Biochimica et Biophysica Acta 818 (1985) 55–60
Elsevier

BBA 72671

Palytoxin-induced permeability changes in excitable membranes

Leander Lauffer *, Siegfried Stengelin, Laszlo Béress ** and Ferdinand Hucho ***

Institut für Biochemie, Arbeitsgruppe Neurochemie, Freie Universität Berlin, Thielallee 63, 1000 Berlin 33 (Germany)

(Received February 12th, 1985)

Key words: Membrane-toxin interaction; Palytoxin, Excitable membrane; Membrane permeability; Acetylcholine receptor

Palytoxin, a toxin isolated from the Caribean corrall *Palythoa caribaeorum*, increases the cation permeability of excitable membranes in vitro. Three membrane systems have been investigated: axonal membranes from crayfish walking leg nerves, membranes rich in nicotinic acetylcholine receptor isolated from *Torpedo californica* electric tissue and, for control, artificial liposomes. Ion permeability of the latter was not affected by palytoxin, but with both biological membranes an increase in cation permeability was observed at a palytoxin concentration of 0.14 μM. Palytoxin-induced cation flow through the axonal membrane was not inhibited by tetrodotoxin, indicating that the voltage-dependent sodium channels were not involved. The effect of palytoxin on the receptor-rich membranes was not blocked by α-bungarotoxin, a competitive antagonist of the nicotinic acetylcholine receptor, nor by triphenylmethylphosphonium, a blocker of the receptor-ion channel. But with both the axonal and the receptor-rich membranes ouabain was an inhibitor of the palytoxin-induced cation flow. Evidence is presented that it is not the $(Na^+ + K^+)$-ATPase which is affected by palytoxin as has been postulated for similar observations with non-neuronal membranes (Chhatwal, G.S., Hessler, H.-J. and Habermann, E. (1983) Naunyn-Schmiedeberg's Arch. Pharmacol. 323, 261–268).

Introduction

Palytoxin is a product from marine coelenterates of the genus *Palythoa* and belongs to the most poisonous animal toxins. With frog myelinated fibres [1] as well as with unmyelinated fibres [2] it causes depolarization by increasing the resting sodium conductance. Depolarization and contraction of cardiac, smooth and striated muscle fibres after palytoxin treatment has been reported (for references see Ref. 3).

Nonexcitable cells too are affected by palytoxin. In particular, it has been shown that palytoxin induces loss of K^+ from erythrocytes followed by haemolysis [4]. This effect could be blocked by the heart glycoside ouabain and it has been proposed that palytoxin acts by transforming the Na^+-, K^+-dependent ATPase ($(Na^+ + K^+)$-ATPase) into a pore allowing the penetration of small ions [5]. Recently, an inhibitory action of palytoxin on $(Na^+ + K^+)$-ATPases of various origins has been described [6,7]. To elucidate the molecular basis underlying the reported effects of palytoxin on excitable cells, we studied the palytoxin-induced cation permeability of membrane preparations of

* Present address: University of California, Department of Biochemistry and Biophysics, Medical School, San Francisco, CA 94143, U.S.A.
** Present address: Institut für Meereskunde an der Universität Kiel, Düsternbrooker Weg 20, 2300 Kiel, F.R.G.
*** To whom correspondence should be addressed.

(i) crayfish walking leg nerves and (ii) Torpedo electric organ; (i) being axonal and (ii) being postsynaptic material. Furthermore, we investigated the effect of palytoxin on the cation-permeability of artificial liposomes.

Materials and Methods

Palytoxin from *Palythoa caribaeorum* was prepared as described in Ref. 8. ^{22}NaCl (200 μCi/ml) was from New England Nuclear, Dreieich, F.R.G. α-Bungarotoxin, gramicidine, methyltriphenylphosphonium, ouabain, tetrodotoxin and phospholipids were obtained from Sigma, München, F.R.G. *Astacus leptodactylus* was purchased locally and *Torpedo californa* came from Pacific Bio-Marine Supply, Venice, CA, U.S.A.

Preparations of axonal membrane fragments from Astacus leptodactylus walking leg nerves and preparation of electroplaque membrane fragments from Torpedo californica. The methods are described in Refs. 9 and 10, respectively.

Preparation of artificial liposomes. The procedure is described in detail in Ref. 10 and is an adaptation of the method developed by Brunner et al. [11]. The composition of the lipids used was the same as that found in a biological membrane [10], i.e., in acetylcholine receptor-rich membranes from *T. californica* electric tissue. A mixture of 2.62 mg phosphatidylcholine/1.76 mg phosphatidylethanolamine/0.80 mg phosphatidylserine/0.40 mg sphingomyelin/0.11 mg phosphatidylinositol/0.85 mg cholesterol is dissolved in 1.5% sodium cholate. After stirring for 30 min at room temperature, the detergent is removed by gel filtration on a column (1.7 cm × 25 cm) of Sephadex G-50. During filtration, liposomes are formed and eluted approximately with the void volume. When Li^+ efflux (see below) is to be measured, the lipids are suspended in Tris-buffer (10 mM, pH 7.4)/100 mM NaCl/300 mM LiCl/1.5 sodium cholate.

$^{22}Na^+$ efflux from membrane fragments. A detailed description can be found in Ref. 10. In general with *Astacus* and *Torpedo* membrane vesicles, small aliquots (50–100 μl) of a concentrated membrane suspension in Tris-buffered (10 mM, pH 7.4) Ringer's solution (160 mM NaCl/5 mM KCL/2 mM $CaCl_2$) preincubated with 20–50 μCi/ml $^{22}Na^+$ are diluted 50-fold in a medium which is isoosmotic but contains no radioactive Na^+. After dilution, 1–2 ml are filtered at distinct time intervals through membrane filters (Schleicher and Schüll, type BA 85) and the radioactivity remaining on the filters is determined with a liquid scintillation counter.

Li^+ efflux from membrane vesicles. Torpedo membranes are incubated in Tris-buffered Ringer's solution containing additionally 200–400 mM LiCl and are diluted 50-fold isoosmotically in a medium containing NaCl instead of LiCl. Aliquots are filtered at distinct time intervals through membrane filters (see above); after filtration the filters are washed overnight with 2 ml 1% Triton X-100 in H_2O and the Li^+ content of the wash solution is determined by flame emission spectroscopy in an atomic absorption spectrophotometer (Varian type AA-275). With artificial liposomes, larger aliquots (500–1000 μl) are diluted and correspondingly larger aliquots are filtered in order to retain enough Li^+ on the filters to be measured by flame emission spectroscopy.

$(Na^+ + K^+)$-ATPase activity. Activity was determined according to Bonting et al. [12], as the fraction of total ATPase activity which can be inhibited by ouabain (10^{-4} M); inorganic phosphate released from ATP was measured in a turbidometric assay developed by Eibl and Lands [13].

Results

Palytoxin and the $^{22}Na^+$ permeability of crayfish nerve membrane vesicles

Nerve membrane vesicles from *A. leptodactylus* walking leg nerves were prepared according to a method developed by Denburg [9]. After differential centrifugation of a hypotonic extract of nervous tissue, axonal and other vesiculated membrane fragments are obtained. Palytoxin induces increased $^{22}Na^+$ efflux from these vesicles. This effect is not inhibited by tetrodotoxin, a specific blocker of the voltage-dependent sodium channel (Fig. 1).

Palytoxin and the cation permeability of membrane vesicles from T. californica electric organ

Acetylcholine receptor-rich membrane fragments from electric organs of the electric rays

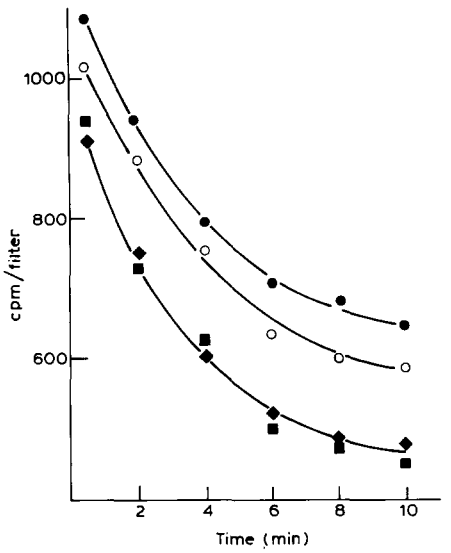

Fig. 1. $^{22}Na^+$ efflux from membrane vesicles of *A. leptodactylus* walking leg nerves in the presence of palytoxin and tetrodotoxin. Protein content of membranes was 4.2 mg/ml. Prior to dilution, aliquots were incubated 30 min at 20°C with 140 nM tetrodotoxin (●——●), 140 nM palytoxin (■——■) or 140 nM of both palytoxin and tetrodotoxin (◆——◆). ○——○, Control, no addition of toxins.

Torpedo spec. are widely used for investigations of the biochemical basis of neuromuscular transmission. For the following experiments, we used vesicle fractions rich in acetylcholine receptor and another fraction rich in $(Na^+ + K^+)$-ATPase activity, both from the *T. californica* electric organ.

The last step in the purification of acetylcholine receptor-rich membrane vesicles according to Ref. 10 is the sedimentation of membrane vesicles in a sucrose gradient. Acetylcholine receptor-rich membrane vesicles sediment in a region of approx. 40%

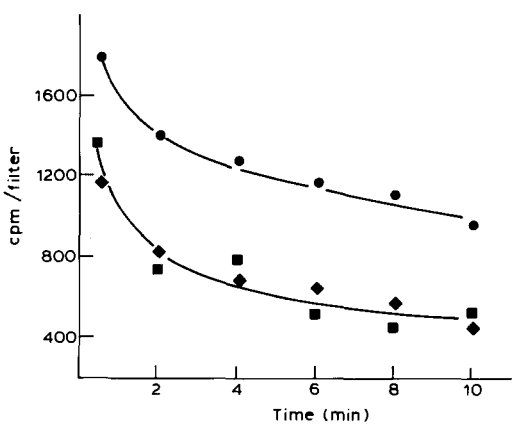

Fig. 2. $^{22}Na^+$ efflux from acetylcholine receptor-rich membrane vesicles in the presence of palytoxin. Protein content of membrane vesicles was 7.2 mg/ml. Prior to dilution, aliquots were incubated 30 min at 20°C with 140 nM palytoxin (■——■) or 140 nM palytoxin together with 180 μM α-bungarotoxin (◆——◆). ●——●, Control, no addition of toxins.

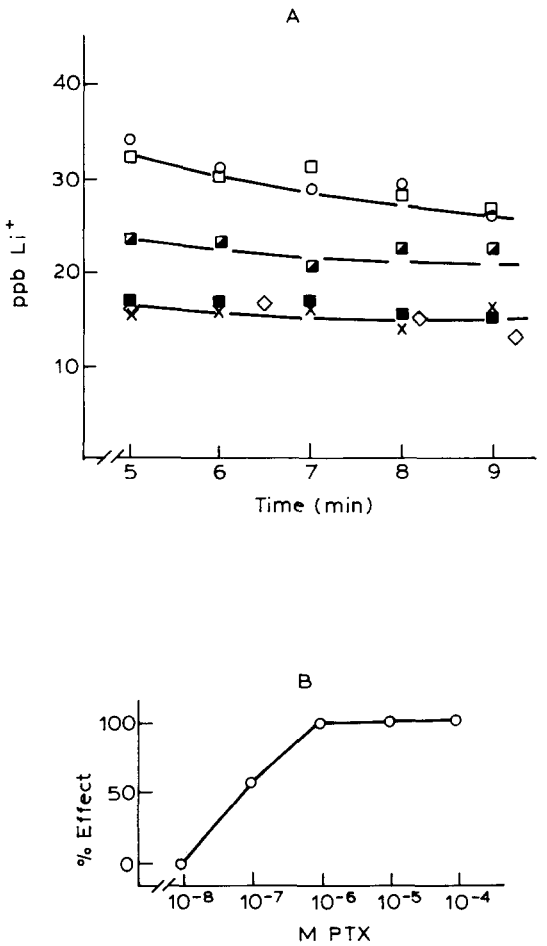

Fig. 3. Li^+ efflux from acetylcholine receptor-rich membrane vesicles in the presence of palytoxin. (A) Direct representation of the data. Li^+ efflux was measured as described in Materials and Methods. Protein content of membrane vesicle suspension was 4 mg/ml. Prior to dilution, aliquots were incubated 30 min at 20°C with palytoxin at concentrations of 10^{-8} M (□——□), 10^{-7} M (□——□), 10^{-6} M (■——■), 10^{-5} M (×——×) and 10^{-4} M (◇——◇). ○——○, Control, no addition of palytoxin. (B) Dose-response curve for the action of palytoxin (PTX) as taken from data shown in (A). For this representation, the amplitudes of the observed Li^+ fluxes were taken (mean of the measured values; the values for 10^{-6} M palytoxin were taken as 100% response).

sucrose, whereas vesicles containing high activities of acetylcholine esterase and/or $(Na^+ + K^+)$-ATPase are of much lower density and thus can be separated [14]. The effect of palytoxin on the cation permeability of both these membrane fractions was investigated: palytoxin enhances $^{22}Na^+$ permeability of acetylcholine receptor-rich membrane vesicles. The potent cholinergic antagonist α-bungarotoxin is unable to block this effect (Fig. 2). This excludes the possibility that palytoxin interacts with the cholinergic binding site, thereby opening the ion channel coupled to it (acting as a cholinergic agonist).

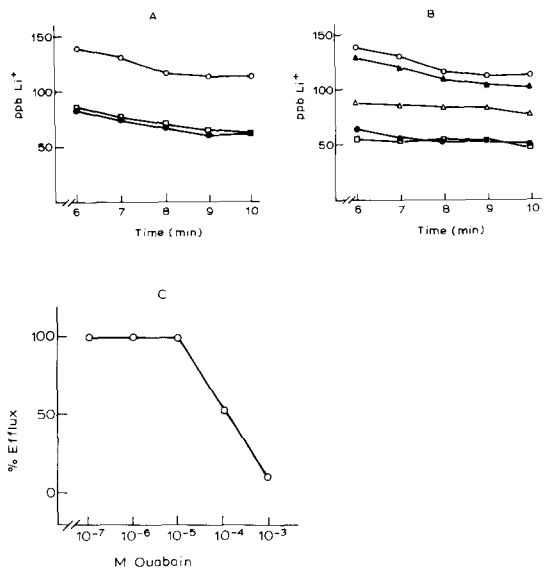

Fig. 4. Li^+ efflux from acetylcholine receptor-rich membrane vesicles. Protein content of membrane vesicles was 6 mg/ml. (A): (○———○) Control, leakage of Li^+ in absence of effectors; (●———●) efflux in the presence of 10^{-5} M carbamoylcholine, and (■———■) membranes pretreated with 10^{-4} M ouabain; efflux in the presence of 10^{-5} M carbamoylcholine. (B) (○———○) Control; (□———□) membranes preincubated with 10^{-6} M palytoxin; (●———●) membranes preincubated with 10^{-6} M palytoxin and 10^{-4} M $TPMP^+$; (△———△) membranes preincubated with 10^{-6} M palytoxin and 10^{-4} ouabain; (▲———▲) membrane preincubated with 10^{-6} M palytoxin and 10^{-3} M ouabain. (C) Dose-response curve for the inhibition of the palytoxin-induced Li^+ efflux by ouabain. The data were compiled from (B) and additional experiments as described in the legend to (B). 0% efflux was determined in the absence of effectors; 100% efflux was taken as mean of the values in the presence of 10^{-6} M palytoxin. The concentration of ouabain was varied in the presence of 10^{-6} M palytoxin.

In similar experiments, we could show that the Li^+ permeability of these vesicles is increased as well by palytoxin, though higher concentrations seem to be required for maximal effect (Fig. 3). The amplitude of the palytoxin-induced Li^+ efflux from these preparations usually is equal to or slightly larger than the one obtained after chemically exciting the acetylcholine receptor complex with the cholinergic agonist carbamoylcholine (cf. Fig. 4). However, the following experiments indicate that palytoxin acts by a mechanism completely unrelated to the acetylcholine receptor-function. Above, we have shown that palytoxin is not interacting with the cholinergic binding site. In previous experiments, we found that the lipophilic cation methyltriphenylphosphonium ($TPMP^+$) is a noncompetitive inhibitor of the acetylcholine receptor and blocks the receptor-regulated ion channel [15]. $TPMP^+$, however, as α-bungarotoxin, is unable to inhibit the effects of palytoxin. The glycoside ouabain on the other hand inhibits

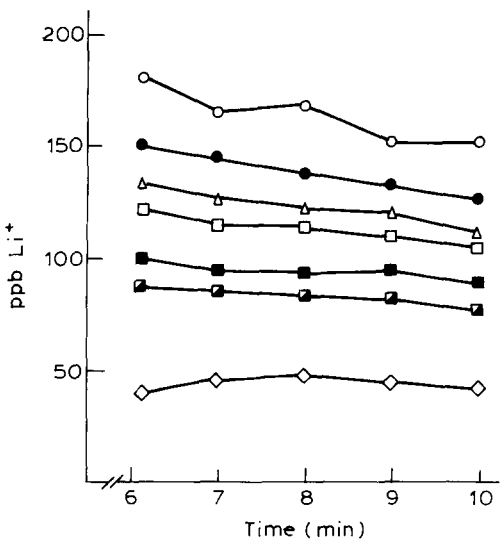

Fig. 5. Li^+ efflux from membranes rich in $(Na^+ + K^+)$-ATPase activity. Protein content of membranes was 3.0 mg/ml. (○———○) Control; (●———●) efflux in the presence of 10^{-4} M carbamoylcholine; (□———□) membranes preincubated with $5 \cdot 10^{-6}$ M palytoxin; (■———■) membranes preincubated with $5 \cdot 10^{-5}$ M palytoxin; (□———□) membranes preincubated with $5 \cdot 10^{-7}$ M palytoxin; (△———△) membranes preincubated with $5 \cdot 10^{-6}$ M palytoxin and 10^{-3} M ouabain; (◇———◇) 'efflux' induced by osmotic shock (dilution of the membrane suspension in distilled water).

palytoxin-induced Li^+ efflux significantly, without affecting carbamoylcholine-stimulated efflux (Fig. 4).

Using $(Na^+ + K^+)$-ATPase-rich membranes, a similar action of palytoxin is observed (Fig. 5) in accordance with Ref. 5. In some of these preparations, as in the one used for the experiment in Fig. 5, a slight carbamoylcholine-stimulated Li^+ efflux is observed, apparently because of a small content of acetylcholine-rich membranes. However, the palytoxin-induced efflux is much more pronounced and is at least partially inhibited by ouabain.

Palytoxin and the Li^+ permeability of artificial liposomes

Not all vesicles in our preparations which can be osmotically shocked are depleted of Li^+ even by high concentrations (0.2 μM) of palytoxin (cf. Fig. 5). One therefore has to assume a component mediating the action of palytoxin which is present only in part of the vesicle population. This component probably is a protein, since palytoxin does not affect the Li^+ permeability of artificial liposomes containing no membrane proteins, whereas in control experiments a large amount of trapped Li^+ is set free by the antibiotic gramicidin (a cation ionophore) or by osmotic shock. A typical experiment is shown in Fig. 6.

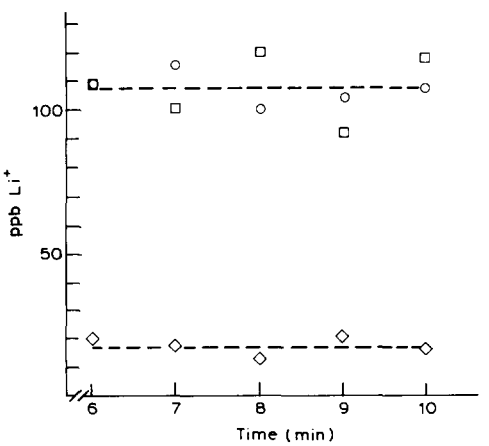

Fig. 6. Li^+ efflux from artificial liposomes. (○——○) Control; (□——□) prior to dilution, liposomes were incubated 30 min at 20°C with 200 nM palytoxin or (◇——◇) with 200 μg/ml gramicidin. Broken lines indicate the arithmetic mean of the Li^+ determinations (amounts to the same value in the control curve and the palytoxin curve).

Discussion

Palytoxin increases the cation permeability of biological membranes such as crayfish nerve membrane fragments of membrane fragments from the electric organ of *T. californica*. In previous work [5] it has been shown that palytoxin causes leakiness of $(Na^+ + K^+)$-ATPase containing membranes, e.g., of erythrocytes, and since in our experiments the palytoxin-induced permeability increase was inhibited by ouabain, a similar effect was considered. But we found that membranes rich in acetylcholine receptor and virtually devoid of ATPase also became permeable to cations in the presence of palytoxin and this permeability too was blocked by ouabain. A mechanism different from the one postulated in Ref. 5 therefore has to be postulated. Several model membranes have been investigated. In the case of the axonal membranes, the observed palytoxin-induced cation flow is not through voltage-dependent sodium channels, because tetrodotoxin does not inhibit it. In the case of the receptor-rich membranes from *Torpedo* electric tissue, palytoxin did not just activate the acetylcholine receptor, because the antagonist α-bungarotoxin had no effect on the cation flow induced by palytoxin. The cations did not flow through the ion channel of the receptor, because the flow could not be inhibited by the proven channel blocker $TPMP^+$. Finally, palytoxin does not form simply holes in the lipid phase of membranes, because artificial liposomes containing no protein did not become leaky in the presence of palytoxin.

At present, we do not have an explanation why in our experiments the permeability increase caused by palytoxin could be blocked by ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase. As mentioned above, similar effects of ouabain with respect to the action of palytoxin have been observed with other cellular and membrane preparations. These results led to the suggestion that palytoxin might act by transforming the $(Na^+ + K^+)$ATPase into a pore which allows the penetration of small ions (as well as sufficiently small uncharged compounds) [5]. Other observations support this notion: palytoxin inhibits the interaction of [^{3}H]ouabain with its binding sites [7,16], and dog erythrocytes, which lack $(Na^+ + K^+)$ATPase, are relatively re-

sistant against palytoxin [16].

At first sight, our results also indicate involvement of the $(Na^+ + K^+)$ATPase in the action of palytoxin. Of all the agents investigated for their inhibition of palytoxin effects, not the blockers of physiological ionophores like the voltage-dependent sodium channel or the acetylcholine-regulated ion channel but only ouabain was found to prevent the permeability increase induced by palytoxin. No effect of palytoxin on artificial lipid bilayers was observed, though it has been reported that palytoxin can bind to these structures [17]. However, in our hands, there is no positive correlation between $(Na^+ + K^+)$ATPase activity in a membrane preparation and the magnitude of the palytoxin permeability increase. With both acetylcholine receptor-rich membranes and membranes enriched in $(Na^+ + K^+)$ATPase activity, palytoxin induces efflux of Li^+, though the former membrane preparation has only very little $(Na^+ + K^+)$ATPase activity.

Either the Li^+ permeability change induced by palytoxin in the acetylcholine receptor-rich membranes is independent of $(Na^+ + K^+)$ATPase and is blocked by ouabain by an unknown mechanism, or there are very few ATPase molecules interdispersed in the receptor membranes. According to this latter hypothesis, these few ATPase molecules would have to be sufficient to deplete the acetylcholine receptor-rich membrane vesicles of Li^+ after interaction with palytoxin. They also would have to be responsible for the ouabain effect. We consider this unlikely, because complete separation of acetylcholine receptor-rich and ATPase-containing membranes by countercurrent distribution has been achieved [18], indicating that the two activities (acetylcholine receptor and ATPase) are truely located on different vesicular membranes. We therefore hesitate to ascribe the palytoxin-stimulated cation efflux from acetylcholine receptor-rich vesicles and its inhibition by ouabain to the presence of ATPase. At present we cannot propose a mechanism for the palytoxin effect and its inhibition by ouabain.

Acknowledgements

Expert technical assistance of J. Zwick is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

References

1 Dubois, J.M. and Cohen, J.B. (1977) J. Pharm. Exp. Ther. 201, 148–155
2 Pichon, Y. (1982) Toxicon 20, 41–47
3 Béress, L. (1983) in Toxins as Tools in Neurochemistry (Hucho, F. and Ovchinnikov, Y.A., eds.), pp. 83–89, Walter de Gruyter, Berlin
4 Habermann, E., Ahnert-Hilger, G., Chhatwal, G.S. and Béress, L. (1981) Biochim. Biophys. Acta 649, 481–486
5 Chhatwal, G.S., Hessler, H.-J. and Habermann, E. (1983) Naunyn-Schmiedeberg's Arch. Pharmacol. 323, 261–268
6 Ishida, Y., Takagi, K., Takahashi, M., Satake, N. and Shibata, S. (1983) J. Biol. Chem. 258, 7900–7902
7 Böttinger, H. and Habermann, E. (1984) Naunyn-Schmiedeberg's Arch. Pharmacol. 325, 85–87
8 Béress, L., Zwick, J., Kolkenbrock, H.J., Kaul, P.N. and Wassermann, O. (1983) Toxicon 21, 285–290
9 Denburg, J.L. (1972) Biochim. Biophys. Acta 282, 453–458
10 Schiebler, W. and Hucho, F. (1978) Eur. J. Biochem. 85, 55–63
11 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 688, 486–494
12 Bonting, S.L., Simon, K.A. and Hawkins, N.M. (1961) Arch. Biochem. Biophys. 95, 416–423
13 Eibl, H. and Lands, W.E. (1969) Anal. Biochem. 30, 51–57
14 Duguid, J.R. and Raftery, M.A. (1973) Biochemistry 12, 3593–3597
15 Lauffer, L. and Hucho, F. (1982) Proc. Natl. Acad. Sci. USA 79, 2406–2409
16 Habermann, E. and Chhatwal, G.S. (1982) Naunyn-Schmiederberg's Arch. Pharmacol. 319, 101–107
17 Habermann, E. (1983) Naunyn-Schmiederberg's Arch. Pharmacol. 323, 269–275
18 Johansson, G., Gysin, R. and Flanagan, S.D. (1980) J. Biol. Chem. 256, 9126–9135