Palytoxin effects through interaction with the Na,K-ATPase in *Xenopus* oocyte

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Abstract Palytoxin (PTX) is known to bind to Na,K-ATPase, to inhibit its activity, and to induce cation conductance, but the mechanism of these effects is still poorly understood. In *Xenopus* oocytes, PTX induced a large cation conductance, an effect that could be prevented or reversed by ouabain for oocytes expressing *Xenopus* Na,K-pumps but not with those expressing *Bufo* Na,K-pumps. In both cases patch-clamp experiments demonstrated a 7–8 pS channel in the presence of PTX. A large PTX-induced conductance could be observed with minimal Na,K-pump inhibition. From the single PTX-induced channel and macroscopic whole oocyte conductance, and the number of Na,K-pumps, we can conclude that PTX-induced conductance occurs through a direct interaction of PTX with a small number of Na,K-pumps.

Key words: Na,K-ATPase; Palytoxin; Ouabain; Single-channel current

1. Introduction

Palytoxin (PTX) is the most potent non-peptidic toxin known to date. PTX is known to induce cell membrane depolarization and an increase of cell membrane permeability to Na^+ and K^+ [6,7,21]. These effects can be attributed to the formation of non-selective cation channels [12,15,17]. Since the specific inhibitor of the Na,K-ATPase, ouabain, is an antagonist of PTX effect, it is likely that Na,K-ATPase is the receptor for PTX [1,3,6]. This view is also supported by the observation of ouabain-sensitive, PTX-induced channels in the lipid bilayer in which purified Na,K-ATPase has been reconstituted [12]. Finally, Scheiner-Bobis et al. [16,18] have shown that yeast cells, which do not express Na,K-ATPase, were resistant to PTX, but that the sensitivity to PTX could be induced by exogenous expression of Na,K-ATPase by cDNA transfection.

There are, however, a number of observations that have been interpreted as indicating that PTX may act through targets other than Na,K-ATPase. In cultured aortic myocytes, PTX-induced channels are sensitive to an amiloride derivative [18]; the single channel conductance varies from 9 to 25 pS, in various cell types [4]; PTX can induce cell acidification [5,14]; PTX treatment augments the intracellular Ca^{2+} concentration [13] and the effect of PTX depends markedly on the presence of extracellular Ca^{2+} [19]. These observations have supported the hypothesis that PTX may act on a molecular target different from Na,K-ATPase, for instance on a low conductance cation channel [4].

In the present work, we have studied the effect of PTX on *Xenopus* oocytes expressing two types of amphibian Na,K-ATPase: the ouabain-sensitive *Xenopus* Na,K-pump and the ouabain-resistant *Bufo* Na,K-pump, in order to extend the observation of PTX effects to non-mammalian target molecules and to better define the kinetics of the interaction of PTX with the Na,K-pump and ouabain.

2. Materials and methods

2.1. Expression Na,K-ATPase in X. laevis oocytes

Most experiments have been performed on native, non-injected oocytes, using the endogenous Na,K-ATPase present at the oocyte membrane. However, in some experiments as indicated in Section 3, cRNA of the α and β subunits of the Na,K-pump was injected to increase the density of the Na,K-pump or to express the ouabain-resistant *Bufo* Na,K-pump. The Na,K-pump was expressed in *Xenopus* oocytes as previously described [9]. Briefly, stage V–VI oocytes were co-injected with 7 ng of α subunit cRNA (*Xenopus* α1 or *Bufo* α1) and 1 ng of β subunit cRNA (*Xenopus* β1 or *Bufo* β1) of the Na,K-ATPase. The oocytes were kept in a modified Barth's solution for 3–5 days to allow the expression of the exogenous Na,K-pump. Before measurement, all oocytes were loaded with Na^+ by overnight incubation in a K^+-free solution as described [22].

Whole oocyte current measurements were performed by the two-electrode voltage-clamp technique, in Na^+-containing (Na^+ 92.4, Mg^{2+} 0.82, Ba^{2+} 5, Ca^{2+} 0.41, TEA 10, Cl^- 22.46, HCO_3^- 2.4, gluconate 80, HEPES 10 mM) or Na^+-free solutions (Mg^{2+} 0.82, Ba^{2+} 5, Ca^{2-} 0.41, TEA 10, Cl^- 22.46, HEPES 10, NMDG 10, sucrose 140 mM). The activity of the Na,K-pump was measured as the outward current activated by 10 mM K^+ [11] at a holding potential of −50 mV, unless otherwise indicated.

2.2. Single-channel current measurements

The oocytes were incubated in a hypertonic solution (K aspartate 200, KCl 20, $MgCl_2$ 1, EGTA 10, HEPES 10 mM, pH 7.4) for several minutes and the vitelline membrane was removed manually. Fire-polished patch pipettes with a resistance of about 10 MΩ were prepared from borosilicate glass (PYERX, Corning, New York) according to Hamill et al. [8] and filled with a solution containing Na^+ 90, Cl^- 80 and Hepes 10 mM (pH 7.4) with or without PTX (2 nM). High-resistance seals were then obtained on the oocyte plasma membrane and single channel currents were studied in the cell-attached configuration. The current signal was amplified and filtered at 3 kHz by means of a List EPC-7 patch-clamp amplifier (List-Medical, Darmstadt, Germany) and recorded on a digital tape recorder (Biologic, France). After filtering at 225 Hz with an 8-pole Bessel filter, current recordings were analyzed using the PClamp software (Axon Instruments, Foster City, CA).

2.3. Chemicals

Palytoxin was purchased from Sigma. Stock solution, 1 μM in water, was kept at −20°C. Final concentrations of palytoxin were obtained by dilution of the stock in bath solution immediately before each measurement. Ouabain was purchased from Sigma and used from a 0.2 M stock solution in dimethylsulfoxide.

All results are expressed as mean ± S.E.M. (n = number of observations)

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3. Results

3.1. Effect of PTX on Xenopus laevis and Bufo Na,K-pump

In non-injected *Xenopus* oocytes, the endogenous Na,K-pump activity measured as the K-induced outward current ranged from 40 to 100 nA. Exposure to 2 nM PTX induced a progressive increase in the whole membrane conductance, as shown in the example tracing of Fig. 1a, from 1.8 ± 0.2 to 18.9 ± 0.6 μS ($n = 30$) in 3 min. This effect could be completely prevented when the Na,K-pump was inhibited by 100 μM ouabain (Fig. 1b). The affinity of ouabain for the *Bufo marinus* Na,K-pump is 3 orders of magnitude lower than that of the *Xenopus* Na,K-pump [10]. Considering the apparent competition between ouabain and PTX [6], it was interesting to determine if PTX would have any effect on the *Bufo* Na,K-pump. To ensure that any observed effect would not be due to PTX interacting with the endogenous *Xenopus* Na,K-pump, we performed the experiment in the presence of a large concentration of ouabain (100 μM) that is about 3 orders of magnitude higher than the K_i of ouabain for the *Xenopus* Na,K-pump [2]. Under these conditions, 5 nM PTX did not induce any detectable increase in conductance in non-injected oocytes or in oocytes expressing the α1β1 *Xenopus* Na,K-pump. However, in oocytes expressing the *Bufo* Na,K-pump

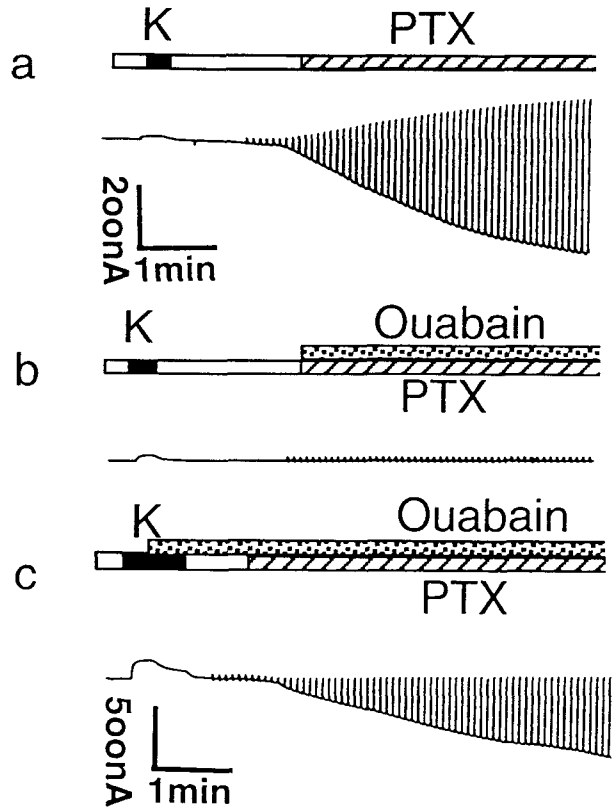

Fig. 1. Conductance change in the presence of PTX. Na,K-pump activity was first measured as the outward current activated by 10 mM K^+ (K), then palytoxin (2 nM) was applied to the oocyte without ouabain (a) or with 100 μM ouabain (b). In the absence of ouabain PTX induced a large change in the current elicited by 1-s 50-mV voltage jumps, while, in the presence of ouabain, PTX did not induce any detectable conductance change. In oocytes expressing the *Bufo* Na,K-pump (c), after exposure to 100 μM ouabain, a concentration sufficient to block completely the endogenous *Xenopus* Na,K-pump and partially the *Bufo* Na,K-pump, PTX still induced a large conductance.

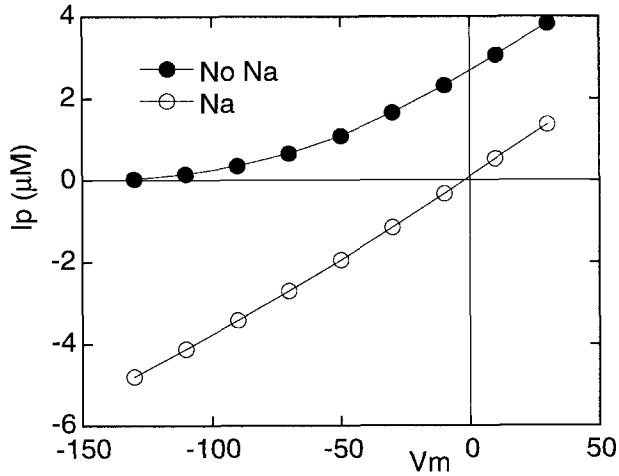

Fig. 2. PTX-induced current–voltage relationship in the presence or absence of external Na^+. The oocyte was held at −50 mV and 500 ms voltage jumps were applied over a −130 to 30 mV potential range; the current at each potential was recorded before and 1 min after application of 2 nM PTX. The figure shows the PTX-induced current (current after PTX minus current before PTX). The experiment was performed in the presence of extracellular Na^+ (open circles) or in the absence of extracellular Na^+ (closed circles). This experiment was performed in a non-injected oocyte.

the same concentration of PTX induced an obvious increase in the conductance (see example in Fig. 1c), even in the presence of 100 μM ouabain, a concentration that might have blocked a significant part of the expressed *Bufo* Na,K-pumps.

Fig. 2 shows the *I-V* curve of the PTX-induced current in sodium-containing and sodium-free solution. With 90 mM Na^+ the *I-V* curve was linear, while in the absence of extracellular sodium and other monovalent small cations, the current showed a strong outward rectification. We also tested the ionic selectivity of the PTX-induced conductance by ionic replacement experiments. Oocytes were first exposed to 2 nM PTX in a K^+-free solution containing 70 mM Na^+ until a large PTX-induced conductance was established and then the chord conductance (0 to −100 mV) was measured before and 10 s after the replacement of Na^+ by the same concentration of Li^+, or K^+. For the change from Na^+ to K^+ the conductance increased to 1.21 ± 0.06 ($n = 7$) times its value in the Na^+ solution and decreased to 0.74 ± 0.04 for the change from Na^+ to Li^+ ($n = 7$). With K^+, however, after the immediate increase there was a slower decrease in the cell conductance with a time course similar to that observed after PTX removal. We then tested the onset of the effect of PTX in a solution containing 10 mM K^+. A much smaller effect of PTX was observed in the presence of external K^+ when compared with the experiments performed in K-free bath solutions. Three minutes after treatment with 2 nM PTX the chord conductance was 5.7 ± 1.3 μS ($n = 8$) with 10 mM K^+ versus 19.7 ± 2.0 μS ($n = 8$), $P < 0.001$, in the K-free solution.

3.2. Kinetics of PTX interaction with the Na,K-ATPase of Xenopus laevis

We then studied the kinetics of the association of PTX with the Na,K-pump by monitoring the whole oocyte conductance. For the *onset rate* of the PTX effect, we were faced with the problem that the effect of PTX was rather variable in amplitude as well as in onset rate, and then both parameters tended

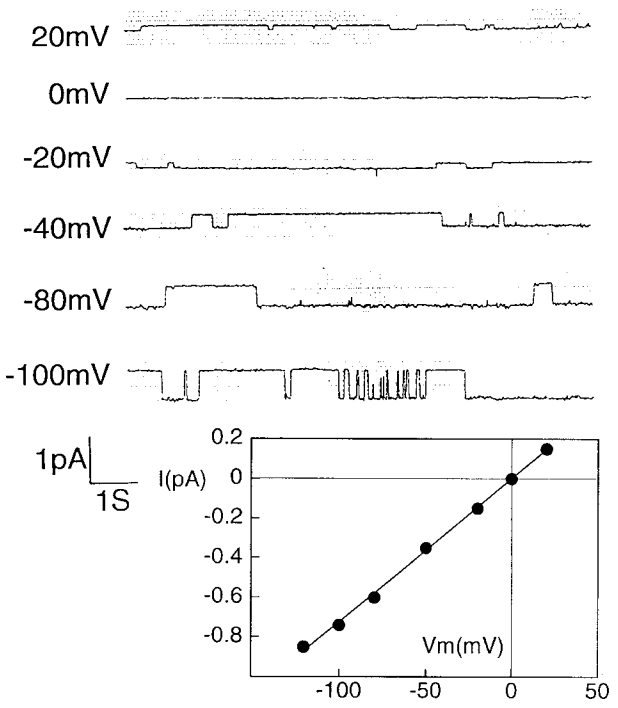

Fig. 3. Palytoxin-induced single channel current. Cell-attached patches were obtained in non-injected oocytes. The pipette solution contained 2 nM PTX. Current recordings at different holding potentials (−V pipette is indicated on the left) show the presence of a channel with slow gating and no obvious voltage dependence of the gating kinetics. The conductance obtained from this single channel *I-V* curve (lower panel) yielded a conductance of 7.3 pS.

to decrease during the course of an experiment. We attributed this problem to the instability of PTX in our solution and/or possibly to binding of PTX to various elements of the perfusion circuit [20], resulting in an uncertain concentration of active PTX in our solution. Therefore, we assumed that we could not know with precision the effective concentration of active PTX in our solutions and we renounced drawing any conclusions concerning the absolute value of the k_{on} or of the affinity. All indicated PTX concentrations should be considered as nominal.

Removal of PTX resulted in an exponential decrease of the whole-oocyte conductance with a time constant (k_{off}) of 0.46 ± 0.04 min^{-1} ($n = 10$). The conductance, however, did not generally return completely to its initial value recorded before exposure to PTX. The residual conductance can be interpreted as indicating a slow dissociation from part of the binding sites, but it seems rather to be due to secondary modifications of other conductive pathways that persisted beyond the interaction of PTX with the Na,K-pump. We also measured the rate of decrease of the PTX-induced conductance when a large concentration of ouabain (100 μM) was added to the PTX-containing solution. The k_{off} values obtained in these experiments ($k_{off} = 0.38 \pm 0.04$ min^{-1} [$n = 8$]) were not significantly different from those obtained by the removal of PTX.

3.3. Single-channel properties of PTX-induced conductance

In the absence of PTX and with a pipette potential of +100 mV in the cell-attached mode, only occasional channels (3 observations in 9 successful patches) could be observed. These channels had a short (a few ms) open time and low open probability (P_o), but no channels with open time longer than 20 ms could be observed. In contrast, in the presence of 2 nM PTX in the pipette a unique type of channel with a P_o larger than 0.3 and slow kinetics was observed in most of the successful patches (12/14). As shown in Fig. 3, the single-channel conductance was 7.1 ± 0.2 pS ($n = 12$) and there was no obvious voltage dependence in the channel gating kinetics. No channel of this type was observed when 10 μM ouabain was present in the pipette with PTX and patches were obtained on non-injected oocytes ($n = 6$). In contrast, we could observe the same channel activity with ouabain and PTX in the pipette in patches obtained on oocytes injected with α1 and β1 cRNA of the *Bufo* Na,K-pump. The single-channel conductance (7.4 ± 0.2 pS, $n = 7$) and gating kinetics of the channel observed with the *Bufo* Na,K-pump were not obviously different from that observed with the *Xenopus* Na,K-pump.

3.4. Channel induction and Na,K-pump inhibition by PTX

Palytoxin has been shown to inhibit the Na,K-pump as well as to induce a cation conductance [6,21]. To evaluate the relationship between these two effects of PTX, we attempted to measure simultaneously the Na,K-pump activity and the PTX-induced conductance as shown in Fig. 4. While PTX induced a large conductance, the Na,K-pump current measured after PTX was only very slightly decreased when compared to that measured before PTX treatment (160 ± 11 nA before PTX versus 146 ± 10 nA after PTX, $n = 11$, $P = 0.03$, Student's *t*-test for paired data).

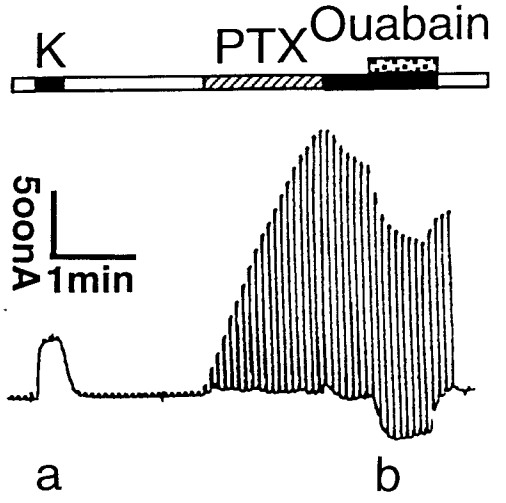

Fig. 4. PTX-induced conductance and Na,K-pump inhibition. Original current recording obtained with an oocyte overexpressing the α and β subunits of the *Xenopus* Na,K-pump. A holding potential close to the reversal potential of the PTX-induced conductance was used in order to minimize holding current. The Na,K-pump current was measured first as the current activated by 10 mM potassium (K, about 380 nA, upward deflection [a]). 2 nM palytoxin (PTX) was then added until a large conductance was induced (about 400 μS after 90 s) as shown by the current deflections due to 5 mV voltage steps. Then, in the presence of K^+ (black bar), the Na,K-pump current was measured as the decrease in current induced by 100 μM ouabain (370 nA, downward deflection [b]). We have shown earlier that the K-induced current and the ouabain-sensitive current are equivalent measures of Na,K-pump activity [11].

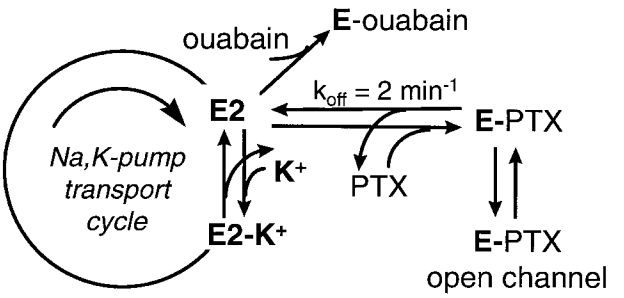

Fig. 5. A model of interaction of PTX with Na,K-ATPase.

4. Discussion

Our results provide an independent confirmation of the fact that the conductance-inducing effect of PTX on the Na,K-pump is due to a direct interaction of PTX with the Na,K-pump. The effect of PTX depends on the presence of functional Na,K-pumps at the cell membrane but not on the pump activity: the effect is present when the Na,K-pump is functional but inactive, as observed in the absence of external K^+. When the Na,K-pump is inhibited by ouabain, the effect is absent and it is also absent or much reduced when the pump is fully activated by a high external K^+ concentration. This apparent competition between PTX and K^+ has been reported by others [1]. Therefore the conductance-inducing effect cannot be explained by a secondary effect of the inhibition of the Na,K-pump and must be due to direct interaction of PTX with the Na,K-pump. This conclusion is further supported by the observation that PTX sensitivity can be restored, in the presence of ouabain, by expression of a ouabain-resistant Na,K-pump form.

All the apparently complex interactions between PTX and ouabain and PTX and K^+ can be explained by the simple mechanism illustrated in the kinetic scheme of Fig. 5. Although it may be incomplete, this model is sufficient to explain all the data that we have observed. PTX creates a cation channel by binding to a specific state of the Na,K-pump, which seems to be the E2 conformation or a substate of the E2 conformation. PTX binding does not occur, or is much slower, when ouabain is first bound, or when the enzyme is driven away from the E2 state by a high external K^+ for instance. Removing PTX from the solution, adding ouabain, or adding potassium results in a decline of PTX conductance with a similar time course, because the rate of this decline is determined by the dissociation of PTX. In the first case the binding rate is zero because PTX is removed, in the two other cases the rebinding of PTX is prevented because the Na,K-pump is immediately driven, by K^+ or ouabain, into a state in which it cannot bind PTX.

The interaction of K^+ with the PTX-treated Na,K-pump is complex. First, K^+ is a conductive ion for the PTX-induced channel yielding a current that depends on the K^+ concentration on both sides of the membrane and the membrane potential. Second, K^+ is also a competitive inhibitor of PTX binding due to its effect on the conformation state of the Na,K-pump. Third, K^+ is also an activator of electrogenic Na,K-pump transport activity as the substrate for the part of the Na,K-pump that has not been inhibited by PTX. These points have to be carefully considered when the effect of K^+ on the PTX-treated Na,K-pump is to be interpreted.

4.1. Relationship between channel induction and Na,K-pump inhibition by palytoxin

The number of active Na,K-pump units at the surface of one *Xenopus* oocyte is about $5–10\times10^9$ in a non-injected oocyte and can be increased to about 50×10^9 after overexpression of exogenous Na,K-ATPase [11]. From the patch-clamp measurement, the single-channel conductance was about 7 pS. On the other hand, the PTX-induced conductance that we observed was of the order of 20–100 μS. A conductance of this size can be produced by about $3–10\times10^6$ open PTX-induced channels. With a single-channel open probability close to 0.5, the observed macroscopic PTX-induced conductance could result from the transformation into channels of 'only' $5–20\times10^6$ Na,K-pump (i.e., less than 1 in 1000 of the active Na,K-pump units at the oocyte surface). Obviously, a much larger proportion of Na,K-pump occupancy by PTX would be needed to produce a sizable inhibition. Indeed, we observed only a small inhibition (less than 10%) of the K-activated Na,K-pump current when the experiments were performed in the presence of concentrations of PTX that induced a large conductance. These results may explain the large difference in the apparent affinity that is observed when the effects secondary to the conductance increase or Na,K-ATPase inhibition are measured [1,17]. Estimation of affinity using an effect such as membrane depolarization may be misleading because occupancy of an extremely low fraction of the potential binding sites by PTX may lead to full depolarization, an apparently maximal effect.

Even though there is a 3-orders-of-magnitude difference in the affinity of ouabain for *Bufo* and *Xenopus* Na,K-ATPase, there was no obvious difference in the effect of PTX on the Na,K-pump of these two species; however, we cannot exclude that small differences exist because of uncertainty in the effective concentration of PTX under our experimental conditions. This indicates that although there is obviously competition between PTX and ouabain for binding to the Na,K-pump, the binding mechanism or binding site may actually be different.

In summary, our results show that PTX acts on the amphibian Na,K-pump in a manner very similar to that observed with other types of Na,K-pumps and that in spite of a very large difference in ouabain affinity, there is no obvious difference between the *Bufo* and *Xenopus* Na,K-pump with regard to the PTX effect. In addition, we have defined the kinetics of the binding of PTX to the Na,K-pump in this system and propose a simple model that explains the interactions between PTX and ouabain or potassium. Our results are entirely compatible with the hypothesis put forward by Rodendo et al. [16] that PTX induces conductance by interacting directly with the Na,K-pump and opens, through the Na,K-pump, a pathway that may share some common structure with the pathway of Na^+ and K^+ when these ions are carried across the membrane by the normal transport cycle of Na,K-ATPase. Along this working hypothesis, it will be possible to use PTX as a tool to study the ion pathway through the Na,K-pump by observing the effects of PTX on isoforms or mutant forms of Na,K-ATPase artificially expressed in the *Xenopus* oocyte.

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References

[1] Bottinger, H., Beress, L., Habermann, E., Biochim. Biophys. Acta 861 (1986) 165–176.
[2] Canessa, C.M., Horisberger, J.-D., Louvard, D., Rossier, B.C., EMBO J. 11 (1992) 1681–1687.
[3] Castle, N.A., Strichartz, G.R., Toxicon 26 (1988) 941–951.
[4] Frelin, C., Vanrenterghem, C., Gen. Pharmacol. 26 (1995) 33–37.
[5] Frelin, C., Vigne, P., Breittmayer, J.P., Mol. Pharmacol. 38 (1990) 904–909.
[6] Habermann, E., Toxicon 27 (1989) 1171–1187.
[7] Habermann, E., Hudel, M., Dauzenroth, M.E., Toxicon 27 (1989) 419–430.
[8] Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., Pflügers Arch. 391 (1981) 85–100.
[9] Horisberger, J.-D., Jaunin, P., Good, P.J., Rossier, B.C., Geering, K., Proc. Natl. Acad. Sci. USA 88 (1991) 8397–8400.
[10] Jaisser, F., Canessa, C.M., Horisberger, J.-D., Rossier, B.C., J. Biol. Chem. 267 (1992) 16895–16903.
[11] Jaunin, P., Horisberger, J.-D., Richter, K., Good, P.J., Rossier, B.C., Geering, K., J. Biol. Chem. 267 (1992) 577–585.
[12] Kim, S.Y., Marx, K.A., Wu, C.H., Naunyn-Schmiedeberg's Arch. Pharmacol. 351 (1995) 542–554.
[13] Monroe, J.J., Tashjian, A.H., Am. J. Physiol. Cell Physiol. 38 (1995) C582–C589.
[14] Monroe, J.J., Tashjian, A.H., Am. J. Physiol. Cell Physiol. 39 (1996) C1277–C1283.
[15] Muramatsu, I., Nishio, M., Kigoshi, S., Uemura, D., Br. J. Pharmacol. 93 (1988) 811–816.
[16] Redondo, J., Fiedler, B., Scheiner-Bobis, G., Mol. Pharmacol. 49 (1996) 49–57.
[17] Rouzaire Dubois, B., Dubois, J.M., Toxicon 28 (1990) 1147–1158.
[18] Scheiner-Bobis, G., Meyer zu Heringdorf, D., Christ, M., Habermann, E., Mol. Pharmacol. 45 (1994) 1132–1136.
[19] Sekler, I., Remis, D., Gimmler, H., Pick, U., Biochim. Biophys. Acta 1142 (1993) 88–92.
[20] Taylor, T.J., Parker, G.W., Fajer, A.B., Mereish, K.A., Toxicol. Lett. 57 (1991) 291–296.
[21] Tosteson, M.T., Halperin, J.A., Kishi, Y., Tosteson, D.C.,J. Gen. Physiol. 98 (1991) 969–985.
[22] Wang, X., Jaisser, F., Horisberger, J.-D., J. Physiol. (London) 491 (1996) 579–594.