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Single potassium channels opened by opioids in rat locus ceruleus neurons

(opiates/morphine/electrophysiology/noradrenaline neurons)

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ABSTRACT **Currents through single-ion channels were recorded in the cell-attached configuration from locus ceruleus neurons enzymatically dissociated from newborn rats. When the selective μ opioid receptor agonist Tyr-D-Ala-Gly-MePhe-Gly-ol was in the patch-clamp electrode, unitary inward currents were observed with conductance of ≈45 pS (measured at zero pipette potential, with 150 mM potassium in the recording electrode). Long silences, lasting many seconds to minutes, separated periods of activity of similar durations. Within such activity periods the distribution of closed times of the channels was best fitted by the sum of two exponential functions (time constants ≈1 and 30 ms), and the durations of channel openings were fit by a single exponential function; mean open time increased from 2 to 120 ms as agonist concentration increased. Channel activity was not seen when high concentrations of opioids were applied to the neuron outside the patch-clamp recording electrode, indicating intimate coupling between receptor and potassium channel. Unitary currents with similar properties were also seen when pipettes contained α_2 adrenoceptor agonists or somatostatin. Taken with previous findings, the results indicate that μ opioid receptors, α_2 adrenoceptors, and somatostatin receptors can couple directly to membrane potassium channels through the local intermediary action of a GTP binding protein.**

Opioid receptors of the μ type are well characterized in the mammalian nervous system (1–3), and their distribution has been mapped (4, 5). This receptor appears responsible for most, if not all, effects of morphine when administered in the therapeutic setting or when self-administered by addicts. The receptor is known to be associated with a *Bordetella pertussis* toxin-sensitive GTP-binding protein (G protein) from biochemical (6) and physiological experiments (7), and its activation is known to lead to an increase in cell potassium conductance, hyperpolarization, and inhibition of firing (7–10). However, little further evidence implicates any common diffusible intracellular second messengers in linking receptor to potassium channel, and neither cAMP nor diacylglycerol seem to be involved (7, 10). Our experiments tested the hypothesis that receptors are closely coupled to potassium channels.

METHODS

Rats aged 1–7 days were decapitated under anesthesia, the brain was removed, and a slice (400 μm) was cut at the level of the locus ceruleus. Experiments with intracellular recording were done as described (10, 11) with glass microelectrodes containing 2 M KCl at 37°C. For patch-clamp recordings, locus ceruleus neurons were dissociated by a method based on that of Huettner and Baughman (12). The nucleus was visualized with a dissecting microscope and cut from the slice with a stainless steel punch (400 μm, i.d.). The punched tissue was incubated with gentle stirring for 15–20 min at 31°C in the following solution: 126 mM NaCl/1.2 mM NaH_2PO_4/1.2 mM $MgCl_2$/1.5 mM $CaCl_2$/2.5 mM KCl/ 25 mM $NaHCO_3$/11 mM glucose/0.5 mM EDTA/1 mM DL-cysteine containing papain at 20 units/ml (Cooper Biomedical), gassed with 95% O_2/5% CO_2. Enzyme action was stopped by adding trypsin inhibitor (1 mg/ml, Sigma) and bovine serum albumin (1 mg/ml, Sigma) (pH 7.3 ± 0.15). The tissue was gently triturated with fire-polished pipettes (tip diameter was first 0.6 mm and then 0.3 mm), and dissociated cells were sedimented (70 × *g* for 10 min) through a solution containing trypsin inhibitor and bovine serum albumin each at 5 mg/ml. The cells thus obtained had primary and secondary dendrites, and the majority of them stained densely with antibody to tyrosine hydroxylase.

Single-channel currents were measured with the Axopatch-1A patch-clamp amplifier in the cell-attached configuration by using fire-polished glass electrodes (0.8–5 MΩ) coated with silicone resin (13). In most experiments the electrodes contained the following: 150 mM potassium gluconate/2.5 mM $CaCl_2$/1.3 mM $MgCl_2$/3 mM tetraethylammonium chloride (TEA), 5 mM Mops·KOH, pH 7.3 ± 0.15 with or without the μ opioid agonist Tyr-D-Ala-Gly-MePhe-Gly-ol (Enk-ol). The cells were superfused at 31 ± 2.5°C in the recording chamber with extracellular solution of 150 mM NaCl/1.3 mM $MgCl_2$/2.5 mM $CaCl_2$/2.5 (or 10) mM KCl/11 mM glucose/5 mM Hepes·KOH, pH 7.3 ± 0.15/0.001 mM tetrodotoxin. The presence of tetrodotoxin and/or 10 mM potassium prevented the occurrence of spontaneous action potentials in the cells, which otherwise could be recorded capacitatively coupled through the membrane at the tip of the patch electrode. Single-channel currents were filtered with 80 db per decade low-pass Bessel filter at 5 or 10 kHz and stored on a video cassette recorder (Sony, SL-2700) using a pulse code modulation converter system (Dass, Unitrade). The stored data were low-pass filtered at 2 kHz, digitized at 100 or 200 s with an A/D converter (Axolab-1, Axon Instruments, Burlingame, CA); analysis was done with FETCHAN and PSTAT (Axon Instruments) programs, after Gaussian filtering with 1.5-kHz cut-off. Unitary currents used for quantitative analysis showed no superpositions during at least 10 min of recording and were assumed to arise from single channels. The event detection threshold was 50% of the mean-channel amplitude, and the dead-time of the recording system was 0.2 ms.

RESULTS

Recording with microelectrodes from neurons in the intact slice showed that the opioid peptide Enk-ol caused a mem-

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Abbreviation: Enk-ol, Tyr-D-Ala-Gly-MePhe-Gly-ol.

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brane hyperpolarization (Fig. 1*A*) (n = 12 cells, rats aged 1–6 days). Amplitude of the hyperpolarization depended on the concentration applied (10–1000 nM); 50 nM caused half-maximal response. With single-electrode voltage-clamp (10), opioids caused an outward current (up to 800 pA) at holding potentials close to the resting level. The effect of Enk-ol or [Met^5]enkephalin was competitively antagonized by naloxone; the dissociation equilibrium constant (K_d) for naloxone calculated by Schild analysis was 2 nM. Agonists selective for δ or κ receptors, Tyr-D-Pen-Gly-Phe-D-Pen (n = 4) and U50488H [*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate, n = 3] (each up to 3 μM), had no effect on resting membrane potential of the neurons. These recordings with microelectrodes suggest that the neonatal locus ceruleus neurons express μ receptors, activation of which increases the cell potassium conductance, as described in detail for adult rats (10, 14).

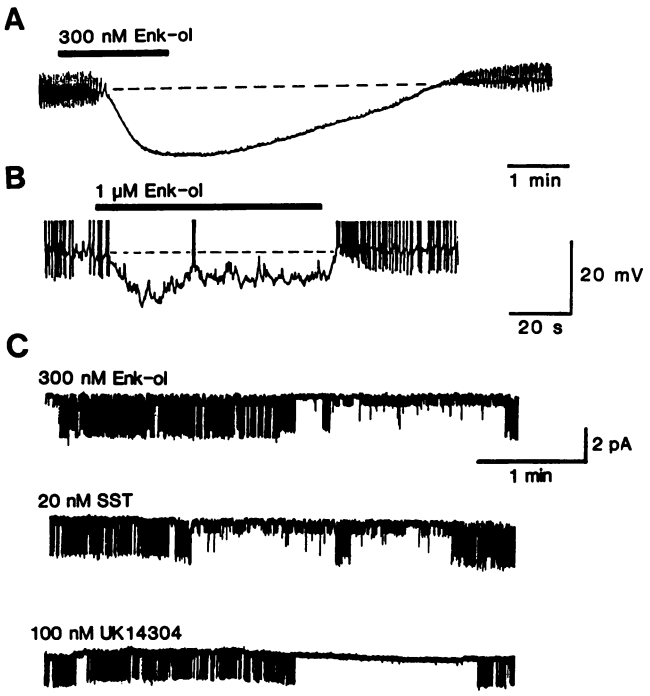

FIG. 1. (*A*) Opioid peptide Enk-ol hyperpolarizes neonatal locus ceruleus neuron. Microelectrode recording of membrane potential of a spontaneously firing neuron in a slice of brain tissue; action potentials (amplitude 80 mV) were not followed by the chart recorder. Enk-ol (300 nM) applied during the period indicated caused a 20-mV hyperpolarization from the resting level, which was −60 mV. (*B*) A similar experiment in the whole-cell configuration on an acutely dissociated locus ceruleus neuron. The recording electrode contained 130 mM potassium gluconate, 20 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1.5 mM ATP, 1 mM GTP, 0.1 mM cAMP, and 10 mM Mops·KOH (pH 7.3 ± 0.15). Normal extracellular solution containing Enk-ol (1 μM) flowed into the chamber from the tip of a plastic tube (diameter 150–200 μm); the cell was moved into this solution during the period indicated by the bar. (*C*) Single-channel currents recorded in cell-attached configuration. In the three records shown the electrode contained Enk-ol, somatostatin (SST), and the α_2 adrenergic receptor agonist UK 14304. Note that a second channel of lower conductance was also active in two patches; such channels were seen whether or not the pipette contained agonist. The potential across the patch in these cases was between −40 and −50 mV. (After recording single-channel currents, the whole-cell configuration was attained, and the resting potential of the cell was measured; the resting potential thus measured ranged from −40 to −60 mV among different patches.) Correction was made for junction potentials at the tip of the patch electrode: if the pipette (containing 150 mM potassium gluconate) was assigned zero potential in normal extracellular solution (2.5 mM potassium) it became 11 mV positive in 150 mM potassium gluconate and 6 mV positive in 130 mM potassium chloride, which approximated the cell interior.

Whole-cell recordings made from acutely dissociated neurons showed that the neurons fired spontaneous action potentials and were hyperpolarized by Enk-ol (Fig. 1*B*). In these respects the cells were very similar to those studied in brain slices removed from adult rats (14).

In the cell-attached configuration discrete inward currents were observed in most patches in which a successful gigaseal was made when the electrode contained Enk-ol (100 nM–1 μM) (Figs. 1*C* and 2*A*). Channel activity occurred for periods of up to several minutes in duration, which were separated by equally long silences. Channels with similar amplitudes as well as intermittent periods of activity were also seen when the recording electrode contained somatostatin (5 and 20 nM, n = 4) or the α_2-adrenoceptor agonist UK 14304 [5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline, 100 nM, n = 2] (Fig. 1*C*). With a larger electrode (≈2 MΩ) two to four channels were sometimes obvious from the frequent occurrence of multiple current levels. Channel activity was observed in 12 of 12 patches when the electrode contained Enk-ol (300 nM), in 0 of 9 patches when the electrode did not contain Enk-ol, and in 1 of 8 patches when the electrode contained Enk-ol (300 nM) and naloxone (30 nM). In contrast, Enk-ol did not open channels when it was applied to the neuron by adding it to the solution that superfused the neuron other than the isolated membrane patch. In five experiments in which the pipette did not contain Enk-ol, the addition of Enk-ol (1 μM) to the superfusing solution did not cause any channel activity; in three experiments in which channel activity was induced by the inclusion of Enk-ol (100 nM) in the pipette, addition of Enk-ol (1 μM) to the superfusing solution did not change channel activity (except to increase slightly the unitary amplitude, presumably as a result of membrane hyperpolarization).

The amplitude of unitary current events was ≈2 pA when the pipette was at ground potential; however, this varied somewhat from cell to cell—presumably because of differences in the resting potential of the neurons (see Fig. 1 legend). When the patch membrane was depolarized (the pipette was made negative with respect to the external solution), the unitary currents became smaller and reversed polarity at about zero membrane potential. The conductance increased to ≈55 pS when the driving force was −80 to −120 mV (Fig. 2*B*). There was no obvious effect of pipette potential on the probability of opening of the channel when the driving force for inward current exceeded about −50 mV; however, for smaller driving forces the probability became lower, and when the currents were outward, their occurrence became very rare. In two experiments the patch-clamp electrode contained 10 mM potassium, in closer simulation of the normal cellular environment; the unitary currents had a conductance of ≈40 pS at 0 mV (outward current) and about 20 pS at −100 mV (inward current); these currents reversed at −50 mV.

The distribution of closed times (excluding the long closed periods mentioned above) was well fitted by the sum of two exponential functions (time constants τ_{c1}, ≈1 ms, and τ_{c2}, ≈30 ms, Fig. 2*D*). There was no obvious correlation between the concentration of Enk-ol in the pipette and these channel closed times (Fig. 3). The open and closed time measurements shown in Fig. 3 were made during the periods of activity that separated the long silences (Fig. 1*C*); these silent periods were so long that sufficient numbers of events could not be obtained for quantitative analysis.

The open time of the channels were always well fitted by a single exponential distribution (Fig. 2*C*), but the mean value (τ_o) was obviously different for different concentrations of Enk-ol in the pipette (Fig. 3). Dependence on the agonist concentration of the mean open time of the channel was

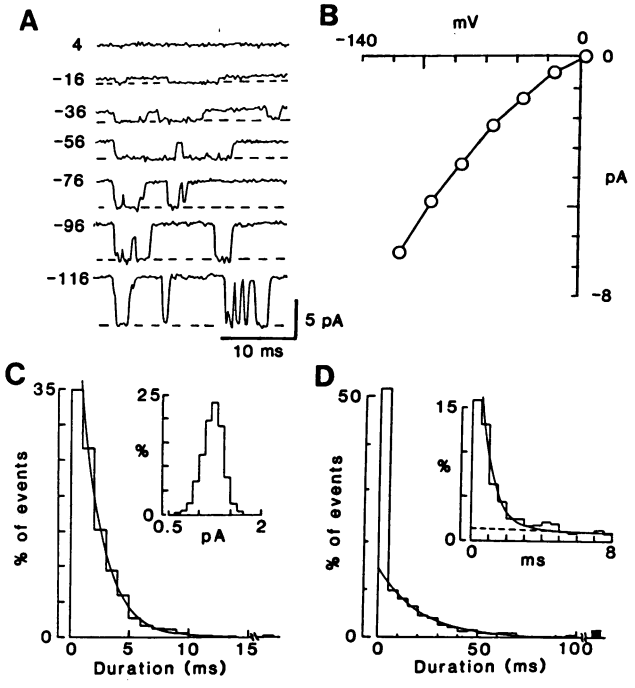

FIG. 2. Currents through a potassium channel activated by μ opioid receptor agonist; the pipette contained 100 nM Enk-ol. (*A*) Typical currents; potential across the patch membrane is indicated beside each trace in mV. (*B*) Current–voltage relation from data of *A*. Single-channel conductance is 47 pS at −36 mV and rises to 78 pS at −100 mV. (*C*) Distribution of open times for a typical channel. Continuous line is fitted by a least-squares minimizing method; open time <1 ms were not used for the fit. It is an exponential function with time constant (τ_o) of 1.9 ms. Total number of events was 1303. (*Inset*) Distribution of amplitudes of the discrete currents recorded at −36 mV. Total number of events was 863. (*D*) Distribution of closed times (1302 events). Line is the least-squares exponential fit to the data, excluding closed times <10 ms; the time constant (τ_{c2}) is 20 ms. (*Inset*) Distribution of closed times for closures <8 ms. Line is a double exponential fit to the data; broken line indicates contribution of slower component. Points in first 0.5-ms bin were not used for fitting. Fast time constant (τ_{c1}) is 0.7 ms.

particularly marked when the brief closures (τ_{c1}) were omitted from the analysis; for these purposes a brief closure was defined as closure lasting $<4\tau_{c1}$ ms (Fig. 3*B*). When the concentration of Enk-ol was 1 μM (which causes close to maximum current in the microelectrode recordings), the channel spent ≈50% of the time open (excluding the very long closures). When the pipette contained Enk-ol (2 μM) and naloxone (20 nM), mean open time was approximately the same as with 300 nM Enk-ol; the dose ratio of ≈7 is reasonable, given our estimate of the naloxone K_d of 2 nM.

DISCUSSION

One conclusion of the experiments is that opioids open potassium channels without the intervention of a freely diffusible second-messenger molecule. This implies that the mechanism of action of μ opioids is fundamentally similar to the action of acetylcholine at M_2 receptors in the heart (15–17). Adenosine and somatostatin have also been shown to open potassium channels by a similar mechanism (17, 18). The properties of the potassium channels opened by muscarinic agonists in the heart and opioids in locus ceruleus neurons are quite similar in terms of unit conductance and the duration of the closed states (15, 19): in both cases a *Pertussis*-sensitive GTP-binding protein intervenes between receptor and channel (7, 17, 20, 21). It seems likely that the potassium channels studied in our experiments are distinct from those that contribute to the background inward rectifier of locus ceruleus neurons (22) because the 40- to 50-pS channels were not active in the absence of agonist, even at hyperpolarized potentials.

In the locus ceruleus neurons, both μ opioid and α_2 adrenergic receptors are coupled to the same population of potassium channels (10). Therefore, the individual channels activated by the α_2 adrenergic agonist UK 14304 would be expected to have the same properties: our limited analysis indicates that this is so and extends the number of receptors to include somatostatin (see also ref. 23). Agonists at many other receptors are also effective to open potassium channels and in most neurons two or three receptors have been shown to couple to the same population of channels through a Pertussis-sensitive G protein. These receptors include α_2 adrenergic (24, 25), somatostatin (18, 23, 26), δ opioid (7), μ opioid (7, 27), muscarinic M_2 (27, 28), $GABA_B$ (GABA is γ-aminobutyric acid) (27, 29–31), and adenosine (32). It would be predicted that single channels activated by agonists at any of this family of receptors (7) would have very similar properties.

The probability that the channel is open reached ≈50% for the higher concentrations of Enk-ol, but it is difficult to relate

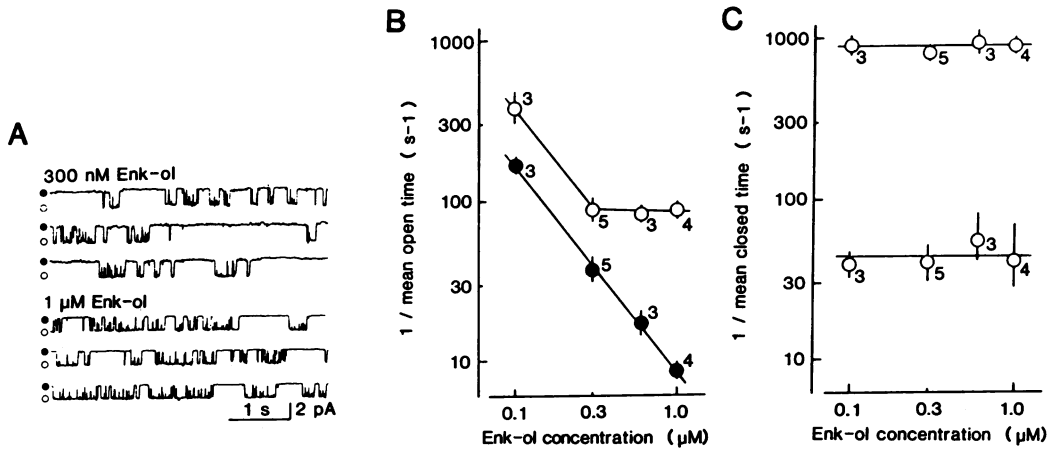

FIG. 3. Effects of different concentrations of opioid agonist (Enk-ol). (*A*) Typical recordings of single-channel activity using two different concentrations. Upper traces, −45 mV; lower traces, −47 mV. (*B*) Mean open time increases with agonist concentration. ○, Mean open times computed from all openings and closings; ●, mean open times measured when brief closures were discounted (for this purpose, a brief closure was any event $<4\tau_{c1}$ ms, which should include 98% of the "short" closures). (*C*) Mean closed times were not different for different agonist concentrations. Data are averaged from the number of channels indicated beside each point. In *B* and *C* very long closures (lasting >300 ms) were ignored. Membrane potentials ranged from −35 to −50 mV.

this to the effective concentrations on the intact cells for three reasons. (*i*) Measurements apply to periods of channel activity typically lasting for many seconds or minutes, but these periods were separated by equally long silences that have not been analyzed quantitatively (Fig. 1*C*). (*ii*) Present measurements have been of inward currents with high concentrations of potassium on both sides of the membrane; the probability of channel opening was less for outward currents when the extracellular potassium concentration was only 10 mM. (*iii*) With the cell-attached configuration that was used some desensitization probably occurs; the whole-cell potassium current shows little desensitization during application of Enk-ol (100 nM) for as long as 2 hr, but with higher concentrations (1–3 μM) the response declines progressively during application (refs. 11, 14, 33; R.A.N., M.J.C., and J. T. Williams, unpublished work).

Our measurements of the open and closed times of the channels during the periods of activity indicate that, within the bandwidth constraints that were used, there are two closed states and one open state. The closed states have average durations of 30 ms and 1 ms; these durations were the same with different agonist concentrations. The dependence of the lifetime of the open state on the agonist concentration (or more markedly the burst of openings that includes the brief closure, see Fig. 3*B*) would be expected if a number of activated G protein molecules could bind to the channel and open it. Kinetic experiments at higher time resolution in the inside-out configuration, in which the channel could be opened by application of activated G protein subunit, may help clarify the further details of channel activation.

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1. Lord, J. A. H., Waterfield, A. A., Hughes, J. & Kosterlitz, H. W. (1977) *Nature (London)* **267**, 495–499.
2. Leslie, F. M. (1987) *Pharmacol. Rev.* **39**, 197–249.
3. Goldstein, A. & James, I. F. (1984) *Trends Pharmacol. Sci.* **5**, 503–505.
4. Mansour, A., Lewis, M. E., Khachaturian, H. & Watson, S. J. (1987) *J. Neurosci.* **7**, 2445–2464.
5. Goodman, R. R., Snyder, S. H., Kuhar, M. J. & Young, W. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6239–6243.
6. Demoliou-Mason, C. D. & Barnard, E. A. (1986) *J. Neurochem.* **46**, 1129–1136.
7. North, R. A., Williams, J. T., Surprenant, A. & Christie, M. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5487–5491.
8. North, R. A. & Tonini, M. (1977) *Br. J. Pharmacol.* **61**, 541–549.
9. North, R. A. (1986) *Trends Neurosci.* **9**, 114–117.
10. North, R. A. & Williams, J. T. (1985) *J. Physiol. (London)* **364**, 265–280.
11. Williams, J. T. & Marshall, K. C. (1987) *J. Neurosci.* **7**, 3687–3694.
12. Huettner, J. E. & Baughman, R. W. (1986) *J. Neurosci.* **6**, 3044–3060.
13. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391**, 85–100.
14. Williams, J. T. & North, R. A. (1984) *Mol. Pharmacol.* **26**, 489–497.
15. Soejima, M. & Noma, A. (1984) *Pflügers Arch.* **400**, 424–431.
16. Yatani, A., Codina, J., Brown, A. M. & Birnbaumer, L. (1987) *Science* **23**, 207–210.
17. Kurachi, Y., Nakajima, T. & Sugimoto, T. (1986) *Pflügers Arch.* **407**, 572–574.
18. Yatani, A., Codina, J., Sekura, R. D., Birnbaumer, L. & Brown, A. M. (1987) *Mol. Endocrinol.* **1**, 283–288.
19. Sakmann, B., Noma, A. & Trautwein, W. (1983) *Nature (London)* **303**, 250–253.
20. Pfaffinger, P. J., Martin, J. M., Hunter, D. D., Nathanson, N. M. & Hille, B. (1985) *Nature (London)* **317**, 536–538.
21. Breitwieser, G. E. & Szabo, G. (1985) *Nature (London)* **317**, 538–540.
22. Williams, J. T., North, R. A. & Tokimasa, T. (1988) *J. Neurosci.*, **8**, 4299–4306.
23. Inoue, M., Nakajima, S. & Nakajima, Y. (1987) *J. Physiol. (London)* **407**, 177–198.
24. North, R. A. & Surprenant, A. (1985) *J. Physiol. (London)* **358**, 17–32.
25. Surprenant, A. & North, R. A. (1988) *Proc. R. Soc. London Ser. B.* **234**, 85–114.
26. Mihara, S., North, R. A. & Surprenant, A. (1987) *J. Physiol. (London)* **390**, 335–356.
27. Christie, M. J. & North, R. A. (1988) *Br. J. Pharmacol.* **95**, 896–902.
28. Sasaki, K. & Sato, M. (1987) *Nature (London)* **325**, 259–262.
29. Lacey, M. G., Mercuri, N. B. & North, R. A. (1988) *J. Physiol. (London)* **401**, 437–454.
30. Andrade, R., Malenka, R. C. & Nicoll, R. A. (1986) *Science* **234**, 1261–1265.
31. Williams, J. T., Colmers, W. F. & Pan, Z. Z. (1988) *J. Neurosci.* **8**, 3499–3506.
32. Trussel, L. O. & Jackson, M. B. (1987) *J. Neurosci.* **7**, 3306–3316.
33. Christie, M. J., Williams, J. T. & North, R. A. (1987) *Mol. Pharmacol.* **32**, 633–638.