Substance P Targets Sympathetic Control Neurons in the Paraventricular Nucleus

Matthew D. Womack, Richard Morris, Thomas C. Gent, Richard Barrett-Jolley

Abstract—The paraventricular nucleus (PVN) contains spinally-projecting neurons implicated in fine-tuning the cardiovascular system. In vivo activity of “presympathetic” parvocellular neurons is suppressed by tonic inhibition from GABA-ergic inputs, inhibition of which increases sympathetic pressor activity and heart rate. Targeting of this specific neuronal population could potentially limit elevations of heart rate and blood pressure associated with disease. Here we show, for the first time, that “presympathetic” PVN neurons are disinhibited by the neuropeptide substance P (SP) acting via tachykinin NK1 receptor inhibition of GABA_\text{\textsubscript{A}} currents. Application of SP to the paraventricular nucleus of rats increases heart rate and blood pressure. In in vitro brain slice experiments, in the presence of GABA, 1\mu mol/L SP increased action current frequency by a factor of 2.7±0.6 (n=5, P=0.05, ANOVA). Furthermore, 1\mu mol/L SP inhibited GABA_\text{\textsubscript{A}} currents by 70±8% (n=8, P=0.005 paired t test). These effects were abolished by NK1 antagonists, but not NK2 and NK3 antagonists. GABA_\text{\textsubscript{A}} inhibition was not reproduced by NK2 or NK3 agonists. The inhibition of parvocellular GABA_\text{\textsubscript{A}} currents by SP was also abolished by a protein kinase C (PKC) inhibitor peptide and mimicked by application of phorbol-12-myristate-13-acetate (PMA), implicating a PKC-dependent mechanism. Single-channel analysis indicates that SP acts through reduction of channel mean open-time (cmot): GABAA cmot being reduced by approximately 60% by SP (P=0.05 ANOVA, Bonferroni). These data suggest that tachykinins mediate their pressor activity by increasing the excitability of spinally-projecting neurons and identifies NK1 receptors as potential targets for therapeutic modulation of the cardiovascular system. (Circ Res. 2007;100:1650-1658.)

Key Words: cardiovascular control ■ GABA ■ substance P

The paraventricular subdivision of the hypothalamic paraventricular nucleus (PVN) contains both hypothalamic-pituitary-adrenal axis control neurons, and spinally-projecting cardiovascular control neurons.\(^1\)\textsuperscript{--}\textsuperscript{6} Estimates suggest that up to 2000 neurons project directly from the PVN to the intermediolateralis (IML) of the spinal cord.\(^7\)\textsuperscript{--}\textsuperscript{9} These projecting parvocellular neurons have been implicated in a number of facets of cardiovascular control. For example, circadian regulation of blood pressure,\(^10\) blood-volume regulation,\(^7,8\) and the cardiovascular response to psychological stress.\(^11\) Furthermore, elevated sympathetic activity is associated with congestive heart failure observed in humans and animals,\(^12\)-\textsuperscript{14} and rat models suggest that this is associated with a diminished GABA sensitivity of PVN neurons.\(^13,15,16\) As such, spinally-projecting PVN neurons are prime targets for therapeutic intervention with the cardiovascular system.

Although there have been, to date, few in vitro studies detailing cellular mechanisms potentially exploitable for therapeutic control of cardiovascular PVN neurons, there have been a number of, largely in vivo, studies investigating their basic control and physiological activity. Early in vivo electrophysiological studies on cats confirmed an anticipated monosynaptic connection between the PVN and spinal sympathetic motor area (the IML).\(^17\) Furthermore, stimulation (electrical or glutamate) of the PVN was shown to give rise to an extremely rapid rise of blood pressure and renal sympathetic outflow in conscious rats.\(^18\) Additional studies show that in vivo, the PVN receives tonic inhibition from GABA-ergic inputs and this maintains their activity at relatively low levels,\(^19\)-\textsuperscript{21} despite the fact that these neurons also receive excitatory glutaminergic inputs.\(^18\) Strikingly, excitation of this nucleus by glutamate, or by blockade of GABA_\text{\textsubscript{A}} receptors with the antagonist bicuculline, increases blood pressure and heart rate.\(^18,22\)

Other physiological inputs to “presympathetic” parvocellular neurons have been, until now, unknown. Potentially, pharmacological inhibition of this neuronal population could limit sympathetically induced elevations of heart rate and blood pressure seen during heart failure or psychological stress.

Increasing evidence from in vivo experiments also suggest that the substance P (SP) family of neuropeptide neurotransmitters (tachykinins) play a major role in cardiovascular control and the expression of a cardiovascular stress re-
response. For example, SP is found at high levels within the PVN. Studies also show that central tachykinins increase efferent sympathetic nerve activity, and injection of tachykinin agonists into the hypothalamus leads to rapid elevation of heart rate and blood pressure. Furthermore, activation of PVN tachykinin receptors increases blood pressure and heart rate. Crucially, central administration of tachykinin antagonists reduce the elevation of blood pressure and heart rate resulting from psychological stress of conscious rats. We therefore sought to investigate whether tachykinins activated PVN spinally-projecting neurons, and if so, by what receptor and mechanism.

**Materials and Methods**

All procedures were performed humanely in accordance with the UK Home Office Regulations.

**Physiological Experiments**

Male Wistar rats (University of Liverpool Biomedical Service Unit) 200 to 300 g were anaesthetized with urethane (1.3 to 1.5 g/kg ip), temperature was monitored by thermocouple and infrared thermometer (Infrascan). The head was placed in a stereotactic frame and a small craniotomy made near to bregma to allow micropipette insertion. The tip of the micropipette was located in the PVN following the coordinates of Paxinos and Watson (1.3 mm posterior, and 0.4 mm lateral to bregma and 7.8 mm below the dura). Post-hoc confirmation of injection site was made either by inclusion of FITC-SP, or by following SP injection by injection of triamethane dye. SP, vehicle (ACSF), or bicuculline were applied unilaterally via a Hamilton syringe (100 nl of 5 mmol/L). Order of application was randomized. Blood pressure (MAP) and heart rate (HR) were recorded over 1200, analogue prefiltering appropriately.

**Brain Slice Preparation**

Spinally-projecting parvocellular neurons can be readily identified in thin fixed brain slices from aged rats, but patch clamp recording from relatively thicker, living slices of aged rats is not practical. Therefore, we used Wistar P14-30 rats in which visualized patch clamp is more feasible. Studies of age related receptor expression in the diencephalon show that most GABA$_A$ subunit expression occurs between E6 and P10. There are smaller changes in expression of a1 to a2/a3 in P10-P16. Furthermore, in vivo studies of tachykinin influence on cardiovascular control used aged rats. Therefore, available evidence suggests that our findings would be similar in experiments on older animals. Brain slice (250 μm) preparation and identification of spinally-projecting neurons by the retrograde-labeling technique has been described previously. Individual parvocellular neurons were identified under near-infrared DIC, with retrogradely labeled neurones being visualized through a G2A filter block (Ex510-560/DM575/BA590 nm) on a Nikon E600FN microscope.

**Electrophysiology**

Our methods for whole-cell recording, action-current frequency (cell-attached-patch), and single-channel (cell-attached-patch) experiments of PVN slices have all been described recently. Slices were superfused with a modified artificial CSF (ACSF) and pipettes filled with an appropriate physiological saline (supplemental Table I, available online at http://circres.ahajournals.org). We used an Axopatch 200B and acquired data at 10 kHz with an Axon DigiData 1200, analogue prefiltering appropriately.

**Analysis: Whole Cell**

Whole-cell current-voltage data were fitted with the Goldman-Huxley-Katz (GHK) current equation:

$$I = \frac{pCl_z \cdot \exp(-zF \cdot Vm/R \cdot T)}{R \cdot T} \left(1 - \exp(-zF \cdot Vm/R \cdot T)\right)$$

Where $Vm$ is the command potential, $pCl$ is the chloride permeability, $R$, $T$, $z$, and $F$ have their usual meanings.

**Single-Channel Analysis**

Membrane potential for cell-attach-patch mode was calculated from:

$$Vm = RMP - Hp$$

Where $Vm$ is the potential across the patch, $RMP$ is the resting membrane potential, and $Hp$ is the holding potential. Predicted reversal potential ($V_{rev}$) was calculated by combining Equation 2 with the Nernst equation:

$$V_{rev} = RMP - 580\log([Cl]_o/[Cl]_i)$$

Where $RMP$ was measured for parvocellular neurons by ourselves as a part of this study ($-62 \pm 1 mV$, $n=4$), $[Cl]_o$ is the extracellular chloride concentration, and $[Cl]_i$ is the estimated intracellular chloride concentration. Kinetic analysis followed previous methods. In the discussion, we assume whole-cell ion channel current to be comparable to the product of number of channels, open probability, and unitary amplitude. For any given holding potential ($Hp$), unitary current is proportional to unitary conductance. In this study we calculate mean open times, rather than $P_o$ specifically.

**Software**

Software for acquisition and analysis were non-commercial. Details (including suppliers) available on our website (http://pcwww.liv.ac.uk/~rjb/RBJ/software.html).

**Statistics**

Statistics were performed with StatsDirect. We used Student paired $t$ tests for single comparisons between means, or, for multiple comparisons, ANOVA with Bonferroni post-hoc analysis as appropriate (stated in the text). Means are given ±SEM, and n is the number of experiments.

**Chemicals**

FITC-SP was obtained from Molecular Probes. Drugs supplied by Sigma-Aldrich, UK, except PMA (Calbiochem), okadaic acid (Calbiochem), SB222200, and MEN10376 (Tocris). In terms of antagonist selectivity, spantide shows 20 fold selectivity for NK2/NK3,36 MEN10376 shows >20 fold selectivity for NK2 over NK1/3,37 and SB222200 shows >1000 fold selectivity for NK3 over NK1/2.38

**Results**

**Intra-PVN SP Increases Blood Pressure and Heart Rate**

Unilateral injection of SP/FITC-SP into the PVN resulted in a 10 mm Hg rise in MAP (from 77±6 to 87±4 mmHg, n=7, P<0.05, paired t test) and 14 BPM rise in HR (from 462±12 to 486±9 BPM, n=7, P<0.05, paired t test; Figure 1). Post-hoc epi-fluorescence analysis of the PVN shows that a proportion of parvocellular neurons took up FITC-SP (indicative of SP-receptor activity).
Figure 2A. This resulted in a significant increase in RSNA (Figure 2B).

**SP Increases Activity of GABA Inhibited Parvocellular Neurons**

To investigate the mechanism of action of SP, we switched to in vitro brain slide recording. We began by measuring the effect of SP on spontaneous action-current frequency. In the presence of ACSF alone, SP had little effect (Figure 3A and 3E). Importantly, however, SP significantly increased frequency against a background of submaximal GABA inhibition. This activation was abolished by the highly selective NK1 antagonist L703606 (Figure 3D and 3H).

**SP Inhibits GABA Whole-Cell Currents in Parvocellular Neurons**

Pressure application of 300 μmol/L GABA evokes large inward currents in these neurons (0.1 to 5 nA, −60 mV). These are inhibited by bath applied SP. Figure 4A illustrates such a reversible inhibition in a retrogradely labeled spinocellular neuron. On average currents were reduced to 30 ± 8% (n=8) of the control current (P≤0.05 paired t test). To confirm the identity of the GABA-evoked currents we constructed current-voltage curves (Figure 4B). These reversed close to the calculated chloride equilibrium potential of 35 mV (Figure 4D) and were powerfully inhibited by the selective GABA<sub>α</sub> receptor antagonist bicuculline (10 μmol/L, Figure 4C and 4E).

**SP-GABA<sub>α</sub> Inhibition Is Mediated by the NK1 Receptor**

Selective NK2 (NKA<sub>4–10</sub>) and NK3 (senktide) agonists and the endogenous NK3 agonist NKB (all 1 μmol/L) failed to inhibit GABA currents (Figure 5A and 5C).

Next, we measured the inhibition of GABA responses by SP in the presence of selective tachykinin antagonists. Antagonists were added to the superfusing ACSF 10 minutes before the addition of SP. Spantide (1 μmol/L), a selective NK1 antagonist, prevented the SP activity (Figure 5Bi and 5D), whereas neither selective NK2 (MEN10376, 1 μmol/L) nor NK3 (SB222200, 1 μmol/L) antagonists prevented the SP effect (Figure 5Bii,iii, and 5D).

**SP-GABA<sub>α</sub> Inhibition Is PKC Dependent**

The NK1 receptor is a classical 7-transmembrane (7-TM) domain G protein–coupled receptor (GPCR) and, although there are exceptions, typically couples to pertussis toxin insensitive G proteins such as Gq/11.40 The GABA<sub>β</sub> and γ-subunits are, in turn, targets for a number of protein kinases, such as PKC.41 Furthermore, the SP-GABA<sub>α</sub> receptor coupling in frog dorsal root ganglia (DRG)42 was mediated by PKC. We therefore investigated whether the PKC activator PMA mimicked the whole-cell effects of SP. Figure 6 (A and C) shows 100 nmol/L PMA bath applied to retrogradely labeled parvocellular neurons significantly inhibits whole-cell GABA currents. Furthermore, when included in the patch pipette, a selective PKC inhibitor peptide (PKC,19–31 1 μmol/L) prevents the effects of SP (Figure 6B and 6D).

**SP-GABA<sub>α</sub> Inhibition Involves Reduction of GABA Channel Open Time**

To further elucidate mechanisms by which SP reduces these whole-cell GABA<sub>α</sub> currents we used cell-attached-patch single-channel recording of parvocellular neurons in the presence and absence of 1 μmol/L extracellular SP. To analyze whether SP alters GABA<sub>α</sub> channel conductance, we
constructed all-point-amplitude histograms of GABA\(_A\) channel events (Figure 7A through 7D). This was done at a range of potentials (with 3 GABA concentrations), with and without SP. Slope conductances were all in the range of 14 to 18pS (Figure 7E and 7F), and there was no difference between any of these, even in the presence of SP (Figure 7F; \(P \leq 0.05\), ANOVA, Bonferroni multi-comparison).

As there is no change in GABA\(_A\) channel conductance, but a striking reduction of whole-cell GABA\(_A\) current, ion channel dogma states that there must be a change in either the

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**Figure 3.** SP increases action current frequency in the presence of GABA. A, 1\(\mu\)mol/L SP bath applied to parvocellular neurons under control conditions. E, Data from 5 similar experiments to that in A. Residual action-current-frequency 92±5%. B, Bath application of 30\(\mu\)mol/L GABA inhibits spontaneous action potential-frequency. F, Mean residual frequency 0.2±0.1 (n=4). C and G, In the presence of bath applied 30\(\mu\)mol/L GABA, SP now increases action current frequency by a factor of 2.7±0.6 (n=5, \(P \leq 0.05\), 2-way ANOVA). D and H, The effect of SP was abolished by the selective NK1 antagonist L703606. Residual frequency: 1.3±0.8% (n=5). L703 indicates 1\(\mu\)mol/L L703609; Cont, control.

**Figure 4.** GABA\(_A\) currents of spinally projecting parvocellular neurons. A, 300\(\mu\)mol/L GABA currents in a (retrogradely-labeled) spinally-projecting parvocellular neuron, inhibited by SP, at a Hp of −60mV. GABA was applied in 3-second pulses. B, 300\(\mu\)mol/L GABA evoked whole-cell currents recorded from the same neuron, but with Hp of −80mV to +60mV. C, GABA 300\(\mu\)mol/L whole-cell currents with and without 10\(\mu\)mol/L bicuculline. D, Data from a number of experiments such as that in B. The smooth line represents the GHK current equation (Equation 1) with chloride permeability constant 0.3. E, Residual whole-cell currents in the presence of 10\(\mu\)mol/L bicuculline (n=5), \(*P \leq 0.005\) (Student paired t test).
number of active channels, or of channel kinetic properties. To analyze which of these mechanisms underlies SP inhibition of GABA<sub>A</sub> current, we undertook kinetic analysis of GABA<sub>A</sub> channel inhibition by SP, again in cell-attached-patch mode. We recorded channel activity at −100 mV (H<sub>p</sub>) with 10 μmol/L or 100 μmol/L GABA with and without 1 μmol/L SP. Dwell times were fit as described in the methods (Figure 8A and 8B). Full kinetic analysis revealed that in the presence of 100 μmol/L GABA there was a significant decrease in burst length (from 63.0 ± 20 ms, n = 3 to 29.4 ± 9 ms, n = 6, P < 0.05) and corrected mean open time (from 3.7 ± 0.3 ms, n = 3 to 1.6 ± 0.2 ms, n = 6, P < 0.05; Figure 8D), compared with those in the presence of 1 μmol/L SP. None of the other parameters tested were significantly altered by SP (Figure 8C and 8D; ANOVA, Bonferroni multi-comparison analysis for all kinetic parameters).

Discussion
In this work we show tachykinins to activate parvocellular neurons in the rat PVN. We show this to be mediated by an interaction between GABA<sub>A</sub> and tachykinin NK1 receptors via a PKC mechanism. Kinetic analysis shows this to be associated with a reduction of single-channel conductance, but not conductance.

“Presympathetic” Parvocellular Neurons
The PVN of the hypothalamus coordinates a number of hormonal and autonomic physiological responses. Indeed, Loewy referred to the PVN as being the “autonomic master controller”. In terms of cardiovascular control, it appears that much of the sympathetic drive is mediated by direct projection of parovascular vasopressinergic neurons to the IML of the spinal cord. Injection of retrograde tracer into the IML has been shown in several studies, to label the spinally-projecting “presympathetic” vasopressinergic neurons, emanating mainly from the ventral medial and lateral parvocellular subdivisions, but also to a lesser degree from the dorsal parvocellular subdivision. Stimulation of the PVN with either glutamate or the GABA<sub>A</sub> antagonist bicuculline increases both heart rate and blood pressure, and this effect can be reduced by the injection of a vasopressin antagonist into the spinal cord. Furthermore, elevated activity of PVN “presympathetic” neurons has been linked to heart failure in rat models. Modulation of these parvocellular neurons at the level of the PVN is therefore of potentially profound importance to the fine control of the cardiovascular system. In vivo, these neurons are tonically inhibited by GABA. We show here that these neurons are activated by SP and, interestingly, this is GABA dependent.
GABA<sub>A</sub> Receptors in Parvocellular Neurons

Despite the great importance of parvocellular “presympathetic” neurons, there have been relatively few studies directly investigating their pharmacological control. A number of studies have demonstrated the presence of GABA<sub>A</sub> receptors in the PVN. More recently, studies by others have shown spinally-projecting parvocellular neurons to also be inhibited by GABA, as predicted from earlier in vivo work. In this study we found retrogradely labeled spinally-projecting neurons to elicit inward currents in response to pressure application of 300 μmol/L GABA. GABA<sub>A</sub> receptors are, however, ubiquitous throughout the central nervous system.

Figure 6. SP inhibition is mediated by PKC. A, Whole-cell GABA<sub>A</sub> currents recorded from spinally-projecting parvocellular neurons before, during, and after the addition of the PKC activator PMA (100 μmol/L) where indicated by the bar and in the presence of 100nmol/L okadaic acid. B and D, GABA<sub>A</sub> currents in control, control + PKCi, then control + PKCi + SP. PKCi prevented the inhibition by SP. C, Summary of 4 experiments such as that in A. D, Summary of 4 experiments similar to B. PKCi indicates PKC inhibitor peptide. SP and PKCi were applied at 1 μmol/L, with GABA at 300μmol/L. Hp = -60mV. *P<0.05 Student paired t test.

Figure 7. SP does not alter the GABA<sub>A</sub> single channel conductance. A, GABA<sub>A</sub> channels recorded in cell-attached mode. B, GABA<sub>A</sub> channels in the presence of 1μmol/L SP. C, All-point amplitude histogram for the patch illustrated in A. D, Amplitude histogram for the patch illustrated in B. In A and B, GABA 100μmol/L. E, Single-channel current-voltage curves with and without SP. Smooth lines represents fits to amplitude means, and calculated V<sub>rev</sub>, 0mV (Equation 3: [Cl]<sub>in</sub> = 15mmol/L, calculated from the Nernst equation after). GABA 1μmol/L (circles), 10μmol/L (triangles), 100μmol/L (squares). F, Mean chord-conductance values, n=4 to 29, P>0.05, ANOVA, Bonferroni multi-comparison test. SP = 1μmol/L SP. Hp = -100mV.
system, and before these parvocellular neurons could be targeted in potential cardiovascular therapies, one would need to identify a more specific pharmacological profile. In previous studies, researchers have investigated whether either orexin or angiotensin II may be direct pharmacological regulators of spinally-projecting parvocellular neurons. Although both neuropeptides have receptors expressed in the PVN and, indeed, angiotensin II directly modulates “medulla-projecting” PVN neurons, neither modulates spinally-projecting neurons themselves. In this study, however, we show that SP acts within the PVN to increase HR, MAP, and RSNA, and we present first direct evidence that spinally-projecting PVN neurons are modulated by SP.

**Inhibition of GABA<sub>\alpha</sub> Ion Channels by SP**

The tachykinins are a family of neuropeptides (SP; neurokinin A, NKA; and neurokinin B, NKB), members of which have been implicated in central cardiovascular control. Several studies have reported high levels of tachykinin receptor expression within the PVN. Although both neuropeptides have receptors expressed in the PVN and, indeed, angiotensin II directly modulates “medulla-projecting” PVN neurons, neither modulates spinally-projecting neurons themselves. In this study, however, we show that SP acts within the PVN to increase HR, MAP, and RSNA, and we present first direct evidence that spinally-projecting PVN neurons are modulated by SP.
Mechanism of Action of SP

We approached this question from two perspectives. (1) What is the intracellular signaling pathway? (2) What are the changes in individual single channel properties, in the presence of SP?

(1) The NK1 receptor frequently couples to PKC via Gq (reviewed40), and PKC phosphorylation of GABA<sub>δ</sub> channel β-subunits (conserved serine–409 and 410 residues) reduces GABA<sub>δ</sub> whole-cell current in cultured cortical neurons.41 In this study, we found that the PKC inhibitor peptide (PKC<sub>19–31</sub>) prevented the SP inhibition of parvocellular GABA<sub>δ</sub> responses. Furthermore, the SP inhibition was mimicked by PMA (a diacylglycerol mimetic), lending further support to the hypothesis that SP inhibits GABA<sub>δ</sub> channels via PKC.

(2) Reduction of whole-cell current generally results from either a reduction of the unitary current (i), channel membrane expression (n), or the open time/probability (cmot(P)). Recent studies have also shown that in some preparations, GABA<sub>δ</sub> channels have an increasing unitary conductance with increasing concentrations of GABA.45 and the archetypal GABA<sub>δ</sub> antagonist bicuculline decreases GABA<sub>δ</sub> conductance.45 Here, we found no change of GABA<sub>δ</sub> channel conductance in the presence of SP. This implies that either the number of active GABA<sub>δ</sub> channels (n) or the open time is reduced by SP. In fact both of these mechanisms have been shown to mediate GABA<sub>δ</sub> inhibition via PKC phosphorylation previously.45,64 In this study, we observed a decrease in corrected mean open time (cmot) in the presence of SP. This decrease in cmot would be large enough to contribute to a large proportion of the inhibition seen in whole-cell experiments. These data also argue against a mechanism solely involving an increase in receptor desensitization, such as that observed by Si et al (SP inhibition of GABA responses in DRG),66 or indeed the PKC driven reduction of the GABA<sub>δ</sub> receptor surface expression.67 Rather, the mechanism appears to involve an allosteric reduction in channel activity, which would be consistent with the inhibitory pathway described by Brandon et al41 and involving phosphorylation of the conserved serine–409 and 410 residues of the β-subunit. The fact that SP inhibition of GABA<sub>δ</sub> current is maintained in cell-attached experiments also demonstrates, unequivocally, that the effect of SP is directly on the target neuron, rather than by causing the release of some intermediary factor from a neighboring neuron. This is because SP exposure is restricted to the area of membrane directly underneath the patch pipette tip.

Significance of SP-GABA<sub>δ</sub> Interaction to Cardiovascular Control

Accumulating evidence shows that spinally-projecting parvocellular neurons exert a powerful modulatory influence on the cardiovascular system. These neurons may be involved with volume regulation,7 circadian regulation of the cardiovascular system,10 the stress response,11 or heart failure.13,15 The current work presents the SP NK1 receptor as a putative target for pharmacological control of the cardiovascular system.

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References


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### Online Table I

The table summarizes the solutions used for electrophysiological experiments and described in the text. Concentrations are given in (mmol/L). *PH'd with KOH, or with +NaOH, "Ohms" refers to the resistance of patch pipettes, when filled.

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<th>NaCHO₃</th>
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