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Potassium Conductance Increased by Noradrenaline, Opioids, Somatostatin, and G-Proteins: Whole-Cell Recording from Guinea Pig Submucous Neurons

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Agonists at α_2-adrenoceptors, δ-opioid receptors, and somatostatin receptors were applied to dissociated guinea pig submucous plexus neurons; whole-cell recordings of membrane current showed that they increased the membrane potassium conductance. The conductance affected showed inward rectification, being described by $G_{ag(max)}/\{1 + \exp((V - V_{0.5})/k)\}$ where $V_{0.5}$ was about −65 mV and $G_{ag(max)}$ was about 10 nS. The agonists were ineffective when the potassium conductance of the neurons had first been increased by intracellular dialysis with purified guanosine 5′-triphosphate (GTP)-binding proteins (G_i or G_o). Agonist actions were prevented by pertussis toxin, applied intracellularly (10–100 ng/ml for several minutes) or extracellularly 1–10 μg/ml for 1 hr); in the latter case, the agonist responses were reconstituted by intracellular dialysis with GTP-binding proteins.

Noradrenergic sympathetic fibers of the autonomic nervous system terminate on and around neurons of the submucous plexus within the intestinal wall. Noradrenaline released from these fibers acts on α_2-adrenoceptors on the neurons, and this leads to a membrane hyperpolarization (the inhibitory postsynaptic potential, or i.p.s.p.). Previous studies with intracellular electrodes have characterized the receptor and have indicated that the i.p.s.p. results from the opening of membrane potassium channels (Hirst and McKirdy, 1975; Surprenant, 1984; North and Surprenant, 1985; Mihara et al., 1986; Surprenant and North, 1988). The potassium conductance exhibits inward rectification and is readily blocked by cesium, rubidium, and low concentrations (1–100 μM) of barium (Surprenant and North, 1988). It was also deduced that a guanosine 5′-triphosphate (GTP) binding protein was interposed between receptor and channel, because the hyperpolarization by noradrenaline became irreversible when the recording electrode contained guanosine 5′-thiophosphate (GTP-γ-S), which is a nonhydrolyzable analog of GTP.

The purpose of the present experiments was to investigate further the properties of the potassium conductance activated by noradrenaline, somatostatin, and opioids in guinea pig submucous plexus neurons, in particular the role of G-proteins in the transduction from receptor to channel. Previous experiments had been made with conventional intracellular microelectrodes, which not only restricts the range of membrane potentials that can be achieved, but also limits the opportunity to introduce substances, such as G-proteins, to the cell interior. We therefore developed a preparation of submucous plexus neurons suitable for patch clamp recording and used the whole-cell configuration.

Materials and Methods

Tissue preparation. Adult guinea pigs (200–300 gm) were anesthetized with ether or halothane and exsanguinated by severing the major neck blood vessels. A thin preparation of submucous plexus from the cecum or ileum was dissected (Surprenant, 1984; Mihara et al., 1987). The tissue was placed in a physiological salt solution of the following composition (mM): NaCl 117, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KCl 2.5, $NaHCO_3$ 25, glucose 11; gassed with 95% O_2 and 5% CO_2. The preparation was next treated with collagenase (Sigma type XI, 1 mg/ml, 30 min at 35°C) in a similar solution that lacked calcium and magnesium. Dissociated ganglia or isolated cells were plated on polylysine-coated glass coverslips and kept prior to use in Eagle's medium (90% vol/vol), 10% fetal calf serum penicillin (100 units/ml), and streptomycin (50 μg/ml).

Recordings were made within 1–32 hr after the cells were plated; the majority of recordings were made within 5 hr. The cells that were most suitable for whole-cell recording remained within isolated ganglia (nodes of the plexus), or within ganglia that had a few remaining short fiber strands in the nodes. Several neurons were stained by including Lucifer yellow (0.01%) in the solution used to fill the recording electrode; subsequent observation of the fluorescence (Zeiss IM35 miscroscope) showed neurons that occasionally had processes up to 50 or 100 μm, but most cells had no obvious long processes.

Recordings. Cells were superfused (15 ml/min, 25–28°C) with the physiological salt solution outlined above, and gigohm seals were made (>5 GΩ; Hamill et al., 1981). Intracellular access was obtained by further suction to the pipette or brief (1 ms) negative voltage pulses; a negative pressure was usually maintained in the pipette throughout the whole-cell recording (10–50 cm water). Pipettes were constructed from Corning glass (7052, outside diameter 1.65 mm, inside diameter 1.55 mm), coated to near the tip with nail polish, and having resistances of 2–5 MΩ.

Whole-cell currents were recorded with an Axopatch-1B amplifier. The largest currents recorded in these experiments were about 1 nA, which would lead to a series voltage drop across the electrode of 2–5 mV. This was 60–80% compensated by the amplifier circuit after the capacitance compensation was adjusted to the verge of oscillation. Voltage command pulses were generated by a stimulator (Grass S88); voltage signals were digitized at 44 kHz (Sony PCM-501ES) and stored on videocassette (Sony SL-2700) for later analysis. The signals were analyzed with IBM-PC AT computer using an Axolab interface and Axess or Pclamp software (Axon Instruments, Burlingame, CA).

The extracellular solution had the following composition (mM): NaCl

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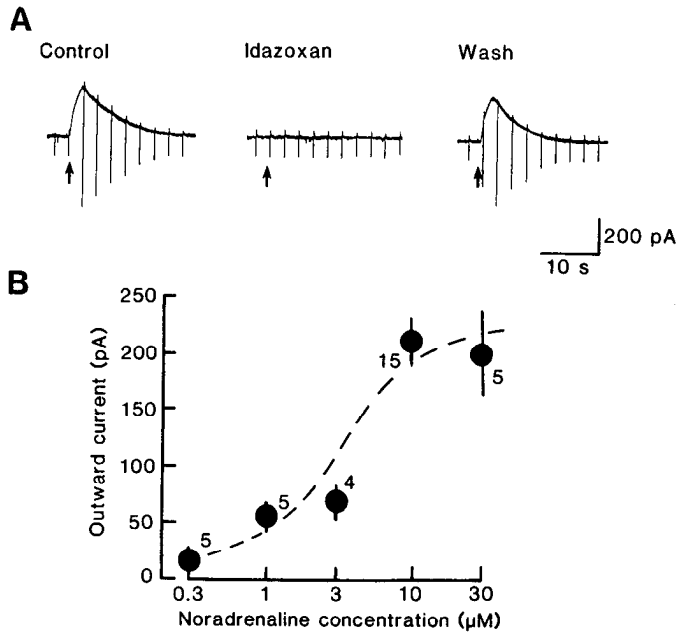

Figure 1. Noradrenaline acts at α_2-adrenoceptors to cause an outward current in dissociated submucous plexus neurons. *A*, Noradrenaline was applied by pressure (pressure pulse 35 kPa for 100 msec). An outward current was evoked, with a very large increase in membrane conductance. No response to noradrenaline was observed when the superfusing solution contained the α_2-adrenoceptor blocker idazoxan (100 nM). Downward deflections are membrane currents during voltage steps to −120 mV. Holding potential was −60 mV. *B*, Outward currents caused by superfusion of different concentrations of noradrenaline. *Numbers* beside each point indicate number of cells tested at each concentration. *Vertical bars* are SEM. Holding potential in all cells was −60 mV.

117, $CaCl_2$ 2.5, KCl 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, and glucose 10.5 (gassed by 95% O_2 and 5% CO_2). The pipette solution contained (mM): KCl 140 (or K-gluconate 140 in 5 cells), $MgCl_2$ 2 (or 1 in some experiments), $CaCl_2$ 1, EGTA 1.1 (or 10 in some experiments), cAMP 0.1, ATP 0.1, GTP 0.1, and 3-[*N*-morpholino]propanesulfonic acid (MOPS, Sigma) 5. GTP and ATP were used because Trussell and Jackson (1987) found that loss of outward current responses caused by adenosine could be prevented by these substances. The recording chamber (volume about 1 ml) was superfused continuously at the rate of 15 ml/min. The superfusing solution was preheated and the temperature of bath was 25–28°C.

G-proteins. G_i was purified from porcine atria as described by Tota et al. (1987) and G_o from bovine brain using the procedure developed by Sternweis and Robishaw (1984) as modified by Haga et al. (1986). Proteins were designated G_i and G_o because analysis of purified proteins by SDS polyacrylamide gel electrophoresis showed bands at 41, 35, and ~10 kDa for G_i and 39, 35, and ~10 kDa for G_o. Specific activities of purified G_i and G_o were about 8 nmol of [^{35}S]GTP-γ-S bound per mg protein. The stock solution of G_i contained 0.2 mg/ml protein (1.6 μM in GTP-γ-S binding sites), cholate (0.9% wt/vol), Tris (10 mM), EDTA (1 mM), benzamidine (1 mM), phenylmethylsulfonyl fluoride (PMSF, 100 μM), dithiothreitol (DTT, 1 mM), and sucrose (10% wt/vol) (pH 8). The stock solution of G_o contained 0.15 mg/ml protein (1.4 μM in GTP-γ-S binding sites) and lubrol (0.6% wt/vol), sucrose (5% wt/vol), DTT (1 mM), Tris (20 mM), EDTA (1 mM), benzamidine (1 mM), PMSF (100 μM), and soybean trypsin inhibitor (1 unit/ml) (pH 8). These proteins were preactivated in $MgCl_2$ (50 mM), GTP-γ-S (1 mM), GTP (100 μM) for 1 hr at 35°C, and diluted to 6 nM or less in the whole-cell solution. [Some experiments were also carried out using a G_i and G_o mixture kindly prepared from bovine brain by Dr. J. Northrup. The solution contained 0.7 mg/ml G-protein (equivalent to about 5.8 μM G_i and 2.5 μM G_o), NaCl 100 mM, K-phosphate 300 mM, Tris 20 mM, dithiothreitol 1 mM, and lubrol 0.1% (pH 8). This protein was preactivated in $MgCl_2$ (50 mM) and GTP (10 mM) for 1 hr at 35°C and diluted 1000 times with the whole-cell solution.]

Pertussis toxin (List) was dissolved in sterile water (125 μg/ml) and stored for up to 2 d at 4°C. Toxin was activated by adding dithiothreitol (5 mM) at 35°C for 15 min. The activated pertussis toxin was diluted to 10 μg/ml with pipette solution containing 20 mM nicotinic acid adenine dinucleotide (NAD). This solution was further diluted in the pipette solution to the final concentration used. In the case of extracellular treatment of pertussis toxin, the activated toxin was simply dissolved in the solution (Eagle's medium) described above.

Drugs. Drugs were applied by adding them to the superfusing solution or by ejecting a few nanoliters of solution from pipette tips positioned about 100 μm from the cell. The superfusing solution flowed at 15 ml/min, and the volume of solution in the recording chamber was typically 1 ml. For pressure ejection, the pipettes had tip diameters of 2–4 μm and contained either noradrenaline (1 mM) or [Met^5]enkephalin (100 μM); pressures of 5–10 psi (1 psi = 6.9 kPa) for 100 msec were typically used (Picospritzer, General Valve). Drugs used were UK14304 [5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK14304, Pfizer], idazoxan (Reckitt), noradrenaline (Sigma), nifedipine (Sigma), NAD (Sigma), dithiothreitol (Sigma), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), somatostatin (Sigma, Peninsula), [D-Pen^{2,4}]enkephalin (DPDPE, Peninsula), [Met^5]enkephalin (Sigma, Peninsula), *N*-bisallyl-Tyr-Aib-Aib-Phe-Leu (ICI174864, Aib = aminoisobutyrate).

Results

Noradrenaline and a_2 receptor agonists

The majority (76 of 129) of neurons showed an outward current when they were held at −60 mV and noradrenaline was applied (Fig. 1) (unless otherwise stated, the neurons were held at −60 mV, which is close to the resting potential). The mean amplitude of the outward current caused by 10 μM noradrenaline was 211 ± 13 pA (this and other values are mean ± SEM, n = 15). The chord conductance (−60 to −120 mV) was much increased by noradrenaline (in control solution 1.7 ± 0.3 nS; in noradrenaline (10 μM) 9.7 ± 0.9 nS, n = 8). The instantaneous current/voltage (I/V) relation of the submucous neurons showed inward rectification in the range −80 to −120 mV (see Surprenant and North, 1988). The additional current caused by noradrenaline also showed inward rectification at −60 mV to −130 mV and reversed from outward to inward at a potential of −100 ± 1.3 mV (n = 11).

The outward current caused by noradrenaline was not significantly different when the recording pipette contained 10 mM EGTA, instead of the usual concentration of 1.1 mM. Noradrenaline caused currents (at −60 mV) of 100 and 480 pA (10 μM, 2 cells) and 170 pA (3 μM, 1 cell). This finding suggests that the intracellular calcium concentration is not a critical determinant of the outward current response. In seven cells, cAMP and ATP were omitted from the pipette solution; noradrenaline caused at outward current of 219 ± 40 pA.

Time-dependent current component. The current elicited by voltage steps from −60 mV to potentials as negative as −150 mV had no obvious time dependence in the majority of cells. However, in the presence of noradrenaline the membrane current negative to E_K had a slowly developing component (in 37 of 60 cells; Fig. 2). The time-dependent component of the inward current induced by noradrenaline was most obvious at the beginning of the superfusion period, before the outward current at −60 mV had reached its steady-state value; as the action of noradrenaline reached a steady state, the time-dependent contribution to the inward current was less marked (Fig. 2*B*). The slowly developing component of the inward current in the presence of noradrenaline reached its maximum amplitude at about −150 mV, and the potential at which it was half-maximally activated was −117.2 ± 5.0 mV (n = 5). When the potassium concentration was increased, the potential at which the time-

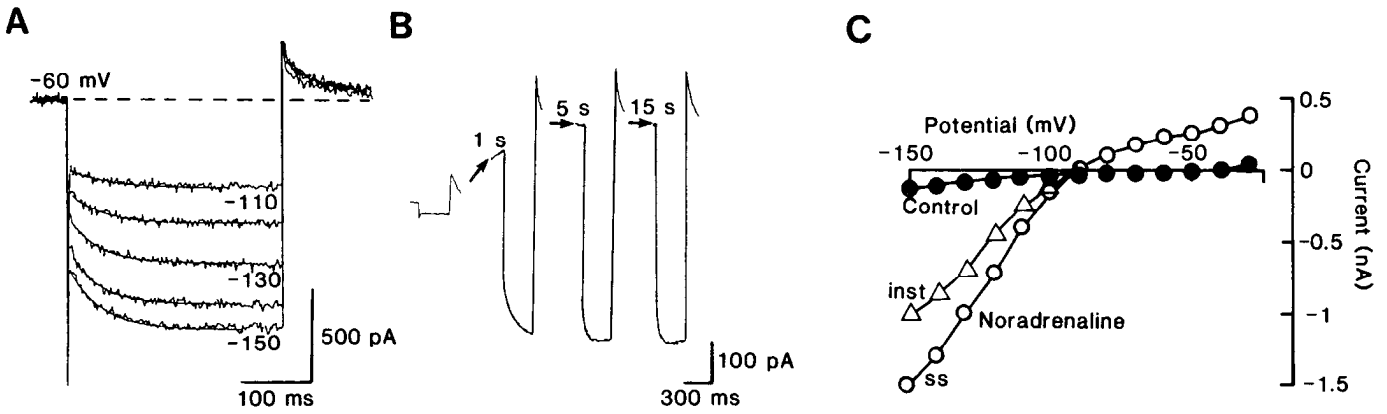

Figure 2. Time-dependent component to inward current caused by noradrenaline. *A,* Membrane currents in response to hyperpolarizing voltage steps from −60 mV to −110, −120, −130, −140, and −150 mV. Noradrenaline (10 μM) was present. *B,* Time dependence of inward current is most marked at onset of noradrenaline action. Five current records are shown, taken before and at the times indicated after the onset of the outward current response to superfusion with noradrenaline (10 μM). Currents were evoked by stepping the potential from the holding level of −70 mV to −130 mV for 300 msec. During the rising phase of the outward current response to noradrenaline (trace labeled 1 s), there is a pronounced time dependence to the inward current, but this has partially disappeared when the outward current reaches its peak (trace labeled 15 s). *C,* Current/voltage relation for one neuron before and during application of noradrenaline. *Filled circles,* Control. *Open symbols,* In noradrenaline (10 μM). *Open circles* (*ss*) indicate the membrane current at the end of a 300-msec voltage step, and *open triangles* (*inst*) indicate the current immediately after settling of the capacitative transient. Time-dependent component is seen only for inward current (negative to E_K). Note inward rectification for the current induced by noradrenaline.

dependent component became evident was shifted to a less negative value. The current developed exponentially, with time constant 46 ± 15 msec ($n = 5$) at −110 mV and 25 ± 11 msec ($n = 7$) at − 150 mV. The time dependence of the inward current evoked by noradrenaline was also observed in sodium-free solution (Tris substitution). [This observation is significant because about 10% of neurons showed an inward current that was activated by hyperpolarization in this voltage range and that had the characteristics described for I_H by Mayer and Westbrook (1983), including marked reduction in sodium-free solutions (H.T. and R.A.N., unpublished observations): these cells were not included in the present report.] The slowly developing component to the inward potassium current was also seen with UK14304 (300 nM or 1 μM, $n = 5$) and [Met]^{5}enkephalin (300 nM or 1 μM, $n = 5$).

Voltage dependence of potassium conductance. The conductance increased by noradrenaline was calculated by dividing the evoked current by $(V - E_K)$ where E_K is the potassium equilibrium potential and V is the holding potential. At −120 mV the instantaneous conductance was 11.1 ± 1.0 nS ($n = 8$) and the steady-state conductance was 16.0 ± 1.5 nS ($n = 8$) (Fig. 3). Barium (10 μM) much reduced the inward currents evoked by the agonists (see Surprenant and North, 1988); in barium the slowly developing component to the inward current was not observed, but most often the inward current declined progressively during the applied voltage command. This may be because of the time dependence of the blocking action of these low concentrations of barium (Standen and Stanfield, 1978; Surprenant and North, 1988; Williams et al., 1988; Uchimura et al., 1989).

The action of noradrenaline was also examined in the voltage range less negative than rest; examples are shown in Figure 3. Noradrenaline (10 μM) evoked an outward current in the voltage range from −105 mV (E_K) to about −15 mV. However, the depolarization itself caused a large outward membrane current at potentials less negative than rest, and the effect of noradrenaline at potentials positive to about −40 mV was to reduce this, thus causing a net inward current at around −20 mV. It was hypothesized that the basic outward rectification might result from a calcium-activated potassium conductance, and that this was reduced because noradrenaline reduced calcium entry. In a calcium-free solution (also containing 3 mM magnesium), this large outward current was indeed reduced. Figure 3*C* shows that in this condition the net current caused by noradrenaline simply declined to zero at about −20 mV without turning inward. In this case, the noradrenaline-sensitive conductance was computed on the assumption that all the current resulted from movement of potassium ions. In 3 cells, the noradrenaline-sensitive conductance (G_{ag}) was well fitted to an expression of the form $G_{ag} = G_{ag(max)}/\{1 + \exp((V - V_{0.5})/k)\}$ where V is the membrane potential, $V_{0.5}$ is the membrane potential at which $G_{ag} = 0.5G_{ag(max)}$, and k represents the steepness of the voltage dependence of the conductance. The values were $G_{ag(max)}$ = 10.6 ± 1.4 nS, $V_{0.5}$ = −64.7 ± 5.6 mV, and k = 16.9 ± 4.0 mV ($n = 4$). In other words, the conductance activated by noradrenaline increased with hyperpolarization, and the center point of the voltage dependence was about −65 mV.

Involvement of α_2 receptor. The amplitude of the current caused by noradrenaline depended on the concentration applied (300 nM − 30 μM), and the concentration causing half-maximal effect was about 3 μM (Fig. 1*B*). This outward current caused by noradrenaline was reversibly blocked by idazoxan [10 nM ($n = 2$), 100 nM ($n = 3$)], which was itself without effect on membrane current. Idazoxan is a competitive antagonist with a dissociation constant at α_2 receptors of 10 nM (Surprenant and North, 1988). UK14304 (300 nM − 10 μM), a selective α_2 receptor agonist (Surprenant and North, 1988), evoked outward currents with all the same properties as those caused by noradrenaline, including a time-dependent component at potentials negative to E_K. The mean amplitude of the outward current (at −60 mV) caused by UK14304 was 180 ± 41 ($n = 3$) for 300 nM and 180 ± 27 pA ($n = 13$) for 1 μM. These values are not different from the peak currents caused by noradrenaline (Student's t test, $p > 0.05$).

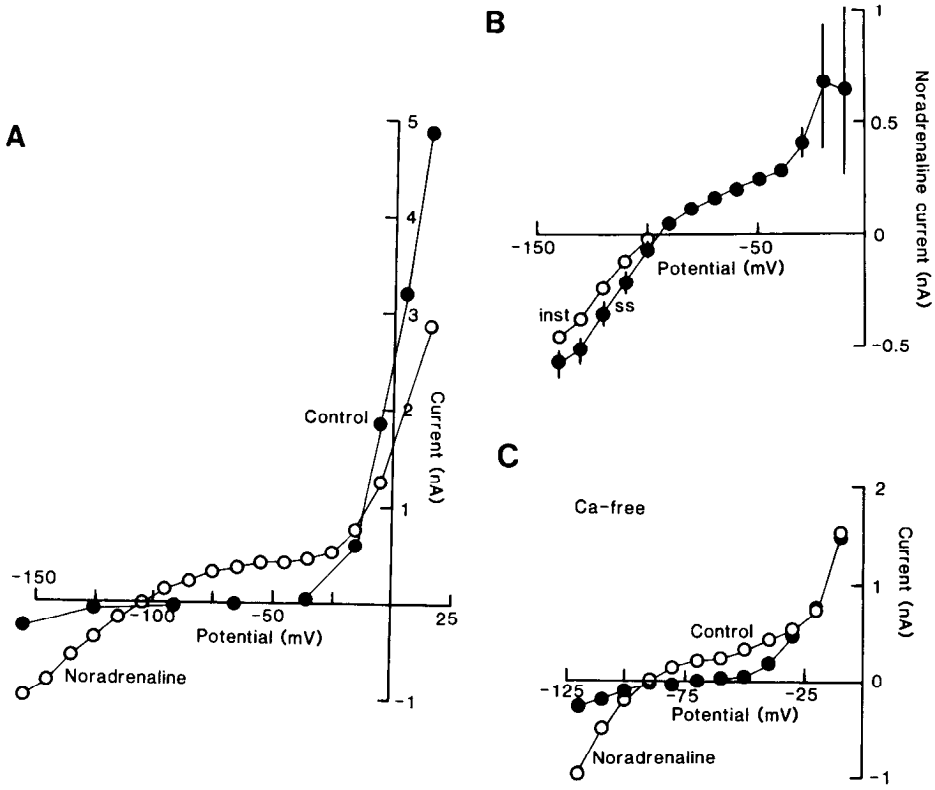

Figure 3. Typical current/voltage relation in the absence (*closed circles*) and presence (*open circles*) of noradrenaline (10 μM). Between −50 and −150 mV noradrenaline causes an inwardly rectifying potassium current. However, at less negative potentials the current caused by noradrenaline becomes less, and in this cell noradrenaline reversed to inward current at −20 mV. *B*, Net current caused by noradrenaline. *Bars* are SEM for 5–8 cells at each point, where these exceed the size of the symbol. Note the large variability in the noradrenaline current at −30 and −20 mV. *C*, In calcium-free solution (also 3 mM magnesium) the action of noradrenaline is little affected in the potential range from −50 to −120 mV. However, the outward rectification of the membrane was reduced in calcium-free solution and the action of noradrenaline disappeared positive to −20 mV.

Effect of GTP-γ-S. When GTP-γ-S (300 μM) was added to the pipette solution, the outward current evoked by a brief application of noradrenaline was greatly prolonged and, in many cases, rendered irreversible (Fig. 4*B*). Subsequent applications of noradrenaline evoked further outward currents which were also sustained ($n = 6$). The sustained current observed in these conditions reversed from outward to inward at −99 ± 3 mV ($n = 3$), indicating that it resulted from an increased potassium conductance. Intracellular dialysis by GTP-γ-S (300 μM) did not obviously change the holding current or membrane conductance without any application of agonist (in 8 of 9 cells).

Effect of pertussis toxin. With a low concentration of pertussis toxin in the pipette (10 ng/ml), the first application of noradrenaline caused the usual outward current, so long as it was applied within a few minutes of entering the whole-cell configuration (Fig. 4*C*); the outward current evoked by noradrenaline disappeared within 10 min ($n = 2$). When higher concentrations of pertussis toxin (100 ng/ml to 10 μg/ml) were used in the pipette solution, the noradrenaline current was blocked even from the first application ($n = 10$). When the pipette solution contained NAD and DTT, but no pertussis toxin, the response to noradrenaline was unaffected ($n = 6$).

No effect of noradrenaline was seen in neurons that had been exposed to pertussis toxin (1 μg/ml) in the external solution (37°C for 30 min, 2 cells; and for 60 min, 23 cells) (Fig. 4*E*). In the case of shorter exposure to pertussis toxin (15 min, 1 cell), the first application of noradrenaline evoked an outward current, but succeeding applications were ineffective. Treatment with pertussis toxin did not obviously change the responses to acetylcholine (100 μM or 1 mM, $n = 3$) [this response was a large (1–2 nA) inward current associated with a conductance increase, presumably from activation of nicotinic receptors], to muscarine (10 μM, $n = 2$) [this response was a small inward current (10pA)], or to substance P (300 nM, $n = 2$) [this response was a small inward current (100pA and 50 pA) associated with a conductance decrease].

Dialysis with G-proteins. These experiments were carried out with purified G_i and G_o, preactivated with 1 mM GTP-γ-S (see Materials and Methods), in the pipette solution. After a delay of about 5 min (6.3 ± 0.9 min in 10 cells) an outward current began to develop. The outward current reached a steady state within a further 5 min and was then sustained for the duration of the recording. The *I/V* relation measured at the beginning of the whole-cell recording intersected that determined later in the recording at the potassium equilibrium potential (−103 ± 8 mV, $n = 10$) (Fig. 5*B*). Under current clamp conditions, the membrane potential went to about −90 mV after prolonged dialysis with the G-protein; however, there were no other obvious changes in the properties of the cell except for the large increase in conductance (e.g., action potentials were unaffected).

The amplitude of the sustained outward current that developed during the cell dialysis was larger when higher concentrations of G-protein were used (Fig. 5*A*). Qualitatively similar effects were observed with both G_i and G_o; the maximal currents were not significantly different. No outward current developed when the pipette contained preactivated G_o and/or G_o (3 nM) that had been boiled (95°C for 2 hr) ($n = 3$). Noradrenaline had no effect when applied to neurons after intracellular application of G_i had caused its maximal outward current ($n = 4$).

Reconstitution of noradrenaline action by G-proteins. Cells were treated with pertussis toxin (1 μg/ml) extracellularly for 1 hr at 37°C and whole-cell recordings were then made with pipettes that contained G_i or G_o preactivated with GTP (see Materials and Methods). A negative pressure of 100 cm water was applied to the recording electrode for several minutes and then reduced to 30 cm. Applications of noradrenaline within the first

few minutes after rupture of the membrane at the tip of the patch electrode did not cause any outward current, but a second application of noradrenaline caused outward current with a large conductance increase at 15 min after recording (Fig. 6). The delay for reconstitution of the outward current response ranged from 5 to 18 min. The amplitude of the reconstituted outward current response (using 10 μM noradrenaline) ranged from 80 to 740 pA at −60 mV. The *I/V* relation in the presence of noradrenaline intersected that determined prior to noradrenaline application at −94 ± 7 mV (n = 6). A time-dependent component to the inward current was not observed in these cells (n = 6).

Enkephalin and δ receptor agonists

[Met⁵]enkephalin caused an outward current in 33 of 75 neurons held at −60 mV (Fig. 7). The amplitude of the outward current became larger when the concentration of [Met⁵]enkephalin was increased (30 nM to 3 μM), becoming maximal at 1 μM. Application of [Met⁵]enkephalin for periods longer than 2 min often caused a gradual decline in the evoked current that was not seen with noradrenaline. For this reason, [Met⁵]enkephalin was usually applied by ejecting it from a pipette tip within 100 μm from the neuron; even in this case the response progressively declined with repeated applications at intervals of less than 20 sec. A similar current was evoked by the stable enkephalin analog that is selective for δ-opioid receptors [D-Pen²,⁵]enkephalin (10 nM to 1 μM); this also declined with repeated applications. The current evoked by [Met⁵]enkephalin (300 nM) was blocked by the selective δ-opioid receptor antagonist ICI 174864 (100 nM; n = 4). The outward current response to [Met⁵]enkephalin (300 nM) was also observed when a higher concentration of EGTA (10 mM) was used in the recording pipette; the mean amplitude was 124 ± 23 pA (n = 5), compared with 121 ± 32 pA (n = 12) when 1 mM EGTA was used.

The overall effect of [Met⁵]enkephalin on the steady-state *I/V* relation was not different from that of noradrenaline described above; the reversal potential of the current was −98 mV ± 1.3 mV (n = 5). In 6 of 33 cells there was an obvious slow component to the development of the inward current, as described above for the action of noradrenaline. This slower component was observed only when the net membrane current was inward, i.e., when voltage command steps negative to −100 mV were applied. In 5 cells, the time constant for development of this slower current component was 94 ± 11 msec at −120 mV. The instantaneous *I/V* relation for the enkephalin-induced current also showed inward rectification in the range −60 mV to −130 mV, as described for noradrenaline above.

When GTP-γ-S was in the pipette solution, the outward current evoked by a brief application of [Met⁵]enkephalin was sustained for several minutes. The *I/V* relation for this sustained outward current showed inward rectification in the range −50 mV to −130 mV and it intersected the control *I/V* relation at −99 ± 1.1 mV (n = 3). The control *I/V* relation was not different from control when the pipettes contained GTP-γ-S.

[Met⁵]enkephalin (1 μM, n = 2) caused no outward current when the pipette solution contained preactivated pertussis toxin (100 ng/ml to 1 μg/ml), although normal responses were obtained if the pipette contained only NAD and DTT ([Met⁵]enkephalin (300 nM) caused current of 107 ± 32 pA in 3 cells). Similarly, dissociated cells pretreated with pertussis toxin (1 μg/ml, 30 min to 1 hr, 37°C), were not responsive to [Met⁵]enkephalin (1 μM, n = 5). Simple addition of the G_i/G_o proteins to the pipette solution did not improve the repeatability of the responses to [Met⁵]enkephalin: only 1 of 8 cells responded to [Met⁵]enkephalin (1 μM) in these circumstances.

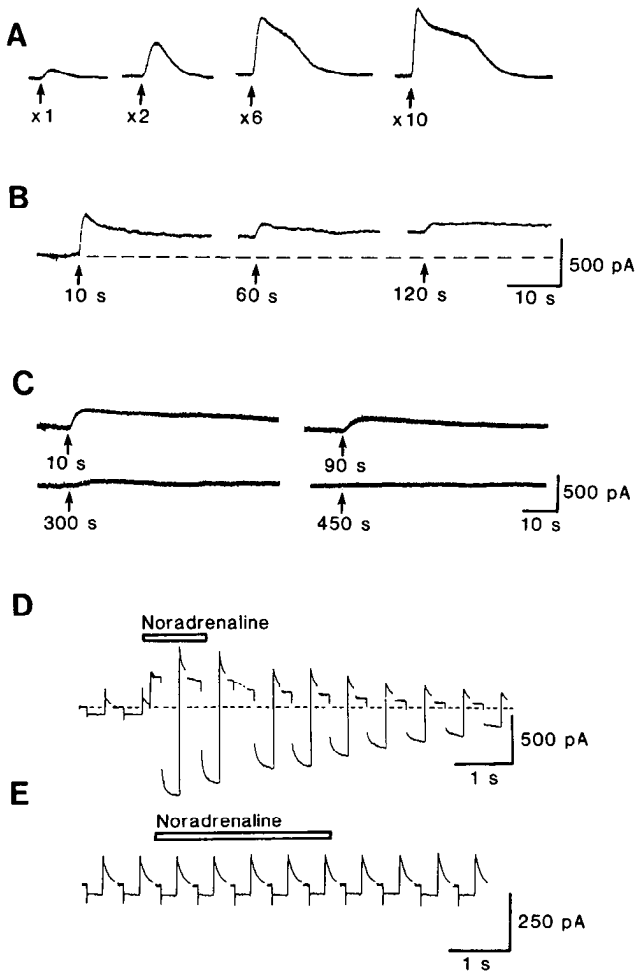

Figure 4. Noradrenaline responses are rendered irreversible by GTP-γ-S and blocked by pertussis toxin. *A*, Response to noradrenaline in a typical neuron voltage-clamped at −60 mV. Noradrenaline was applied by pressure pulse (pressure was 35 kPa, pulse duration was 5, 10, 30, and 100 msec from left to right). *B*, A similar experiment in which the recording pipette contained GTP-γ-S (300 μM). The first outward current response (evoked 10 sec after the whole-cell recording began) persisted partially. Second and third applications of noradrenaline 1 min and 2 min after breaking into the cell caused only small irreversible outward currents. *C*, Noradrenaline was applied by pressure (35 kPa, 100 msec) on 4 occasions at different times after breaking into the whole-cell configuration. The recording pipette contained pertussis toxin (10 ng/ml). Note the progressive loss in the response to noradrenaline (compare *A*). *D*, Recording from another cell in which superfusion with noradrenaline (10 μM) caused an outward current of about 200 pA at the holding potential of −60 mV. Voltage steps to −120 mV (300 msec duration) were evoked at 10 sec intervals. *E*, A similar experiment to that shown in *D* but in a neuron that had been exposed for 1 hr at 37°C to pertussis toxin (1 μg/ml).

Somatostatin

Somatostatin (3–30 nM) caused an outward current in 9 of 25 cells; many of the cells that were not responsive gave typical responses to noradrenaline. The mean amplitude of the current evoked by 10 nM somatostatin (at −60 mV) was 102 ± 30 pA (n = 3) and the current reversed polarity at −107.4 ± 4.9 mV.

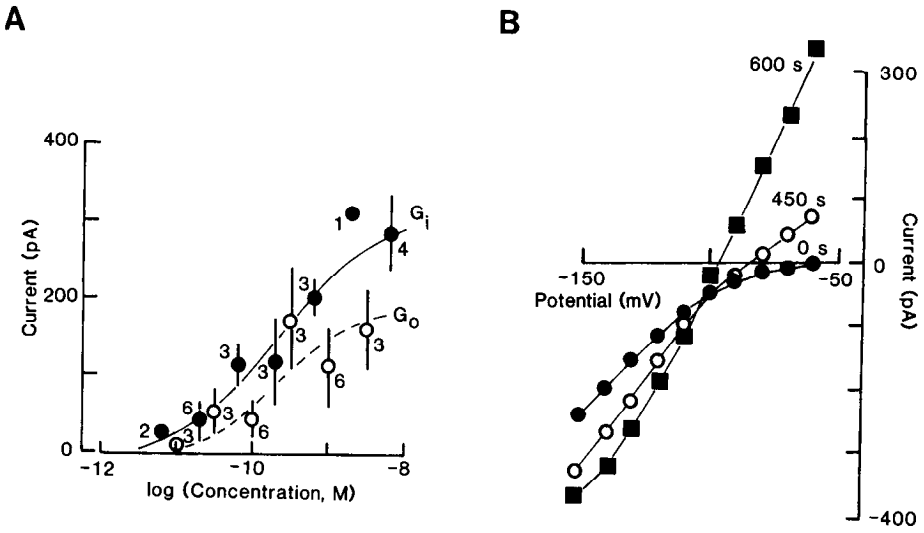

Figure 5. Potassium currents evoked by intracellular dialysis with G-proteins. *A,* Membrane current recorded 10 min after breaking into the whole-cell recording mode. *Filled circles,* Experiments with pipettes containing G_i. *Open circles,* Cells recorded with pipettes containing G_o. *Bars* are SEM and *numbers* are cells tested at each concentration. Lines drawn by eye. Holding potentials −60 mV. *B,* Voltage dependence of current caused by G-protein. Recording electrode contained G_i (2 nM). *Filled circles* show current/voltage relation immediately after breaking into the cell (resting potential was −50 mV). *Open circles* and *filled squares* show currents after 7.5 and 10 min recording; note that the resting potential of the cell had reached about −90 mV. The current/voltage relation after dialysis with G-protein showed no inward rectification, but rather slight outward rectification. The current-voltage relation at 10 min persisted for the remainder of the recording, up to 15 min in this case.

Somatostatin was ineffective when the pipette contained pertussis toxin (100 ng/ml or 10 μg/ml, $n = 4$).

Discussion

Application of whole-cell recording methods to the submucous neurons has allowed several further conclusions with regard to the consequences of activating α_2-adrenoceptors, δ-opioid receptors, and somatostatin receptors. First, the persistence of the effects with a highly buffered internal calcium concentration implies that internal calcium seems not to play a critical role in the transduction from receptor to potassium conductance, in contrast to inferences made on the basis of earlier studies (Morita et al., 1981; Morita and North, 1981, 1982). However, the present results do not exclude the possibility that the potassium channels involved can also be gated by changes in internal calcium concentration (Morita et al., 1981). Second, the results confirm that the potassium conductance increased by the agonist shows inward rectification (Surprenant and North, 1988) but clearly distinguishes it from the background inward rectifier of these neurons. The conductance increased by noradrenaline was best-fitted by a Boltzmann function centered around −65 mV, whereas the basic inward rectification of the neurons is centered around −90 mV (in 5 mM potassium; Surprenant and North, 1988). This result is similar to that described for the action of opioid agonists in neurons of the rat locus ceruleus (Williams et al., 1988). A third feature of the current induced by the agonists that had not previously been observed was the time-dependent component to the inward current seen in about half the cases. This was clearly distinguishable from the H-current that is seen in about 10% of submucous plexus neurons (H.T., R.A.N., K.-J. Shen, and A. Surprenant, unpublished observations), being faster in onset and unaffected by low sodium concentrations. The time-dependent component to the inward current induced by the agonists was very similar to that reported in amphibian myocytes for the current induced by acetylcholine (Simmons and Hartzell, 1987; Breitwieser and Szabo, 1988). The time-dependent component to the inward current usually became less as the outward current evoked by the agonist reached its steady state (Fig. 2); this might imply that there are 2 distinct potassium conductances increased by noradrenaline but the first (time-dependent) component undergoes rapid desensitization whereas the second (time-independent) component does not.

The second major advantage afforded by the whole-cell recording method is the ability to introduce large-molecular-weight molecules into the cell interior. Thus, the present experiments confirmed and extended studies with conventional microelectrodes in the following ways. First, intracellular dialysis of pertussis toxin at low concentrations (10 ng/ml) rapidly blocked agonist responses. We had previously shown that extracellular application of much higher concentrations of pertussis toxin would block hyperpolarizations caused by noradrenaline. Second, intracellular dialysis with GTP-γ-S had little effect on membrane conductance by itself but rendered irreversible the outward currents evoked by any of the agonists (Fig. 4); in a previous study it was found that the hyperpolarization caused by somatostatin became irreversible when the intracellular electrodes contained 20 mM GTP-γ-S (Mihara et al., 1987). In amphibian myocytes, intracellular dialysis with the nonhydrolyzable GTP analogs caused a progressive outward current even in the absence of applied agonist (in that case, acetylcholine) (Breitwieser and Szabo, 1988), and this developed more rapidly when the ratio of concentrations of nonhydrolyzable analog to those of GTP was high. However, in the majority of our experiments the pipette contained GTP-γ-S (300 μM) and no added GTP but no outward current was observed unless agonist was applied.

Intracellular dialysis with purified G-proteins (G_i or G_o) which had been preactivated with GTP-γ-S (see Materials and Methods), caused a slowly developing increase in potassium conductance (Fig. 5). It is unlikely that this action results from residual GTP-γ-S used for preactivation; at the highest concen-

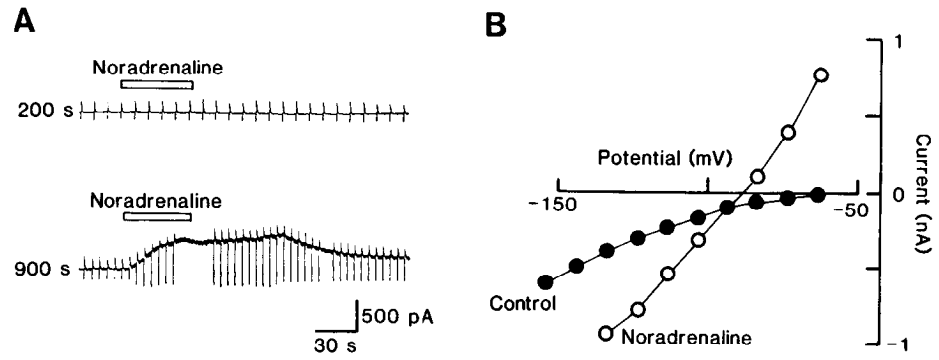

Figure 6. G-proteins reconstitute outward current responses to noradrenaline. *A,* Neuron was treated with pertussis toxin (1 μg/ml for 1 hr at 37°C) prior to recording. After whole-cell recording (time zero) began, a negative pressure of 100 cm H_2O was applied to the pipette. After 200 sec of recording, noradrenaline (10 μM) had no effect; *top trace* shows membrane current at −60 mV, and during 300 msec steps to −120 mV. The negative pressure was reduced to 10 cm H_2O immediately after the noradrenaline application, and noradrenaline (10 μM) was reapplied after 15 min (*lower trace*). Noradrenaline now caused a very large outward current response associated with an increase in membrane conductance. The recording pipette contained a mixture of G_i and G_o (2 nM). Break in downward current steps indicates time at which current/voltage relation was made (see *B*). *B,* Current/voltage relation for current induced by noradrenaline for another neuron. Note the lack of inward rectification (compare with Figs. 2 and 3).

trations of G-protein used (6 nM) the GTP-γ-S concentration would be about 3 μM, and even 300 μM GTP-γ-S caused no outward current in the absence of agonist (see above). After several minutes of such dialysis, noradrenaline no longer evoked any outward current; a simple interpretation is that the potassium channels are not opened by noradrenaline because they are already opened by the G-protein. There are suggestions, however, that dialysis with G-protein may have additional or more complicated actions. Membrane currents evoked by hyperpolarizing commands in cells exposed to intracellular G-protein did not show any time-dependent component, as did the current induced by the agonists in at least some cells. The membrane current that developed in neurons dialysed with G-proteins did not exhibit the strong inward rectification typical of the agonist induced current (compare Fig. 5*B* with Fig. 2*C*). Similar observations have been reported for cardiac myocytes dialyzed with $G_{\alpha i}$ (Szabo, personal communication). There is evidence in rat hippocampal neurons that a single species of G-protein can activate distinct potassium channels; both purified G_o and a recombinant $G_{o\alpha}$ subunit opened at least 4 different types of potassium channel in excised membrane patches (VanDongen et al., 1988). Under more physiological conditions, with intact cells, agonists seem to open one predominant species of potassium channel (Soejima and Noma, 1984; Miyake et al., 1989).

Dialysis of the neurons with G-protein preactivated with GTP did not cause any significant change in membrane current. However, the reconstitution of the agonist current by intracellular dialysis with G-proteins strongly supports the interpretation that the pertussis toxin is working by the ADP ribosylation and hence

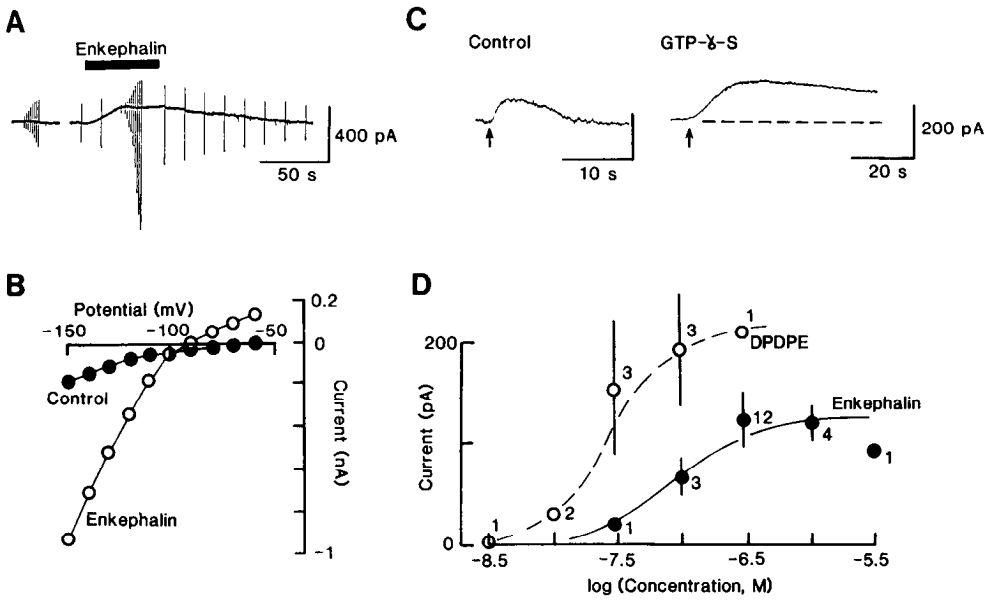

Figure 7. Potassium currents caused by enkephalin in submucous neurons. *A,* Superfusion with [Met⁵]enkephalin (300 nM) caused an outward current at −60 mV. Downward deflection indicates membrane current responses to 300 msec hyperpolarizing pulses to −120 mV. The closely spaced deflections are current responses to similar voltage steps, but delivered at 10 sec intervals and each increasing by −10 mV. *B,* Current/voltage relation in absence (*filled circles*) and presence (*open circles*) of enkephalin. Data are from cell in *A*. *C, Left trace*: Response of a normal cell to brief application of [Met⁵]enkephalin lasts about 10 sec (pipette for drug application contained 100 μM [Met⁵]enkephalin and pulse was 100 msec). *Right trace*: A similar experiment on a neuron when the recording electrode contained GTP-γ-S (300 μM). In this case a similar application of enkephalin (35 kPa, 100 msec) evoked an outward current that only partially passed off even after 1 min. *D,* Concentration-response curves for [Met⁵]enkephalin and DPDPE on dissociated submucous plexus neurons.

inactivation of the native G-protein. Currents evoked by agonists in pertussis-treated neurons after internal dialysis with a G-protein also showed some differences to those observed in untreated neurons; they showed no inward rectification and no time dependence, again suggesting imperfect reconstitution.

Noradrenaline normally acts at α_2-adrenoceptors on submucous plexus neurons to bring about a hyperpolarizing postsynaptic potential; this results from opening of a set of inwardly rectifying potassium channels. The main result of the present experiments is that a potassium conductance can also be opened by the intracellular application of G-proteins, with both G_o and G_i being effective. These and other findings are consistent with the hypothesis that the agonists at α_2-adrenoceptors (as well as the δ-opioid and somatostatin receptors) normally open potassium channels by catalyzing the formation of activated G-protein. They do not address the issue of which G-protein is responsible for the transduction in normal cells; that question might be resolved by the intracellular application of selective antibodies.

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