ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 345–354

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

# Comparison of electrochemical detection of acetylcholine-induced nitric oxide release (NO) and contractile force measurement of rabbit isolated carotid artery endothelium

Emrah Kilinc [a], Gunay Yetik [b], Tayfun Dalbasti [c], Mehmet Ozsoz [a,*]

[a] *Analytical Chemistry Department, Faculty of Pharmacy, Ege University, 35100 Bornova, Izmir, Turkey*
[b] *Pharmacology Department, Faculty of Pharmacy, Ege University, 35100 Bornova, Izmir, Turkey*
[c] *Neurosurgery Department, School of Medicine, Ege University, 35100 Bornova, Izmir, Turkey*

Received 3 August 2001; received in revised form 19 September 2001; accepted 29 September 2001

**Abstract**

Since the identification of nitric oxide (NO) as an endothelial-derived relaxing factor, it became very important to quantify NO in biological models eventhough it is present in very low concentrations with a very short half-life. The use of electrochemistry as an alternative detection method is quite promising and electrochemical probes are now being developed to detect NO. This paper consists of an amperometric, bi-polymer modified, platinum–iridium microelectrode (Pt 90%–Ir 10% alloy, multistranded, total diameter 130 μm) design and its application for NO detection in acetylcholine (Ach) introduced, rabbit isolated carotid artery endothelium model. In a pH range of 3.0–10.0. pH 3.0 was found to be the optimum pH. As the pH values increased up to 10.0, the response current decreased as the oxidation of NO is catalyzed by $H^+$ in the acidic media. Temperature effect was checked at 25 °C (room temperature), 30 and 40 °C. An increasing trend was observed in sensor response with the increasing temperature. Most common biological interferences as ascorbic acid, uric acid and glucose were eliminated via bi-polymer coatings of four layers of Nafion and a layer of 50 mM *o*-phenylenediamine (OPD). When S/N ratio was accepted as 3, limit of detection was calculated as 15 nM. NO release from carotid artery endothelium was also determined by measuring response force in thermostatic isolated organel baths. Obtained force responses (mg) were compared with the electrochemical (nA) sensor responses. 

*Keywords:* Electrochemistry; Nitric oxide; Acetylcholine; Carotid artery; Endothelium

## 1. Introduction

In 1980, Furchgott and Zawadski [1] for the first time identified the endothelium-derived relaxing factor, later identified as nitric oxide (NO) [2,3]. NO plays an important role in many physio-

* Corresponding author. Tel.: +90-232-388-4000/1353; fax: +90-232-388-5258.
*E-mail address:* ozsozs@eczacilik.ege.edu.tr (M. Ozsoz).

0731-7085/02/$ - see front matter 
PII: S0731-7085(01)00621-5

logical and pathophysiological processes that have been previously reviewed [4–9].

Because of its importance, new accurate and specific methods for nM NO determination in biological systems are being developed [10–14].

The desire to measure small amounts of NO release in situ has created an active field of research involving the design of microelectrochemical sensors. The use of electrochemistry as an alternative detection method is very promising and electrochemical probes are now being developed for in situ NO detection. In such probes a case to highlight is the need for the modification of the electrode surface for making the electrode material more selective for NO. Various polymers and transition metals have been used for the surface modification. Shortly after its characterization by Malinski et al. [15], nickel-porphyrine became one of the most common catalysts used [16–25]. The usage of iridium, as alloys in platinum, for surface modification has also been reported by Ichimori et al. and the prepared electrode's been electrochemically characterized [26]. Nafion and *o*-phenylenediamine, alone or with some co-polymers, have also been used in surface modification against some common interferences [13,27–30].

In our study we designed an amperometric bi-polymer (Nafion and OPD) coated microprobe for in situ quantification of NO in Acetylcholine induced, rabbit isolated carotid artery endothelium model. Current responses were obtained via successive Ach injections into the 10 ml electrochemical cell. Obtained current responses (nA) were compared with that of contractile force responses (mg) obtained in isolated organel baths via Ach increments of the same concentration. The aim of our study was to employ two different measurement techniques on the same sample for the comparison of the data. To our best knowledge such an approach of data on the same sample with two different measurement techniques has not been recorded yet. Thus the concept and the comparison of the two sets of results will be a valuable contribution to the literature we believe. Other studied experimental parameters were the effect of pH, temperature on sensor response, elimination of biological interferences and limit of detection.

## 2. Experimental

### 2.1. Apparatus

Electrocemical detection was performed with a three-electrode system. A multistranded Pt–Ir alloy wire (Pt 90%–Ir 10%, 130 μm total diameter) served as the working electrode after modifications. Ag–AgCl electrode (Model RE-1, BAS) was used as the reference and an ordinary platinum wire (1 mm id) as the auxiliary electrode. All electrochemical processes and in vitro determinations were carried out in an electrochemical cell (10 ml Fig. 1) with a magnetic stirrer providing 400 rpm convective transport at room temperature. The potentiostats used were: EG&G PAR Model (Princeton Applied Research) 264 A Voltammetric Analyzer and 626 Polarecord Metrohm Voltammetric Analyzer/Recorder. An Orion 290 A pH-meter was used in buffer preparations.

### 2.2. Chemicals

A NO saturated solution was obtained by bubbling NO gas through deoxygenated 0.1 M phosphate buffer for 30 min, having a value of $\sim 2$ mM (1.9 mmol/l) concentration at saturation [31]. A series of standart NO solutions were prepared by diluting the NO saturated solution and were used in sensor calibration. $K_2HPO_4$ and $KH_2PO_4$ were purchased from Sigma. Phosphate buffer (0.1 M, pH 7.4) was prepared daily via bi-distilled water. Glucose, ascorbic acid, uric acid, acetylcholine and *o*-phenylenediamine(1,2-diaminobenzene) were obtained from Sigma. Teflon-insulated, multistranded Pt–Ir alloy wire (total diameter 130 μm) (10% Ir–90% Pt) was purchased from Medwire®. Ninety-five to ninety-eight percent (w/w) $H_2SO_4$ stock solution and $NaNO_2$ were obtained Merck and used without extra purification. Other chemicals were at least reagent grade quality and used as received. The aqueous solutions were prepared with bi-distilled water.

### 2.3. Microelectrode preparation

All the working electrodes (microelectrodes) were prepared from Teflon-insulated Pt–Ir alloy wire (Pt 90%–Ir 10%, 130 μm total diameter) by removing 3 mm of the Teflon coating (from the tip of the wire) using ordinary flame. Pt–Ir alloy was used because of the reported electrocatalytic effect of iridium in oxidation reactions [32]; on the other hand pure platinum is more fragile than the Pt–Ir alloy, which makes it harder to use in real sample applications. Electrodes were then preconditioned in 0.5 M $H_2SO_4$ solution by applying a constant potential of + 1.9 V for 30 s followed by a cyclic voltammetry between the ranges − 0.25– + 1.1 V with a scan rate of 100 mV/s for 10 min. Prepared electrodes were dip-coated four times (four layers) with 1/10 diluted Nafion solution each dried at 200 °C for 4 min as indicated [13]. Electropolymerization of 50 mM OPD was performed on the Nafion-coated electrodes by applying + 0.65 V (vs. Ag–AgCl) for 10 min. The formation of poly(phenylenediamine) films was checked with cyclic voltammetry (not shown). Electrodes were calibrated individually before use.

### 2.4. Procedure

Freshly obtained rabbit carotid arteries were cut into two equal segments of 0.5 mm in length and were kept in Krebs solution at 4 °C till use. One portion was used in electrochemical studies in the electrochemical cell while the other was placed in thermostatic organel bath for force measurements.

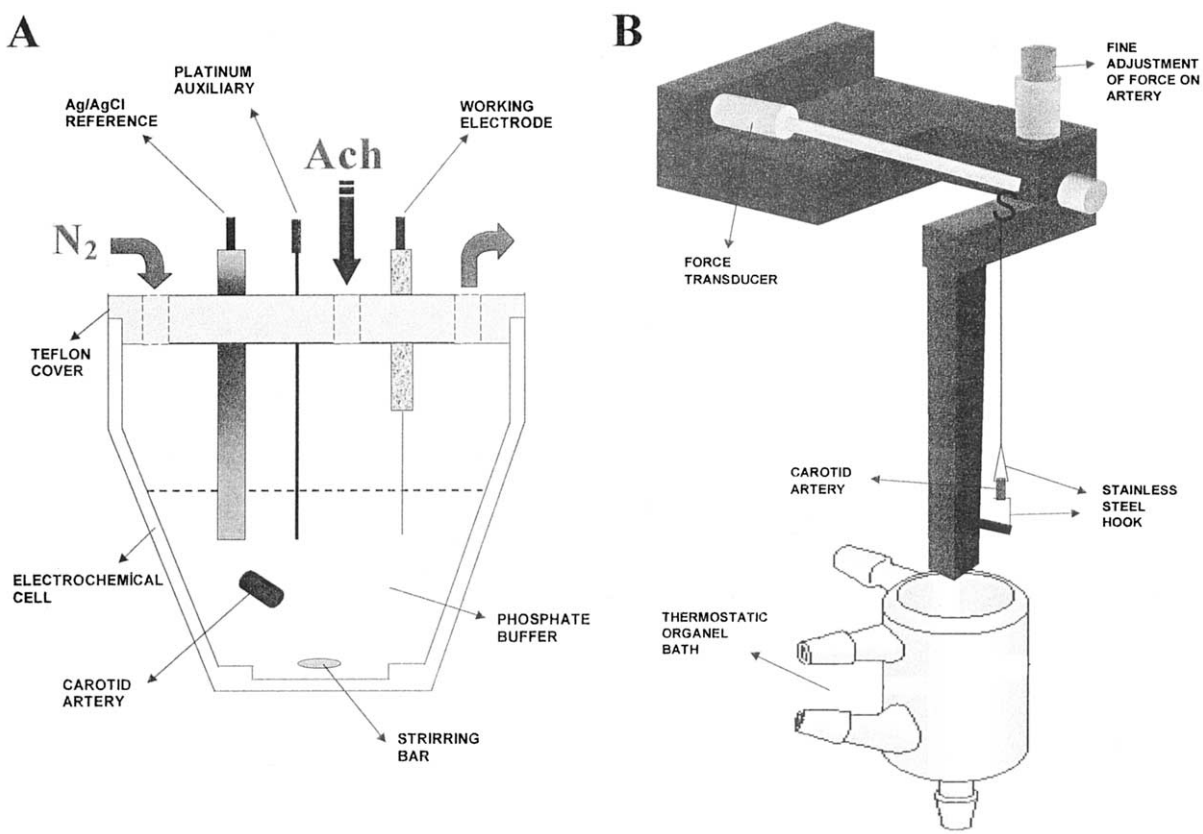


Fig. 1. Schematic drawing for electrochemical (A) and force measuring (B) experimental instrumentation for nitric oxide (NO) and vascular relaxation detection of carotid artery. In electrochemical and force measuring methods, 0.05 M phosphate buffer solution (PBS) (pH 7.4) and Krebs solution was used, respectively as the supporting electrolyte.

*2.4.1. Current measurements*

Fresh, isolated, rabbit artery was placed in electrochemical cell containing 10 ml 0.1M phosphate buffer (pH 7.4) solution (PBS). PBS was the supporting electrolyte as the electrode calibration was done in PBS. Current reponses in PBS and Krebs media were checked and found to be the same. Ag–AgCl reference, Pt auxiliary electrode and multistranded Pt–Ir microelectrode (working electrode) were placed through the holes in its teflon cover (Fig. 1). Fifty microliter of Ach stock was injected under $N_2$ atmosphere and 400 rpm convective transport with the Ach final concentration being $10^{-9}$, $3 \times 10^{-9}$, $10^{-8}$, $3 \times 10^{-8}$, $10^{-7}$, $3 \times 10^{-7}$, $10^{-6}$, $3 \times 10^{-6}$, $10^{-5}$, $3 \times 10^{-5}$, $10^{-4}$ M, respectively. Simultaneously current–time recordings were recorded at room temperature and then %Resp–log[Ach] plots were obtained.

*2.4.2. Force measurements*

Fresh, isolated, rabbit carotid artery segment was placed in 25 ml thermostatic organel bath in 10 ml Krebs solution, between the stainless steel hooks. Ach final concentrations were the same of current measurements after injections with the increments in logarithmic scale. Dose–response recordings were obtained and were used in graphing the logarithmic %Resp–log[Ach] plots. All force measurements were recorded and edited by the software VbPoly®, connected to the force transducers via RS-232 A/D convertor card.

## 3. Results and discussion

The experimental setup and instrumentation used during current (A) and force (B) measurements are displayed in Fig. 1. Freshly obtained rabbit carotid artery was cut into two equal segments (0.5 mm in length) and placed both in electrochemical cell and the thermostatic organel bath. In electrochemical cell under pure nitrogen atmosphere in 0.1 M phosphate buffer (pH 7.4), after the settlement of the three electrode system (working, reference and the auxiliary electrode), operational potential ($+0.90$ V) was applied and steady-state values of current was obtained before the injections of Ach with the final concentrations being; $10^{-9}$, $3 \times 10^{-9}$, $10^{-8}$, $3 \times 10^{-8}$, $10^{-7}$, $3 \times 10^{-7}$, $10^{-6}$, $3 \times 10^{-6}$, $10^{-5}$, $3 \times 10^{-5}$ and $10^{-4}$. This concentration range was chosen where the carotid artery segments displayed the best relaxations and contractions. At the same time simultaneously current–time recordings were obtained with a convective transport of 400 rpm. In the thermostatic organel bath the carotid artery segment was placed between stainless steel hooks with one end connected to the force transducer so that desired force could be applied and the change in contractile force could be measured during relaxation when NO was released. The force transducer system was computerized with a RS-232 A/D converter and by using the software (VbPoly®) Dose–Response (Relaxation) recordings could be obtained.

Fig. 2 shows the electrochemical characterization of the electrode using the experimental parameters examined. After the modification of the electrode with four layers of Nafion and 50 mM OPD, the permeability of the membrane was checked with 2 mM NO using differential pulse voltammetry (Fig. 2A). Here A′, B′, C′ represent the voltammograms (scan rate 10 mV/s) of phosphate buffer with membrane modified electrode, 2 mM NO with unmodified electrode and 2 mM NO with membrane modified electrode, respectively. It is obviously clear that 2 mM NO response with (c) and without (b) membranes is the same in NO saturated 0.1 M phosphate buffer (pH 7.4). As can be seen from all these three voltammograms, modification of the electrode with membranes has no negative effect on NO permeability. The selectivity of the membrane modified electrode was also studied (Fig. 2B). While the unmodified electrode gave high responses to $10^{-2}$ M glucose (b), $10^{-2}$ M ascorbic acid (c) and $10^{-2}$ M uric acid (d), the modified electrode did not respond to any of these interferences. Therefore, the modified electrode may easily be used in a biological matrix media in the presence of such biological interferences. The dependence of the electrode response on temperature is displayed in Fig. 2C. With the increase in temperature the response current also showed an increase trend. The response for $5 \times 10^{-4}$ M NO at 25 °C ($n = 6$, coefficient of variation (CV%) =

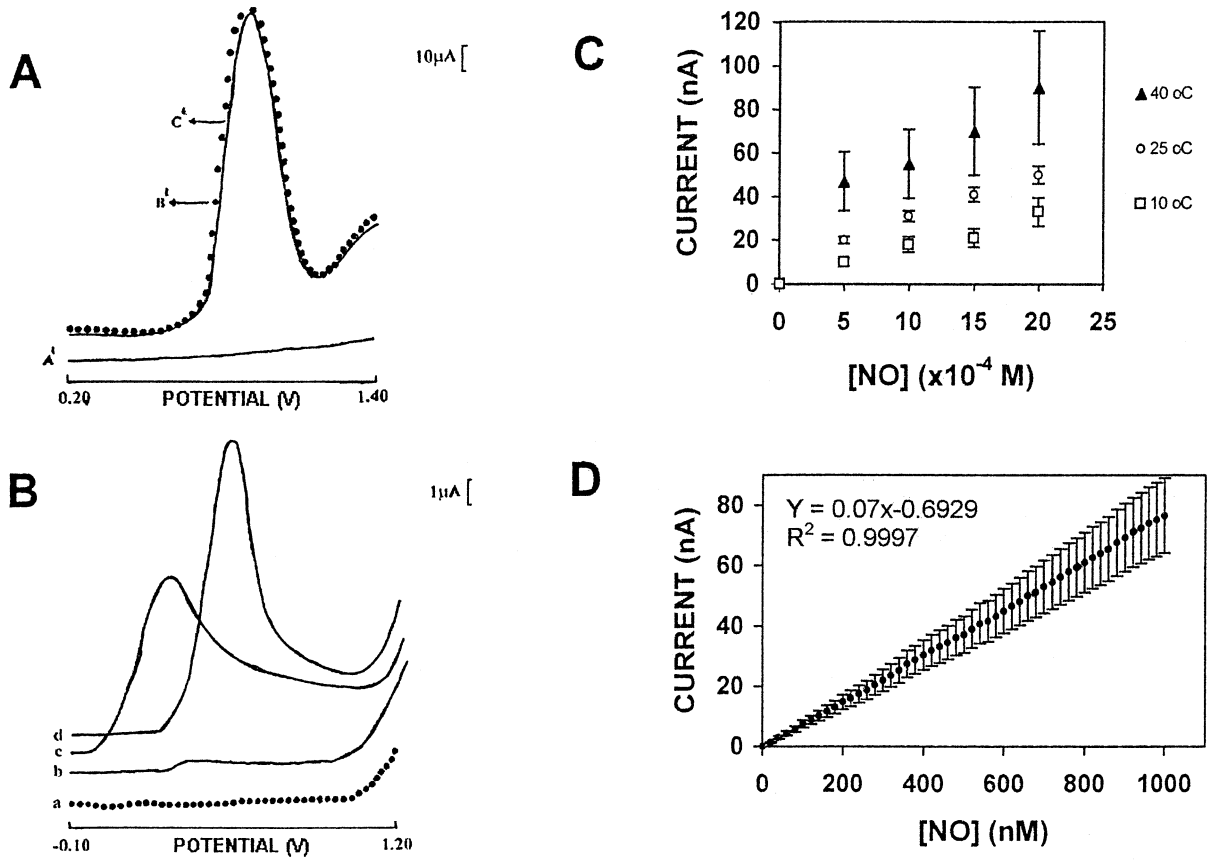


Fig. 2. NO permeability (A), selectivity (B), temperature dependence (C), calibration plot (D) of the membrane modified electrode. Differential voltammograms (scan rate +10 mV/s) of PBS with membrane modified electrode (A′), 2 mM NO with unmodified electrode (B′) and 2 mM NO with membrane modified electrode (C′) (Fig. 2A). Differential pulse voltammograms (scan rate +10 mV/s) of $10^{-2}$ M glucose (b), ascorbic acid (c), uric acid (d) with unmodified electrode and membrane modified electrode (a) (Fig. 2B). Other conditions as in Fig. 1.

8.10) is 2.35 times smaller than the one at 40 °C and 2.00 times bigger than the one at 10 °C, which is parallel to previous remarks in literature [33]. In Fig. 2D, the calibration plot for the membrane modified electrode was obtained and linearity was checked in the operational concentration range up to 1 μM NO and no departure from linearity was observed. The equation and the $R^2$ value of the calibration plot was found out to be $y = 0.077 \times -0.6929$ and $R^2 = 0.9997$, respectively. In the concentration range examined, the reproducibility of the electrode responses were also checked and CV% ($n = 10$) was calculated as 16.33%. The reproducibility seems not to be so good this may be due to the stability problems of NO in aqueous solutions due to its gaseous state. It is indicated that at 4 °C saturated aqueous solution of NO is stable for 48 h [31] but the diluted solutions are expected to have shorter stability and the recordings have been done at room temperature which induces the decrease in the NO concentration.

The prepared electrodes, as they were not enzyme electrodes and do not contain any biological component, the stability was not especially checked, but polymer (bi-polymer coatings of four layers of Nafion and a layer of 50 mM *o*-phenylenediamine-OPD) modified electrodes are

stable for 10 days at 4 °C (same response current obtained for 10 days for 2 mM NO via differential pulse voltammetry).

The effect of pH and temperature was also investigated in the range pH 3.0–10.0 (Fig. 3) with increasing NO concentration in 20 nM steps. In the range pH 3.0–6.0 the media was 0.05 M acetate buffer where as for the range pH 7.0–10.0 was 0.05 M phosphate buffer. The optimum pH range was found to be 3.0–5.0. Acidic media was the suitable range as expected as the oxidation of NO needed protons. As the pH values increases to basic state decrease in current values were observed as expected. The maximum response was obtained at pH 6.0 with a poor reproducibility (CV% as 63.92). The best reproducibility was obtained at pH 7.4 (CV% calculated as 14.31). For pH 9.0 and 10.0 no response was obtained. Calculated CV% values for the range pH 3.0–8.0 are as follows, respectively ($n = 4$); 16.00, 18.26, 30.60, 63.92, 14.31 and 142.30. As the best reproducibility was obtained with satisfactory response at pH 7.4, membrane modified electrode is applicable to biological media where the pH value is known to be around 7.4.

When S/N ratio was accepted as 3 the limit of detection on the designed electrode in determined as 15 nM (not shown), which indicates that the prepared electrodes can be employed for in vitro NO monitoring on rabbit carotid arteries and other real samples since the maximum NO concentration released from endothelium cells may be up to 0.5–1.0 μM [13,18,34]. Same designed electrodes were also used for NO detection in 5-aminolaevulinic acid based photodynamic therapy in rat cerebellum [35] and very similarly designed electrodes (difference in OPD–Nafion ratio) modified with glucose oxidase used for glucose mapping in experimental focal epilepsy [36].

Fig. 4 is the comparison of the draft data obtained by two different techniques; measuring force (Fig. 4A) and measuring current (Fig. 4B) on the equal pieces of the same rabbit carotid arteries. During force measurement, initial response forces measured before the injections of Ach were 6510 mg (a), 7920 mg (b), 10 060 mg (c), 8820 mg (d), 3425 mg (e), respectively. With the injections of Ach, relaxation was observed and the force began to decrease in each artery, making a minima at $3 \times 10^{-6}$ M (a), $3 \times 10^{-6}$ M (b), $3 \times 10^{-5}$ M (c), $10^{-4}$ M (d) and $3 \times 10^{-5}$ M (e), respectively. The current results obtained (Fig. 4B, a–e) have the similar response trend to Ach injections. The increasing NO formation trend was observed during Ach injections. The membrane modified electrode does not respond to Ach injections in the absence of carotid artery segment (not shown).

Percentage response–pAch($-\log$[Ach]) graphs were plotted in Fig. 5 to see the overview of the response trend during force (●) and current (▲) measurements of different arteries (a–e). The maxima values obtained during measurements were accepted as 100% and Ach concentrations were plotted as pAch($-\log$[Ach]) values in $x$-axis. During force measurements, carotid arteries began relaxation starting usually at $3 \times 10^{-8}$ till $3 \times 10^{-5}$ M. Following this concentration range, the rate of NO release began to decrease leading to contraction of the artery as expected. While measuring current, NO oxidation started at around the same concentration value of $3 \times 10^{-8}$ M Ach. The same increase trend was observed all during the Ach injections (Fig. 5, a–e).

## 4. Conclusion

In this paper, we have displayed the fabrication, electrochemical characterization of a bi-polymer modified amperometric platinum microelectrode and its application for in vitro NO measurements from rabbit isolated carotid artery endothelium. At the same time we have obtained dose–response curves in thermostatic organel bath for the same rabbit isolated carotid artery endothelium. To our best knowledge no effort has been recorded to employ both techniques on the same sample to quantify NO release and compare the trends in the two sets of results. Thus we believe, making an overview on the two detection techniques, and the comparison of our obtained data of the same sample will be a valuable contribution to the literature.

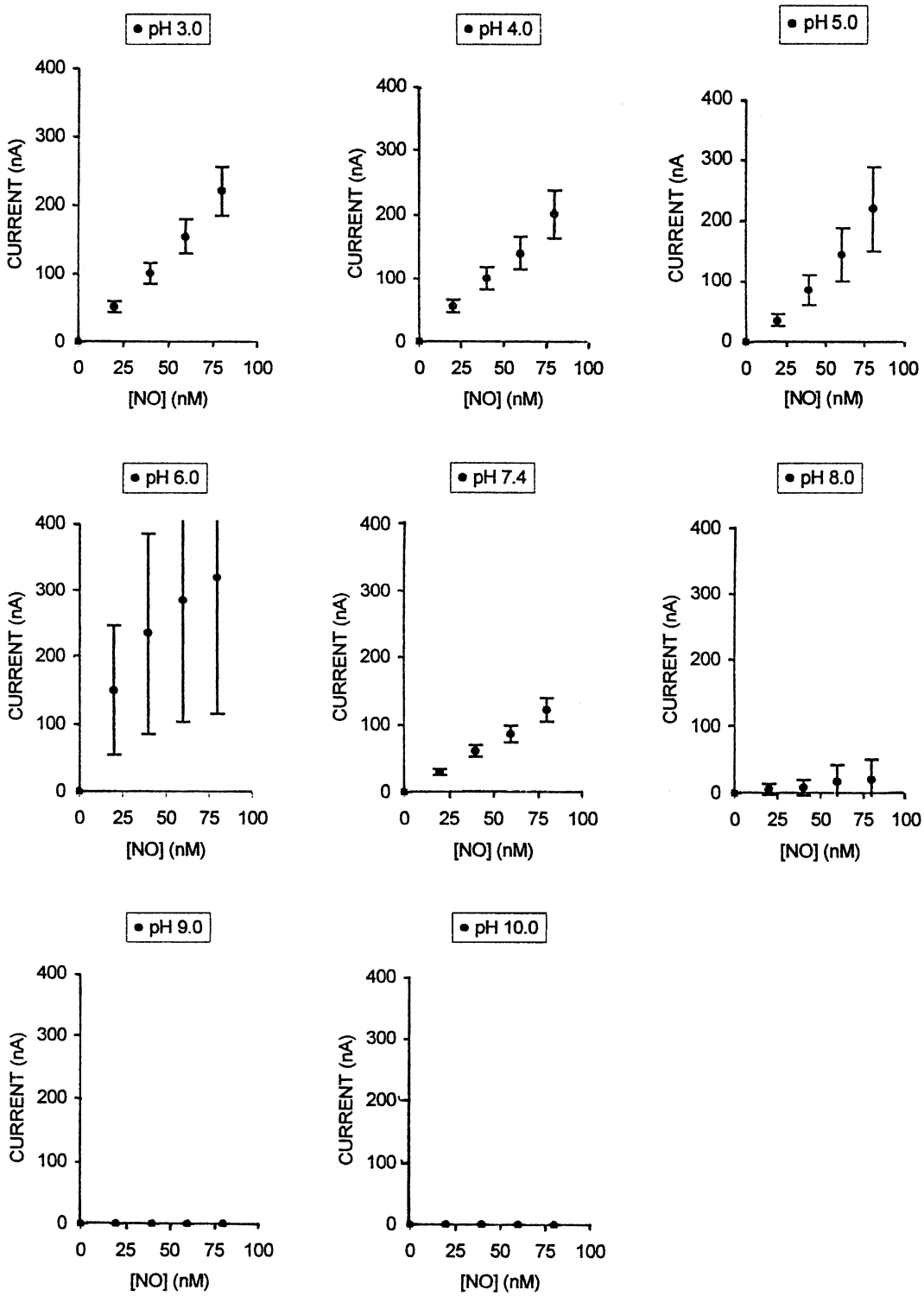


Fig. 3. Dependence of electrode response on pH. 0.05 M acetate buffer for pH range 3.0–6.0 and PBS for the range 7.0–10.0. Other conditions as in Fig. 1.

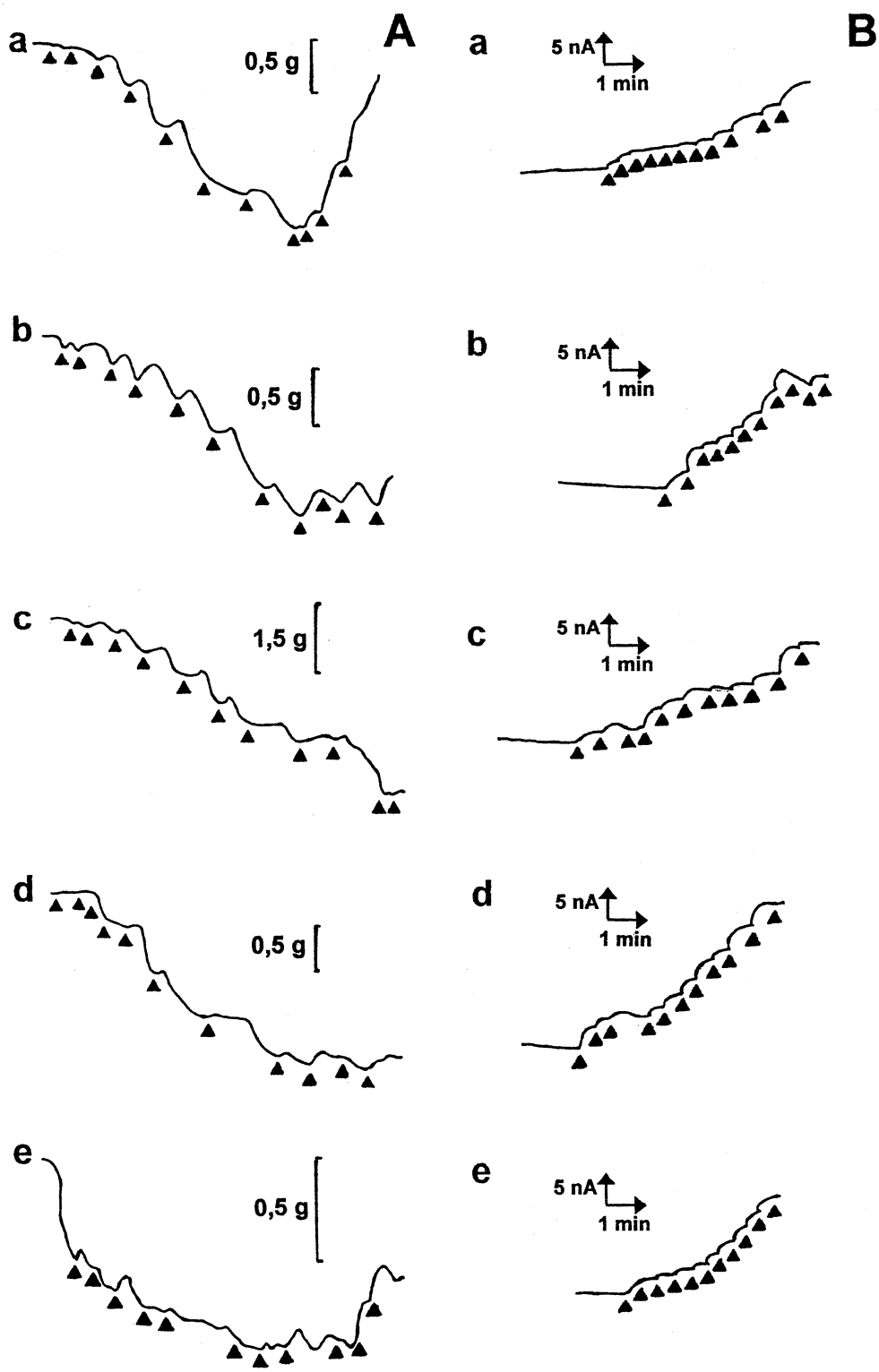


Fig. 4. Draft data of force (A) and current (B) measurements of various carotid arteries (a–e). Triangles (▲) indicate the acetylcholine (Ach) injections in the range $10^{-9}$–$10^{-4}$ M. Other conditions as in Fig. 1.

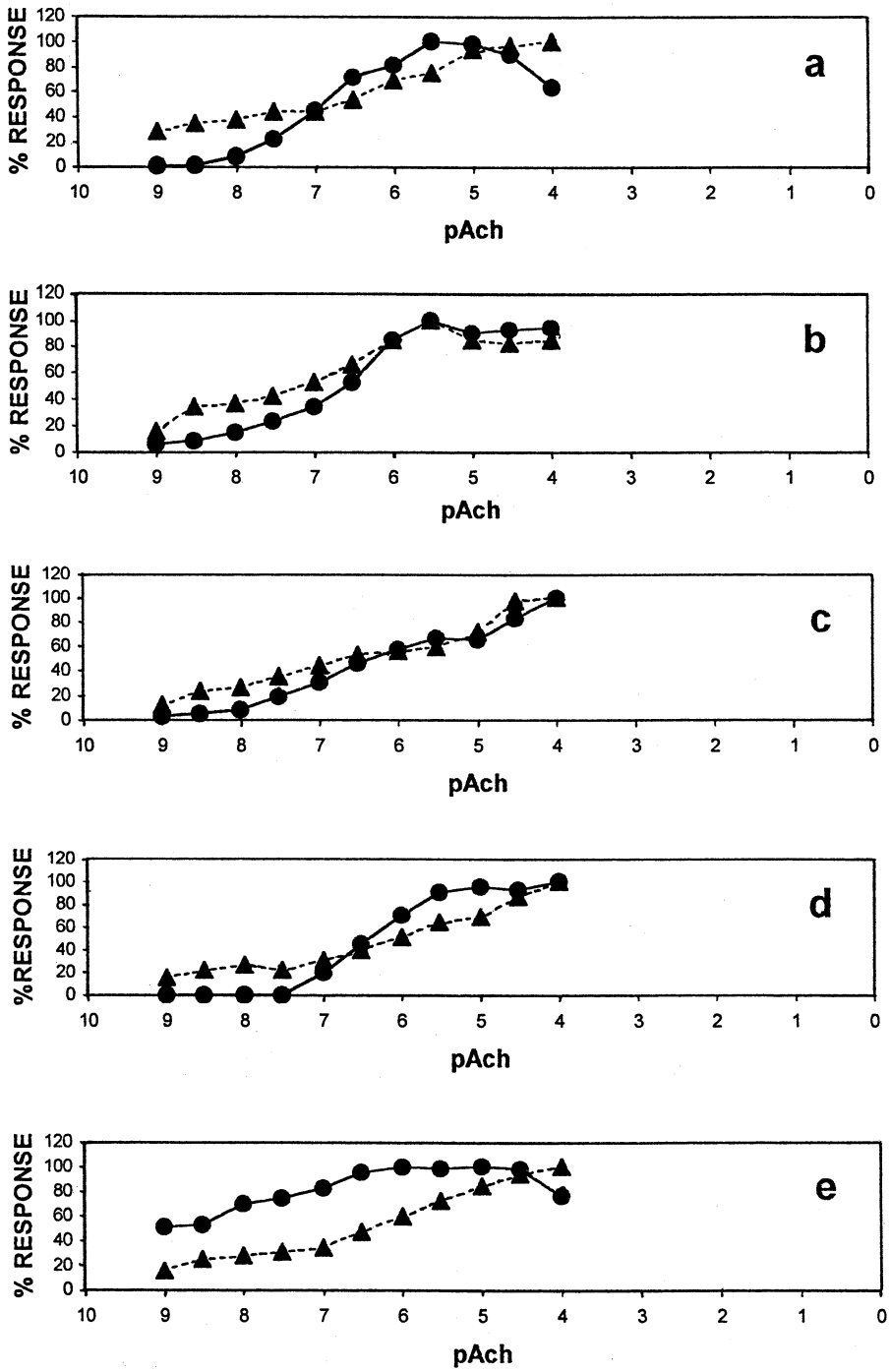


Fig. 5. Percentage response–pAch ( − log[Ach]) graphs obtained from the draft data of Fig. 4 to see and compare the overview of the response trend during force (●) and current (▲) measurements. Conditions as in Fig. 1.

## References


[1] R.S. Furchgott, J.V. Zawadski, Nature 288 (1980) 373–376.
[2] R.M.J. Palmer, A.G. Ferrige, Nature 327 (1987) 524–526.
[3] L.J. Ignarro, G.M. Buga, K.S. Wood, R.E. Byrns, G. Chaudhuri, Proc. Natl. Acad. Sci. USA 84 (1987) 9265–9269.
[4] C.F. Nathan, FASEB J. 6 (1992) 3051–3064.
[5] R.G. Knowles, S. Moncada, Biochem. J. 298 (1994) 249–258.
[6] S. Moncada, Acta. Physiol. Scand. 145 (1992) 201.
[7] M.S. Mulligan, S. Moncada, P.A. Ward, J. Pharmacol. 107 (1992) 1159.
[8] C.R. Lyons, G.J. Orloff, J.M. Cunningham, J. Biol. Chem. 267 (1992) 6370.
[9] Y. Izumi, D.M. Clifford, C.F. Zorumski, Science 257 (1992) 1273.
[10] S. Archer, FASEB J. 7 (1993) 349–360.
[11] M. Feelisch, in: M. Feelisch, J.S. Stamler (Eds.), Methods in Nitric Oxide Research, Wiley, Chichester, 1996.
[12] T. Malinski, L. Czuchajowski, in: M. Feelisch, J.S. Stamler (Eds.), Methods in Nitric Oxide Research, Wiley, Chichester, 1996, pp. 319–339.
[13] M.N. Friedemann, S.W. Robinson, G.A. Gerhardt, Anal. Chem. 68 (1996) 2621–2628.
[14] B. Fethi, S. Trevin, J. Devynck, Electroanalysis 8 (12) (1996) 1085–1091.
[15] T. Malinski, A. Ciszewski, J. Bennett, J.R. Fish, L. Czuchajowski, J. Electrochem. Soc. 138 (7) (1991) 2008–2015.
[16] S. Mesaros, S. Grunfeld, A. Mesarosova, D. Bustin, T. Malinski, Anal. Chim. Acta 339 (1997) 265–270.
[17] S. Trevin, F. Bedioui, J. Devynck, Talanta 43 (1996) 303–311.
[18] T. Malinski, Z. Taha, Nature 358 (1992) 676–678.
[19] F. Bedioui, S. Trevin, J. Devynck, J. Electroanal. Chem. 377 (1994) 295–298.
[20] A. Ciszewski, G. Milczarek, Electroanalysis 10 (11) (1998) 791–793.
[21] F. Lantoine, S. Trevin, F. Bedioui, J. Devynck, J. Electroanal. Chem. 392 (1995) 85–89.
[22] F. Bedioui, S. Trevin, J. Devynck, F. Lantoine, A. Brunet, M.A. Devynck, Biosens. Bioelectron. 12 (1997) 205–212.
[23] C. Privat, F. Lantoine, F. Bedioui, E. Millanvoye van Brussel, J. Devynck, M.A. Devynck, Life Sciences 61 (12) (1997) 1193–1202.
[24] S. Trevin, S. Andre, J. Devynck, J.L. Boucher, F. Bedioui, Anal. Comm. 34 (1997) 69–71.
[25] T. Malinski, Z. Taha, S. Grunfeld, A. Burewicz, P. Tomboulian, F. Kiechle, Anal. Chim. Acta 279 (1993) 135.
[26] K. Ichimori, M. Ishida, M. Fukahori, H. Nakazawa, E. Murakawi, Rev. Sci. Instrum. 65 (1994) 2714.
[27] A.M.Y. Lin, L.S. Kao, C.Y. Chai, J. Neurochem. 35 (1995) 2043–2049.
[28] W.A. Cass, N.R. Zahniser, K.A. Flach, G.A. Gerhardt, J. Neurochem. 61 (1993) 2269–2278.
[29] D.A. Smith, A.F. Hoffman, D.J. David, C.E. Adams, G.A. Gerhardt, Neurosci. Lett. 255 (1998) 127–130.
[30] F. Pariente, J.L. Alonso, H.D. Abruna, J. Electroanal. Chem. 379 (1994) 191–197.
[31] S. Mesaros, S. Grunfeld, A. Mesarosova, D. Bustin, T. Malinski, Anal. Chim. Acta 339 (1997) 265–270.
[32] J. Wang, G. Rivas, M. Chicharro, Electroanalysis 8 (5) (1996) 434–437.
[33] K. Shibuki, Neurosci. Res. 9 (1990) 69–76.
[34] Y. Kitamura, T. Uzawa, K. Oka, Y. Komai, H. Ogawa, N. Takizawa, H. Kobayashi, K. Tanishita, Anal. Chem. 72 (2000) 2957–2962.
[35] T. Dalbasti, S. Cagli, E. Kilinc, N. Oktar, M. Ozsoz (2001) unpublished data.
[36] T. Dalbasti, E. Kilinc, A. Erdem, M. Ozsoz, Biosensors. Bioelectron. 13 (7–8) (1998) 881–888.