)M) was added to the bath. This muscarinic blocker produced a significant \((F_{14,154} = 30.4, P < 0.05)\) gradual decline in \([\text{Ca}^{2+}]_i\), to basal levels.
In 12 cells (2 with retrograde label) that showed increased levels of \([\text{Ca}^{2+}]_i\) to application of ACh, the increased level was gradually reversed (Fig. 5B) after the application of 4-DAMP (RBI; 100 µM).

Finally, we examined whether the presence of \(\text{Ca}^{2+}\) was necessary in the extracellular fluid for the increase in \([\text{Ca}^{2+}]_i\) after ACh (1 mM) application. In 13 cells examined (1 with retrograde label), there was only minimal, if any, effect when the 1.8 mM CaCl\(_2\) was excluded from the usual bath solution and 1 mM EGTA was added. However, a robust increase in \([\text{Ca}^{2+}]_i\) was observed in the presence of extracellular \(\text{Ca}^{2+}\) (Fig. 6).

Immunocytochemical treatment of tissue with primary M\(_1\) muscarinic receptor antibody revealed there was a high density of staining in the five sets of sections (Fig. 7). However, there was no indication of immunoreactivity in the SFOs of the two control sets of sections that were incubated under identical conditions but where the M\(_1\) antibody was omitted.

**DISCUSSION**

The present findings indicate that neurons of the SFO are retrogradely labeled when DiI is injected into the SON and its perinuclear region. The pattern of retrograde labeling in the SFO is annular. Backlabeled neurons from the SFO can be cultured and studied with \([\text{Ca}^{2+}]_i\) imaging techniques. Both cultured retrogradely labeled neurons (i.e., efferents of the SFO to the SON) and unlabeled cells failed to show changes in \([\text{Ca}^{2+}]_i\) to increases in extracellular osmolality (−10%). Also, only a few of the dissociated cells of the SFO, labeled or unlabeled, responded to ANG II. However, a failure to detect changes in \([\text{Ca}^{2+}]_i\) is not necessarily indicative of complete lack of cellular response to the manipulations; e.g., the manipulations may influence K\(^+\) channels producing only a weak depolarization and

Fig. 6. The effects of ACh (1 mM) in the presence of \(\text{Ca}^{2+}\) in the bath solution (1 of 13 cells labeled) or in a \(\text{Ca}^{2+}\)-free bath solution. There is a rapid and robust increase in \([\text{Ca}^{2+}]_i\), when \(\text{Ca}^{2+}\) is present and only a weak and delayed response in the absence of \(\text{Ca}^{2+}\) and the addition of 1 mM EGTA. There is a significant \((F_{7,84} = 6.7, P < 0.05)\) difference in the pattern of response between the 2 conditions.

Fig. 7. M\(_1\)-muscarinic receptor immunoreactivity in the SFO. When the section was not treated with the primary antibody (M\(_1\) receptor), no positive immunoreactivity was seen (A). However, when sections were treated with the primary antibody, dense immunoreactivity was seen (B and C).
possibly no change in $[Ca^{2+}]_i$; if, for example, the change is dependent on action potentials.

A relatively high number of cells, both labeled and unlabeled, increased $[Ca^{2+}]_i$, to the cholinergic agonist, carbachol. Basal levels of $[Ca^{2+}]_i$ could be restored after carbachol was washed off the cells, and the carbachol response could be repeated, although at a diminished level. In addition, the carbachol-induced increase in $[Ca^{2+}]_i$ could be blocked by the muscarinic receptor antagonist atropine. The normal endogenous cholinergic receptor agonist ACh also increased $[Ca^{2+}]_i$ in labeled and unlabeled cells. Again, atropine was effective in blocking the response and basal levels could be restored after washes. However, the repeated response to the agonist was also diminished compared with the original response. When more specific muscarinic subtype receptor antagonists were used, both pirenzepine and 4-DAMP were effective antagonists of the ACh-induced increase in $[Ca^{2+}]_i$, indicating that ACh is acting on $M_1$-muscarinic receptors. The ACh-induced increase in $[Ca^{2+}]_i$ was largely dependent on the presence of extracellular Ca$^{2+}$, indicating that Ca$^{2+}$ entry is a critical event in the signaling process. Finally, immunocytochemical evidence indicates the presence of $M_1$-muscarinic receptors in the SFO.

The pattern of retrograde label in the SFO with DiI is similar to previous studies using injections in the perinuclear SON area (17); i.e., it is annular or concentrated around the periphery of the organ with a relative paucity of label in the middle (41). However, the annular pattern is much less distinct when DiI is used as the retrograde label compared with rhodamine-labeled microspheres (17). This may due to the fact that DiI, both under fluorescent microscopy and photography, tends to “bleed” and produce a relatively diffuse image. The annular pattern of DiI label of the SFO with DiI is notable relative to the fact that immunolabeled cells in the SFO with $M_1$ receptors tended to be homogeneous across the SFO. The latter may explain why, in addition to labeled neurons, a large number of unlabeled cells responded to cholinergic manipulation.

Previous research has shown that $M_1$ receptors are present in the forebrain of primate and rat (8, 11). The present study found $M_1$ receptors in the hypothalamus and septum consistent with the findings of the previous reports. The present study found $M_1$ receptor immunoreactivity also is present in the SFO where cholinergic effects have been localized by pharmacological and physiological studies (27, 38, 40).

The annular pattern of retrograde label (see Fig. 1) may explain the failure of ANG II to produce an increase in $[Ca^{2+}]_i$ in retrogradely labeled cells. Numerous studies indicate that the SFO is sensitive to ANG II (26, 28, 39). However, the localization of ANG II receptors in the SFO appears mainly in the core of the structure (21; i.e., an area that may overlap with the area of paucity of retrograde label). Such an argument would not explain, however, why the unlabeled cells were also relatively nonresponsive. Perhaps SFO neurons maintained in tissue culture downregulate or internalize their ANG receptors. Another possibility is that membrane-bound receptors are lost during the dissociation process, thus rendering the cells unresponsive to specific neurochemicals. Ferguson and Bains (9) found that electrophysiological recordings obtained from slices do not differ in their basic properties from those obtained from dissociated cells. Nevertheless, the dissociation process may affect the cells’ reactivity to specific drugs.

Studies have found mecano/osmosensitive cells in areas of the nervous system that are involved in body fluid regulation and cardiovascular homeostasis (2). There is both functional (13, 23) and electrophysiological (37) evidence to suggest that SFO neurons are osmo- and/or Na$^+$ sensitive. However, the present study failed to detect any response in SFO cells to a 10% increase in osmolality of extracellular fluid. It is possible that SFO osmosensitive cells lose their sensitivity to increased extracellular osmolarity in the course of culture or that input is transduced through mechanisms not involving increased $[Ca^{2+}]_i$. It should be noted that in studies of the supraoptic nucleus in which electrophysiological effects are detected given osmotic changes of 10% or less, these increases are referenced from physiological osmotic pressure (1, 3, 31). In the present study, the 10% percent increase from a hypoosmotic reference would only have been 3–4% hyperosmotic compared with a physiological reference, but studies have detected neuronal responses with lower osmotic changes (34).

The present study found a robust response of $[Ca^{2+}]_i$ to cholinergic stimulation of the dissociated SFO cells consistent with in vivo studies of the SFO’s sensitivity to cholinergic stimulation (27, 38, 40). The receptors mediating the cholinergic response are clearly muscarinic in nature, given the efficacy of atropine blockade. Furthermore, the receptors appear to be of the $M_1$ subtype, and as deduced from the efficacy of pirenzepine, an $M_1$-receptor antagonist, and 4-DAMP, an $M_1/M_3$-receptor antagonist, (6) they block the effects of cholinergic receptor agonist. Finally, the immunocytochemical evidence of the presence of the $M_1$ receptor in the SFO argues for an important role for SFO $M_1$ receptors.

Although the present study clearly indicates that extracellular Ca$^{2+}$ is critical for a significant ACh-induced increase in $[Ca^{2+}]_i$, it is not clear from the present data whether the substantive amount of Ca$^{2+}$ producing the increase is due to a large membrane flux of external Ca$^{2+}$ inward or a relatively small flux of Ca$^{2+}$ inward that then mediates a large release of internal stores of Ca$^{2+}$.

**Perspectives**

The SFO is one of three sensory circumventricular organs (15) that senses blood-borne extracellular humoral factors (e.g., osmotically activate solute, ANG II, atrial natriuretic peptide; Ref. 15). Such blood-borne factors and their actions through sensory circumventricular organs are key mechanisms in body-brain communication that are necessary for the control of effec-
tor mechanisms that collectively maintain body fluid and cardiovascular homeostasis. The findings of the present study indicate that cells located at the periphery of the SFO projecting to the SON are sensitive to cholinergic receptor agonists, but insensitive to increased extracellular osmolarity and ANG II. The results suggest that the cells in the perimeter of the SFO may receive input from the central core of the SFO and thereby act as integrative interneurons. The SFO is a highly complex structure with many different cell types, and its morphology is consistent with the idea that substantial neural processing takes place in the SFO. An understanding of the cellular organization of the SFO and the flow of information through the SFO will be important to gain a full understanding of the processing of body fluid/cardiovascular-related sensory input into the central nervous system.

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