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Oxytocin and cAMP stimulate monovalent cation movements through a Ca^{2+}-sensitive, amiloride-insensitive channel in the apical membrane of toad urinary bladder

(ion selectivity/monovalent cations/permeability/noise analysis/ion channel)

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ABSTRACT **The effects of oxytocin and cAMP on ion transport were investigated in toad urinary bladders incubated with Ca^{2+}-free solutions on the apical side. Under these conditions both oxytocin and cAMP markedly stimulated the movements of Na^+, K^+, Rb^+, Cs^+, Li^+, and NH_4^+ through a pathway that is insensitive to amiloride. The amiloride-insensitive currents were inhibited by the addition of Ca^{2+}, Sr^{2+}, or Mg^{2+} to the apical solution. The movement of the monovalent cations was associated with a spontaneous Lorentzian component in the power spectrum of the fluctuation in short-circuit current. The plateau of the Lorentzian component was enhanced by oxytocin and cAMP and was depressed by divalent cations. Methohexital inhibited the stimulation of monovalent cation movements caused by oxytocin. These findings suggest that oxytocin and cAMP activate at least two kinds of ionic channels in the apical membrane of toad urinary bladder: the well-known amiloride-sensitive channel and an amiloride-insensitive channel that allows the movement of several monovalent cations and is blocked by Ca^{2+} and other divalent cations.**

Until quite recently it was widely thought that the effects of neurohypophysial hormones on transepithelial ion transport are due almost exclusively to the activation of an amiloride-inhibitable Na^+ channel in the apical membrane (1). However, recent studies from our laboratory have shown that oxytocin and cAMP also stimulate ion movements through K^+-selective channels in both the apical (2) and basolateral membranes (3, 4) of amphibian tight epithelia. These findings indicate that the physiologic effects of neurohypophysial hormones may involve the activation of several ionic channels. The purpose of the present experiments was to examine the effects of oxytocin and cAMP on an ionic channel of amphibian epithelia that is detected when Ca^{2+} is removed from the apical solution. After Ca^{2+} removal, a Lorentzian component appears in the noise spectrum of the apical membrane of the frog skin (5). This Lorentzian component is due to the activity of a channel that is freely permeable to a large variety of monovalent cations and is not inhibited by amiloride.

MATERIALS AND METHODS

The procedures followed in our laboratory have been described in detail (6). In brief, urinary bladders of the toad (*Bufo marinus*) were mounted with minimal edge damage in an Ussing-type chamber, which allowed continuous perfusion of both surfaces of the tissue. The membrane area exposed to the bathing solutions was 0.5 cm^2. The transepithelial potential was clamped to zero with a low-noise voltage clamp (7). Transepithelial currents (I_{sc}) flowing from the apical towards the basolateral compartment were considered as positive. The fluctuation in I_{sc} was recorded and analyzed as described (6, 7). In the present study, the fundamental frequency of our analysis was 1 Hz and the maximum frequency plotted in the figures and used in our final analysis was 800 Hz. Many studies have shown that the random open-close process of ion-selective channels causes fluctuations in current, which give rise to a Lorentzian component in the power-density spectrum (8). The mathematical equation that describes the frequency dependence of this noise component is: $S(f) = S_o/[1 + (f/f_c)^2]$, where S_o, the plateau value, represents the power density at the lower frequency end of the spectrum, and f_c, the corner frequency, is the frequency where $S(f) = S_o/2$. The noise spectra recorded from biological membranes usually contain a $1/f$-noise component, which becomes dominant at the low-frequency end of the spectrum (see Fig. 4 *Lower Left* and *Right*). In addition, at high frequencies, the instrumentation noise surpasses the Lorentzian noise from the preparation. The Lorentzian parameters (S_o and f_c) were determined by nonlinear least-square curve fitting (9) of the spectral data.

Solutions. The basolateral side was always exposed to NaCl Ringer's solution with the following composition: 115 mM NaCl/2.5 mM $KHCO_3$/1 mM $CaCl_2$, pH 8.0. The composition of the apical NaCl Ringer's solution was 115 mM NaCl/2.5 mM $KHCO_3$/1 mM $CaCl_2$/8 mM Hepes/9.4 mM Tris, pH 8.0. Composition of Ca^{2+}-free apical solutions was 115 mM XCl/8 mM Hepes/11.1 mM Tris, pH 8.0/0.5 mM EGTA, where the main cation, X, was one of the following monovalent cations: Na^+, K^+, Rb^+, Cs^+, NH_4^+, or Li^+. We calculated the concentration of free Ca^{2+}, following the method of Fabiato and Fabiato (10). When we assumed that the water and solutes used to prepare the solutions contained 10 μM Ca^{2+}, the calculations showed that the solutions contained <0.1 μM free Ca^{2+}. Na_2SO_4 Ringer's solution had the following composition: 57.5 mM Na_2SO_4/2.5 mM $KHCO_3$/1 mM $CaSO_4$/8 mM Hepes, pH 8.0. For K_2SO_4 Ringer's solution Na_2SO_4 was replaced by K_2SO_4 on an isomolar basis. Aliquots of an aqueous stock solution of amiloride (10 mM) (a gift from Merck Sharp & Dohme) were added to the apical Na^+ or Li^+ Ringer's solution (final concentrations are indicated in the legends of the figures). Oxytocin and cAMP [8-(4-chlorophenylthio)-cAMP, sodium salt] were purchased from Sigma. Both substances were dissolved in distilled water and added to the basolateral solutions. The sensitivity of I_{sc} to different divalent cations (Sr^{2+}, Mg^{2+}, Ca^{2+}) was tested by adding the chloride salts to the apical solution (0.5 mM); EGTA was omitted from the

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Abbreviation: I_{sc}, transepithelial current(s).

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apical solution when Sr^{2+} or Ca^{2+} were used but was included when Mg^{2+} was added.

RESULTS

It is well known that oxytocin and cAMP stimulate transepithelial salt transport by increasing the movements of Na^+ through amiloride-sensitive channels in the apical membrane of tight epithelia. The left-hand portion of Fig. 1 illustrates this effect. The toad urinary bladder was incubated with Na^+ Ringer's solution on both sides before oxytocin (0.1 unit/ml) was added to the basolateral solution. The hormone elicited a marked increase in I_{sc}. Both the hormone-stimulated and the resting I_{sc} pass mostly through amiloride-sensitive channels because addition of the diuretic (50 μM) to the apical solution caused the characteristic near-complete and reversible inhibition of I_{sc} before and during exposure to oxytocin. Once the tissue had recovered from the inhibitory effects of amiloride, the apical side of the tissue was perfused with Ca^{2+}-free Na^+ Ringer's solution. The removal of apical Ca^{2+} produced a marked stimulation of I_{sc}. When amiloride was added to the Ca^{2+}-free apical solution, it did not block I_{sc} completely as in Ca^{2+}-containing solutions. Close inspection of Fig. 1 shows that the amiloride-inhibitable component of I_{sc} in the presence and absence of Ca^{2+} is comparable. Hence, a large I_{sc}, similar in size to that caused by removing Ca^{2+}, still persisted in the presence of amiloride. This amiloride-insensitive current component was markedly inhibited when Ca^{2+} was added to the apical solution. Similar results were obtained in four other bladders. In other experiments we examined the effects of adding ouabain (10 mM) to the serosal solution or of using Cl^--free solutions on the amiloride-insensitive currents. Ouabain (n = 3) inhibited >90% of the current through the amiloride-insensitive pathway. When the tissues were incubated in Na_2SO_4 Ringer's solution on both surfaces, oxytocin produced a stimulation of the amiloride-insensitive current after Ca^{2+} was removed from the apical solutions.

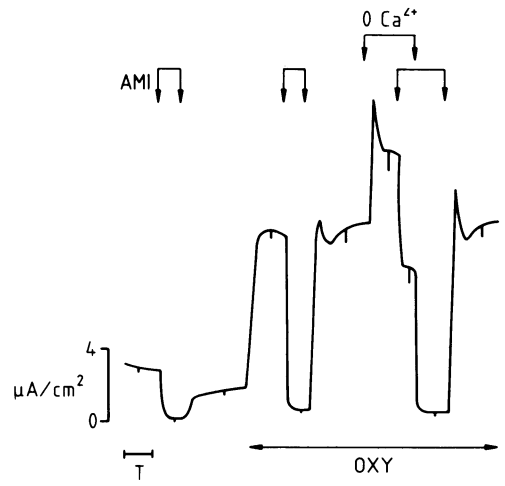

FIG. 1. Effects of the removal of Ca^{2+} from the apical solutions on the stimulation of I_{sc} by oxytocin. Both apical and basolateral compartments were perfused with Na^+ Ringer's solution throughout the experiment except for the period labeled "0 Ca^{2+}," when the apical compartment was perfused with Ca^{2+}-free Ringer's solution. The periods when amiloride (50 μM) was present in the apical solution are marked by the pairs of vertical arrows. The period when oxytocin (0.1 unit per ml) was present in the basolateral solution is shown at the bottom of the graph ("OXY"). The vertical deflections in the current traces of this and the following figures were caused by briefly changing the clamping potential from 0 to −3 mV. The time (T) calibration equals 12 min.

So far, these experiments reveal the existence of substantial movements of ions through an amiloride-insensitive pathway in oxytocin-stimulated bladders perfused with Ca^{2+}-free Ringer's solution on the apical side. Since the current is observed when the tissue is incubated in Cl^--free solutions, it is reasonable to assume that it is carried by the inward movements of Na^+. We have not yet measured whether there is any substantial flux of cations from serosal to mucosal solution during the stimulation of I_{sc} caused by oxytocin in Ca^{2+}-free solutions. It is possible then, that I_{sc} is actually smaller than the current carried by the inward Na^+ flux through the amiloride-insensitive channel.

To further characterize the Ca^{2+}-sensitive pathway, experiments were undertaken (*i*) to examine the permeability of this pathway for K^+, (*ii*) to investigate the effects of amiloride on the movements of K^+, and (*iii*) to study the inhibitory effects of divalent cations. In the experiment shown in Fig. 2, we first show the effects of 5 and 50 μM amiloride on a bladder perfused with Na^+ Ringer's solution on both sides. These concentrations of amiloride produce their characteristic large and rapidly reversible inhibition of I_{sc}. Then the apical side was perfused with a solution in which all of the Na^+ had been replaced by K^+ and in which Ca^{2+} had been removed. With this solution, a small inward-oriented I_{sc} was recorded, which was rapidly and reversibly blocked when 0.5 mM Sr^{2+} was added to the apical solution. The inward current could be abolished by substituting large organic cations (choline, tetraethylammonium) for K^+ (not shown). The Ca^{2+}-sensitive currents were also observed when bladders were incubated in Cl^--free solutions (SO_4^{2-} Ringer's solution). Therefore, it is reasonable to assume that the currents in the right-hand portion of Fig. 2 were carried by K^+. When oxytocin was added to the basolateral solution, I_{sc} was markedly stimulated. The oxytocin-activated current also was reversibly inhibited by the addition of Sr^{2+} to the apical solution, whereas amiloride had only a small inhibitory effect. Analysis of the dose–response curves showed that ≈35 μM Sr^{2+} was required to inhibit 50% of I_{sc} after oxytocin treatment. Similar effects on the inward K^+ current were produced by Mg^{2+} and Ca^{2+} in other experiments (see Fig. 4). The current component depressed by 0.5 mM Ca^{2+} averaged 3.1 ± 1.1 $\mu A/cm^2$ before and 16.3 ± 4.3 $\mu A/cm^2$ after oxytocin treatment (n = 6). In contrast, the addition of amiloride produced only a small and slowly developing inhibition of I_{sc}. The average depressions of I_{sc} caused by amiloride were 0.24 ± 0.14 and 0.52 ± 0.22 $\mu A/cm^2$ for 5 and 50 μM amiloride (n = 3), respectively.

So far it is clear that Na^+ as well as K^+ pass through the Ca^{2+}-sensitive, oxytocin-activated, amiloride-insensitive pathway. Fig. 3 illustrates an experiment in which we tested whether other monovalent cations permeate this pathway. The tissue was first incubated with Ca^{2+}-free K^+ Ringer's solution as the apical solution. Oxytocin elicited a large stimulation of I_{sc}. Once the stimulatory effects of oxytocin were fully developed, the apical side was perfused with Ca^{2+}-free solutions in which Na^+, Rb^+, Cs^+, Li^+, or NH_4^+ successively constituted the main cation. The Na^+ and Li^+ solutions also contained amiloride (20 μM) to prevent movements of these ions through the amiloride-sensitive channels. All of the ions tested appeared to pass through the Ca^{2+}-sensitive, oxytocin-activated pathway. The currents were similar for Na^+, K^+, and Rb^+, but Cs^+ and Li^+ produced about half as much current. With NH_4^+ we always observed a progressive increase in I_{sc} and a large increase in conductance that might be related to NH_4^+ accumulation in the cells. During perfusion with each ion, Ca^{2+} was briefly added to the apical solution to test whether I_{sc} passed through a divalent cation-sensitive pathway; in all cases, a rapidly reversible inhibition of I_{sc} was observed. Because we do not have yet determinations of the electrochemical gradients across the

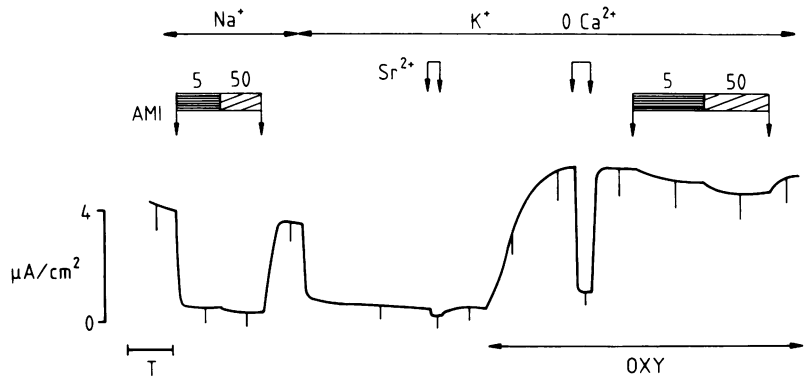

FIG. 2. K^+ movements through the Ca^{2+}-sensitive channel and their modification by oxytocin and amiloride. First, the inhibitory effect of amiloride (AMI) on the mucosa to serosa Na^+ current was recorded while the urinary bladder was incubated with Na^+ Ringer's solution on both sides. During the remaining part of the experiment, the apical side was perfused with Ca^{2+}-free K^+ Ringer's solution. $SrCl_2$ (0.5 mM) and amiloride (5 and 50 μM) were added to the apical solutions at the indicated periods. Oxytocin (0.1 unit per ml) was added to the basolateral perfusion medium during the period indicated "OXY." The time (T) calibration equals 12 min.

cell membranes, these experiments only show which cations pass through the amiloride-insensitive pathway without providing a quantitative measurement of channel selectivity.

In order to avoid movements of ions through the amiloride-sensitive channels, we performed the experiments described below with KCl Ringer's solution as the apical medium. Fig. 4 *Left* illustrates an experiment in which we recorded I_{sc} and the power-density spectra of the fluctuation in I_{sc} before and during the treatment of the tissue with oxytocin. Prior to the hormonal treatment, the spectrum shows a Lorentzian component with a corner frequency (f_c) of ≈450 Hz. The addition of Ca^{2+} (0.5 mM) to the apical medium depressed the Lorentzian component completely. Similar spectra were recorded with Na^+ or Rb^+ as the main apical cation. The presence of a Lorentzian component in the power-density spectrum indicates that the pathway for monovalent cations consists of channels that open and close randomly. The addition of oxytocin to the basolateral medium enhanced the Lorentzian plateau considerably but did not significantly alter the corner frequency: $f_c^{CTR} = 462.2 \pm 43.0$ Hz; $f_c^{OXY} = 480.4 \pm 13.3$ Hz ($n = 3$; CTR = control; OXY = oxytocin). This finding suggests that oxytocin does not modify the channel gating but increases the number of conductive channels in the apical membrane. The Lorentzian noise component recorded during oxytocin treatment was completely abolished by 0.5 mM apical Ca^{2+}.

Neurohypophysial hormones markedly increase cytoplasmic levels of cAMP; therefore, it was interesting to determine whether the effects of oxytocin could be mimicked by cAMP. Fig. 4 *Right* illustrates an experiment in which the effects of cAMP on the Ca^{2+}-sensitive I_{sc} and its fluctuation were analyzed. Initially, when the apical side of the tissue was exposed to Ca^{2+}-free KCl Ringer's solution, an inward-oriented I_{sc} was recorded that was almost completely blocked by addition of 0.5 mM Mg^{2+} to the apical solution. Analysis of the fluctuation in I_{sc} revealed a Lorentzian component in the power-density spectrum, which was completely abolished by apical Mg^{2+} (0.5 mM). I_{sc} and the associated Lorentzian noise component were drastically elevated when 1 mM cAMP was added to the basolateral solution. Also the activated I_{sc} and its fluctuation were blocked by the addition of Mg^{2+} to the apical solution. As in the experiments with oxytocin, during the activation of the Lorentzian noise component, the corner frequency did not change significantly: $f_c^{CTR} = 539.4 \pm 54.5$ Hz; $f_c^{cAMP} = 509.4 \pm 47.8$ Hz ($n = 5$).

It is known that basolateral methohexital (0.3 mM) inhibits the increase in water permeability caused by antidiuretic hormone in the toad urinary bladder, while the increases in urea and Na^+ movements remain essentially unaltered (11–13). Methohexital also blocks the increase in capacitance (13) and the appearance of the characteristic intramembrane particles (14) that seem to be associated with antidiuretic hormone-stimulated water movements. We examined the effects of methohexital on the oxytocin-stimulated current of the Ca^{2+}-sensitive channel (Fig. 5). Methohexital (0.3 mM) in the basolateral solution had little effect on the resting I_{sc} or its modification by Sr^{2+}. Addition of oxytocin to the methohexital-treated tissue caused an increase in I_{sc}. However, when methohexital was removed from the basolateral solution, a further increase in I_{sc} occurred, showing that the effects of oxytocin were markedly depressed in the presence of methohexital. In five experiments, the oxytocin-stimulated I_{sc} in methohexital-treated bladders averaged 25.5 ± 6.3% of the effect observed after removal of methohexital.

DISCUSSION

The most interesting feature of these experiments is the finding that oxytocin and cAMP stimulate ionic movements through a channel that drastically differs from the well-studied amiloride-sensitive Na^+ pathway. Indeed, the experiments described here show that when the toad urinary bladder is incubated in Ca^{2+}-free solutions on the apical side,

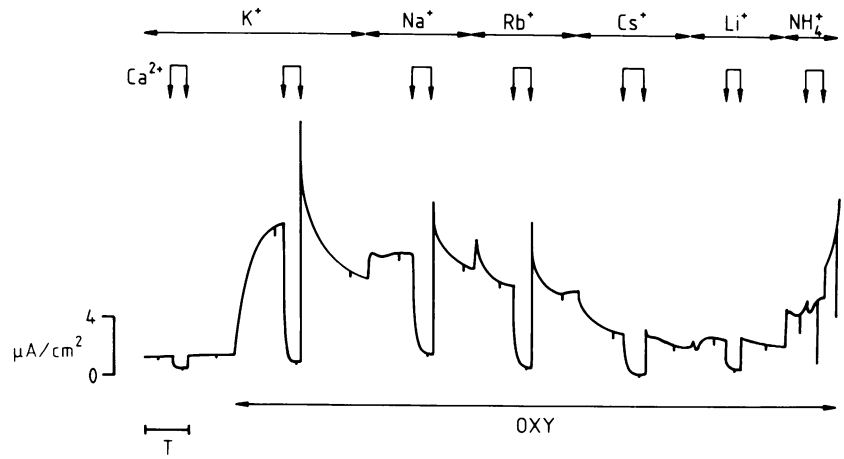

FIG. 3. Movements of different monovalent cations through the oxytocin-stimulated bladder. In this experiment the apical side was perfused throughout with Ca^{2+}-free solutions, each prepared with the chloride salt of the monovalent cation indicated. During perfusion with each monovalent cation, $CaCl_2$ was briefly added to the apical solution. Oxytocin (0.1 unit per ml) was added to the basolateral medium as indicated ("OXY"). The time (T) calibration equals 15 min.

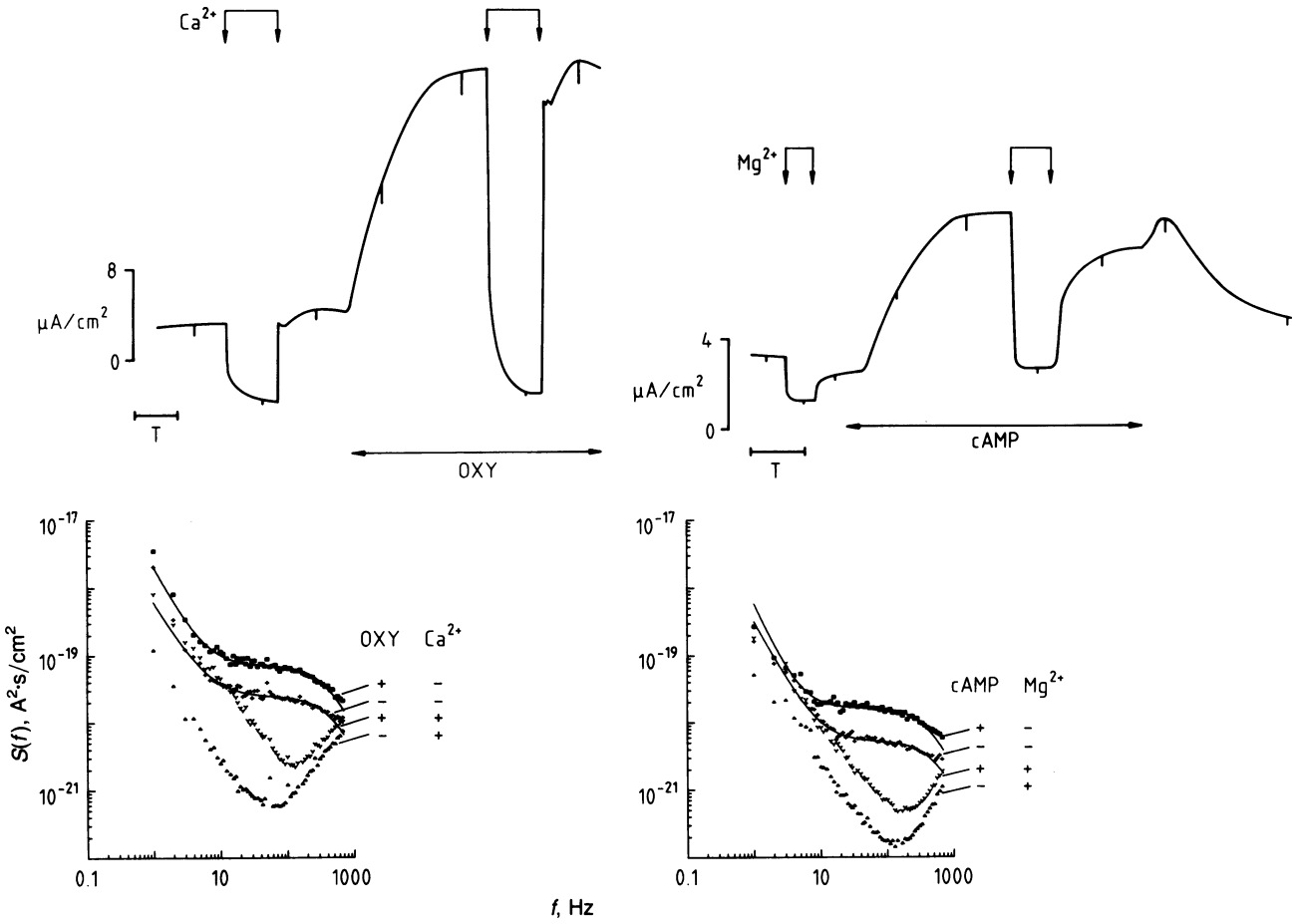

FIG. 4. Effects of oxytocin (OXY) (*Left*) and cAMP (*Right*) on I_{sc} and its spontaneous fluctuation. The inhibitory effects of Ca^{2+} and Mg^{2+} are shown as well. Records in *Upper Left* and *Upper Right* show the I_{sc} from which spectra shown in *Lower Left* and *Lower Right*, respectively, were recorded. Spectra recorded before the addition of oxytocin or cAMP: +, control; ▲, in the presence of 0.5 mM Ca^{2+} (for oxytocin) or Mg^{2+} (for cAMP). Spectra recorded during the treatment of the bladder with oxytocin or cAMP: ■, control; Y, in the presence of 0.5 mM Ca^{2+} (for oxytocin) or Mg^{2+} (for cAMP). The Lorentzian parameters in *Lower Left* were $S_o = 24.0 \times 10^{-21}$ A^2·s/cm^2 and $f_c = 427.5$ Hz before oxytocin addition and $S_o = 69.5 \times 10^{-21}$ A^2·s/cm^2 and $f_c = 360.5$ Hz during oxytocin treatment. Lorentzian parameters in *Lower Right* were $S_o = 5.19 \times 10^{-21}$ A^2·s/cm^2 and $f_c = 486.2$ Hz before cAMP addition and $S_o = 16.5 \times 10^{-21}$ A^2·s/cm^2 and $f_c = 377.6$ Hz during cAMP treatment. Throughout the experiment, the tissue was incubated with Ca^{2+}-free K^+ Ringer's solution (0.5 mM EGTA) and Na^+ Ringer's solution as the apical and basolateral bathing medium, respectively. The time (T) calibration equals 10 min in *Upper Left* and 12 min in *Upper Right*.

oxytocin and cAMP stimulate the movements of a variety of monovalent cations that do not pass through the amiloride-sensitive channel. Moreover, in Na^+ solutions an amiloride-insensitive component of I_{sc} appears when Ca^{2+} is removed from the apical solution. All of these amiloride-insensitive movements are markedly and rapidly inhibited when Ca^{2+}, Sr^{2+}, or Mg^{2+} are added to the apical solution. Three lines of evidence suggest that we are dealing with ion movements through a cell membrane pathway rather than through the paracellular shunt. (*i*) The association of ion movements through the Ca^{2+}-sensitive channels with a spontaneous Lorentzian component in the power spectrum indicates that we are dealing with channels that switch randomly between the open and closed conducting state. Such transitions are characteristic of membrane channels, whereas, thus far, similar noise components have not been identified for paracellular pathways. (*ii*) For the Na^+ experiments, the currents were recorded with no electrical potential gradient across the tissue and nearly identical solutions on both sides (the only difference is the absence of Ca^{2+} in the apical solution). (*iii*) The Na^+ current is abolished by ouabain. In addition, two observations suggest that the channel is located in the apical membrane of the epithelium. First, changing divalent cation concentration in the apical solution has rapid and reversible effects on the channel. Since these cations hardly move across either the apical membrane or the whole epithelium, the effects are likely to be due to an effect on the apical border. Second, under our experimental conditions, noise signals originating in the basolateral membrane are likely to be attenuated below levels of detection (15).

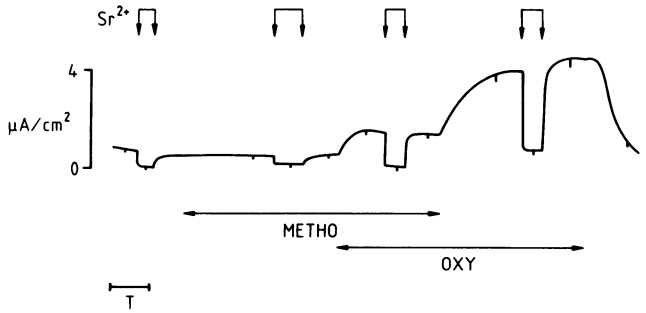

FIG. 5. Effect of methohexital (METHO; 0.3 mM) on the response to oxytocin (OXY). Throughout the experiment, the toad urinary bladder was perfused with Ca^{2+}-free K^+ solution (no EGTA) on the apical side and Na^+ Ringer's solution on the basolateral side. Sr^{2+} (0.5 mM) was added to the apical solution to estimate the current that is sensitive to divalent cations. The time (T) calibration equals 12 min.

These considerations suggest that oxytocin and cAMP increase the movements of ions across the apical membrane

of toad urinary bladder through two separate pathways: one, the amiloride-sensitive channel that is permeable to Na^+ and Li^+; and the other, a pathway inhibited by alkaline-earth metal ions that allows large movements of several monovalent cations and that is only slightly modified by amiloride. The monovalent cation selectivity and the sensitivity to divalent cations of the second channel are similar to those of the Ca^{2+}-sensitive channel previously identified in the skin of *Rana catesbeiana* (5). The major difference appears to concern the channel kinetics detected during the analysis of the spontaneous fluctuation in current: the value of the corner frequency for the channel described here was 462.2 Hz, whereas in frog skin it had a value of 120.5 Hz (5). The association of movements of ions through the Ca^{2+}-sensitive channels with a spontaneous Lorentzian component in the power spectra further differentiates the Ca^{2+}-sensitive pathway from the amiloride-sensitive channels. Ion movements through the latter are not associated with a spontaneous Lorentzian component either under control conditions or after treatment with oxytocin (8). The requirement of Ca^{2+}-free apical solutions to observe these channels also distinguishes them from other amiloride-insensitive pathways that are increased by neurohypophysial hormones, since the other pathways were observed in Ca^{2+}-containing solutions (2, 13, 16).

The Ca^{2+}-sensitive channels described here have two features in common with the Ca^{2+} channels found in a wide variety of other cell types (17–20): first, Ca^{2+} removal is necessary to induce the movement of a variety of monovalent cations (17–19); and second, movement of ions is markedly enhanced by oxytocin and cAMP. In other tissues it has been suggested that cAMP is necessary to maintain Ca^{2+} channel function (21, 22). In spite of these similarities, we have not yet been successful in establishing for the Ca^{2+}-sensitive channels of the toad urinary bladder three major features of the Ca^{2+} channels of other tissues (4): (*i*) permeability to Ca^{2+} and other divalent cations, (*ii*) activation by membrane depolarization, and (*iii*) modification by dihydropyridine compounds, most of which have a blocking action on the channel, although some analogues can activate it (23–25).

As far as the physiological role of the channel is concerned, previous experiments with methohexital showed that this drug selectively blocks the oxytocin-induced increase in hydraulic conductance (11, 12), hence the blocking effects of this drug hint that activation of the channel may be related to an event that occurs simultaneously with the increase in hydraulic conductivity. Activation of the channel could be associated with increased hydraulic conductivity in several ways. For example, the pathway could be a constituent of the extra membrane that becomes inserted into the apical surface during the stimulation of water permeability by the hormone (14). Another possibility is that activation of the channel could be part of the mechanism that regulates the oxytocin-induced fusion of cytoplasmic vesicles with the apical membrane. However, the available evidence is not sufficient to resolve these issues.

Although we have not yet established the physiologic role of the Ca^{2+}-sensitive channel, the identification of a pathway that is under the control of cAMP and neurohypophysial hormones is of importance because it reveals an unrecognized component in the regulation of apical membrane properties. Moreover, modification by cAMP-activated kinases is an important feature to be examined during the isolation of apical membrane transport proteins. Hence, a full description of cAMP-sensitive processes is essential information in the purification of these proteins.

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