Biochimica et Biophysica Acta, 1070 (1991) 321–331

BBAMEM 75429

The use of the potential-sensitive fluorescent probe bisoxonol in mast cells

Christian Bronner and Yves Landry

Laboratoire de Neuroimmunopharmacologie, Université Louis Pasteur Strasbourg I, Illkirch (France)

(Received 1 May 1991)

Key words: Bisoxonol; Oxonol; Exocytosis; Mast cell; Membrane potential; Sodium pump; Potassium

The regulation of the plasma membrane potential of rat peritoneal mast cells at the resting state and during activation was investigated using bisoxonol as a potential-sensitive fluorescent dye. Fluorescence microphotography showed that this negatively charged probe was not only present in the plasma membrane, but was also distributed in the cytoplasm. The intracellular localization of bisoxonol was confirmed by conducting experiments which showed that bisoxonol fluorescence was not enhanced in ATP-permeabilized mast cells. Rotenone (10^{-7} M) and oligomycin (10^{-6} M) did not change the fluorescence of bisoxonol showing, therefore, that mitochondrial depolarization was not recorded with bisoxonol and suggesting that bisoxonol may represent a useful probe to study plasma membrane potential changes in the absence of exocytosis. We showed that, in non-stimulated mast cells, the blockade of the sodium pump enhanced the fluorescence of bisoxonol as did gramicidin a non selective ionophore used to fully depolarize the cells. High concentration of potassium (30 mM) as well as different ionic channel blockers did not significantly change the fluorescence intensity of bisoxonol, suggesting that ionic channel permeabilities were not involved in maintaining the resting plasma membrane potential of mast cells. Mast cells stimulated by compound 48/80 completely lost the fluorescence, shown by fluorescence microphotography, suggesting that exocytotic phenomena might induce a dye redistribution which is not only due to changes in the plasma membrane potential. In mast cells pretreated with pertussis toxin, which blocks mast cell-exocytosis, compound 48/80 induced a delayed (2 min) decrease of bisoxonol fluorescence which was shown to be dependent on the activity of the sodium pump. Considering that bisoxonol is a useful potential-sensitive probe in exocytosis-deprived mast cells, our results suggest that the sodium pump is mainly involved in the changes of plasma membrane potential of mast cells.

Introduction

Histaminocytes include mast cells and basophilic leucocytes. These cells secrete inflammatory mediators upon aggregation of cell surface receptors for immunoglobulin E (IgE). Some types of mast cells, such as connective tissue mast cells also called serosal mast cells, respond to various peptides by a non-receptorial mechanism (see review in Ref. 1). Whatever the triggering agent may be, the early events including the exocytotic process are not fully known. One of these events might be changes in the plasma membrane potential. However, changes in the membrane potential during mast cell activation are a matter of controversy. Indeed, by recording the membrane potential of rat mesentery mast cells with a microelectrode, Tasaka et al. [2] showed that mast cells stimulated with compound 48/80 underwent depolarization as a consequence of degranulation. In contrast, it was shown that mast cells activation by non-immunological secretagogues involves membrane hyperpolarization due to a chloride current [3]. This hyperpolarization was proposed to increase calcium influx by providing electrical driving force, and therefore to play a role in the elevation of cytosolic calcium [3]. IgE-dependent degranulation of mast cells does not require the opening of ionic channels [4], whereas compound 48/80 involves cadmium-sensitive calcium channels [5,6] and chloride channels [3].

Lindau and Fernandez [7] showed that the resting potential of mast cells fluctuated between 0 and −30

Correspondence: C. Bronner, Laboratoire de Neuroimmunopharmacologie, Université Louis Pasteur Strasbourg I, B.P. 24, 67401 Illkirch Cedex, France.

mV, with a very small whole-cell conductance (10–30 pS) and an input resistance of 30–100 GΩ. These values were supposed to be due to the imperfect pipette membrane seal. However, the authors could not exclude a resting potential caused by the electrogenic activity of the sodium pump or by the Na^+/Ca^{2+} exchanger, which might have been short-circuited by the leakage conductance of the seal. Such ionic exchanges are interesting candidates for the regulation of the plasma membrane potential changes during mast cell-activation.

Considering that patch-clamp techniques were not sufficient to record membrane current below 1 pA which can effectively be generated by the sodium pump [7], we used an other technique based on the behaviour of the potential-sensitive fluorescent probe bisoxonol. Oxonol dyes have been shown to be useful probes for investigating the electrogenic properties of ionic translocating systems [8–10]. However, the use of fluorescent probes in mast cells can lead to numerous misinterpretations, as these cells undergo fast exocytosis which cannot be easily dissociated from early biochemical events. For instance, studies on membrane fluidity changes induced by compound 48/80 with trimethylaminodiphenylhexatriene [11,12] and cytosolic calcium monitoring with fura-2 or quin-2 [13,14] were shown to be drastically influenced by the exocytotic process itself. Nevertheless, it was shown that bisoxonol is a useful probe for studying plasma membrane potential changes in rat basophilic leukemia cells [15,16] in that it does not monitor mitochondrial depolarization and in that changes in plasma membrane potential changes occur largely before the degranulation process starts.

Therefore, to establish the usefulness of bisoxonol in stimulated mast cells we engaged studies on the distribution of this probe in mast cells, as well as on the influence of mitochondrial depolarization on the fluorescence signal. The present paper proposes bisoxonol as a potential-sensitive probe that can be used in mast cells only in the absence of exocytosis. In these conditions we showed that the plasma membrane potential of mast cells is mainly controlled by the activity of the sodium pump. This enzyme appears to be involved in the changes of mast cell membrane potential during activation which, however, appears as a consequence of mast cell stimulation.

Materials and Methods

Chemicals

Pertussis toxin, 4-aminopyridine (4-AP), mast-cell-degranulating-peptide (MCD), deoxyglucose, dinitrophenol, rotenone, oligomycin, compound 48/80, ATP, gramicidin and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were obtained from Sigma Chemical Co (St. Louis, MO). 1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) and bis(1,3 diethylthiobarbiturate)trimethine oxonol (bisoxonol) were purchased from Molecular Probes (Eugene, OR). Tetraethylammonium chloride (TEA) was obtained from Merck-Schuchardt (Hohenbrunn, F.R.G.). Ethanol was used to solubilize gramicidin (100-fold concentrated) and oligomycin (10000-fold concentrated). Dimethylsulfoxide was used for rotenone (10000-fold concentrated), and bisoxonol (1000-fold concentrated). TMA-DPH was solubilized as a stock solution of 5 mM in dimethylformamide.

Cell system

Male Wistar rats (Iffa-Credo, L'Arbresle, France) weighing 350 to 400 g were killed by stunning and bled. 12 ml of balanced salt solution containing (mM): NaCl, 137; KCl, 2.7; $CaCl_2$, 1; $MgCl_2$, 1; NaH_2PO_4, 0.4; glucose, 5.6; and Hepes, 10; NaOH (pH 7.4), supplemented with 0.05% gelatin were injected into the peritoneal cavity. The peritoneal fluid was collected and centrifuged for 1.5 min at $180 \times g$. The pellet was resuspended in the same solution and the mast cells were purified using a bovine serum albumin gradient as described previously [17,18]. Mast cells were washed immediately after the purification step with the corresponding buffer, i.e., in some experiments (Figs. 3, 5 and 9), potassium and/or calcium were omitted and replaced with equivalent amounts of sodium. Otherwise the concentrations of potassium chloride and calcium were 2.7 mM and 1 mM, respectively. In the experiment shown in Fig. 9 purified mast cells (> 98%) were resuspended and adjusted with the buffer to a concentration of 10^6 cells/ml and were pretreated with 300 ng/ml of pertussis toxin for 2 h, at 37 °C and then, washed with the buffer.

Bisoxonol fluorescence measurements

The mast cell suspension (2.5 ml containing $4.5 \cdot 10^5$ cells/ml) was placed in 1 cm quartz cuvettes. The cuvettes were preincubated for 2 min, at 37 °C, in the thermostated cuvette-holder of a Jobin-Yvon Spectrofluorimeter (JY 3D) with gentle stirring. Changes in the plasma membrane potential were monitored using bisoxonol as previously described [18]. Briefly, bisoxonol was added to obtain a final concentration of 100 nM and fluorescence intensities were recorded continuously at 540 and 580 nm. To quantify the membrane potential in millivolts, the valinomycin K^+ null-point is usually recommended [9]. Although, the fluorescence of bisoxonol has been correlated to the plasma membrane potential, this null-point method was not possible since valinomycin binds to oxonol dyes. Gramicidin, a non-selective cation ionophore, was used to abolish the transmembrane gradient of monovalent cations, i.e., to induce maximal cell depolarization.

Mast cell permeabilization

Mast cells were permeabilized as described elsewhere [19]. Briefly, mast cells were purified and resuspended (2.5 ml containing $4.5 \cdot 10^5$ cells/ml) in a calcium- and magnesium-free medium in the presence of EDTA (20 μM). The addition of ATP (18 μM) allows the immediate opening of ionic channels which are believed to allow the influx of molecules which are normally impermeant [20]. Under these conditions the effect of mast cell permeabilization was studied on the fluorescence of bisoxonol. This effect was compared to that obtained with the fluorescence of TMA-DPH which was previously shown to be selectively localized in the plasma membrane under resting conditions [17].

Bisoxonol fluorescence microphotography

The localization of bisoxonol in intact mast cells and in compound 48/80-stimulated mast cells was assessed by performing micrographs using a Nikon Optiphot-2 photomicroscope equipped for epiillumination (DM 580) with filters at 510–560 nm for excitation and at 590 nm for observation. The original magnification was 400 ×; 400 ASA KODAK Tmax films were used for recording. Because of the rapid photobleaching of bisoxonol, it was difficult to obtain fluorescence microphotographs clearer than those shown in Fig. 2.

Results

Preliminary controls

The effects of varying bisoxonol concentration and mast cell density on the fluorescence intensities of bisoxonol are shown in Fig. 1. Increasing the concentration of bisoxonol from 25 to 250 nM led to the appearance of a dose-dependent fluorescence signal, which stabilized within 2 min. The concentration of 100 nM or 150 nM of bisoxonol were usually recommanded [15,16,21]. Here, we showed that in the presence of $4.5 \cdot 10^5$ mast cells/ml, the concentration of 100 nM of bisoxonol is appropriate because it does not saturate the binding sites for bisoxonol since higher concentration enhanced the fluorescence signal (Fig. 1A). Increasing the mast cell density in the presence of a fixed concentration of bisoxonol is shown in Fig. 1B. The fluorescence signal was proportional to the mast cell density indicating a partition equilibrium between the dye in the extracellular aqueous phase and the dye in the cells. Therefore, in the presence of $4.5 \cdot 10^5$ mast cells/ml, 100 nM is an accurate concentration for bisoxonol because there is still bisoxonol available in the aqueous phase which is supposed to transduce changes in the state of polarization of the plasma membrane of mast cells.

The cellular localization of bisoxonol in mast cells

The cellular localization of bisoxonol was approached by two types of experiments, i.e., fluorescence microphotography and mast cell permeabilization. Fig. 2B shows that the fluorescence was distributed in the whole unstimulated cell except in the middle part which may correspond to the nucleus. A similar distribution was also observed for rat basophilic

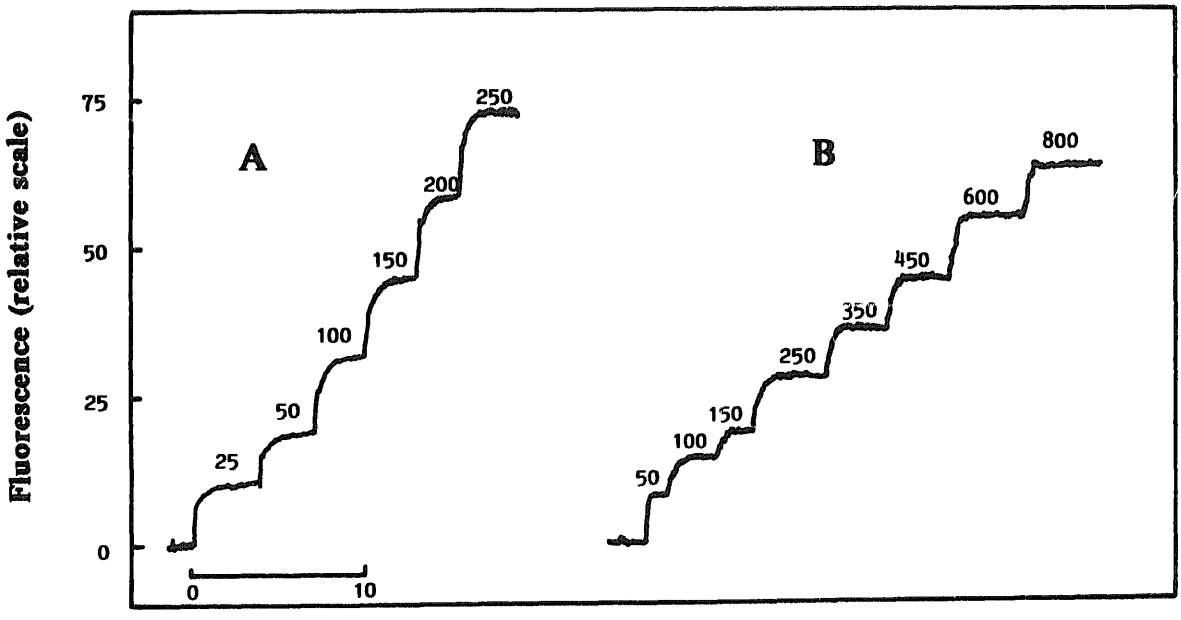

Fig. 1. Fluorescence intensities of bisoxonol according to the dye concentration and to the mast cell density. (A) Mast cells ($4.5 \cdot 10^5$/ml) preincubated at 37 °C in the quartz cuvette for 5 min before adding the various doses of bisoxonol which were given on the trace in nM. (B) Bisoxonol (100 nM) was preincubated at 37 °C for 5 min before adding mast cells from a concentrated suspension of $5 \cdot 10^6$ mast cells/ml. The numerals on the trace indicate the mast cell density in cells/ml $\times 10^{-3}$. Traces were representative of one experiment repeated twice. The scale indicates the time in min.

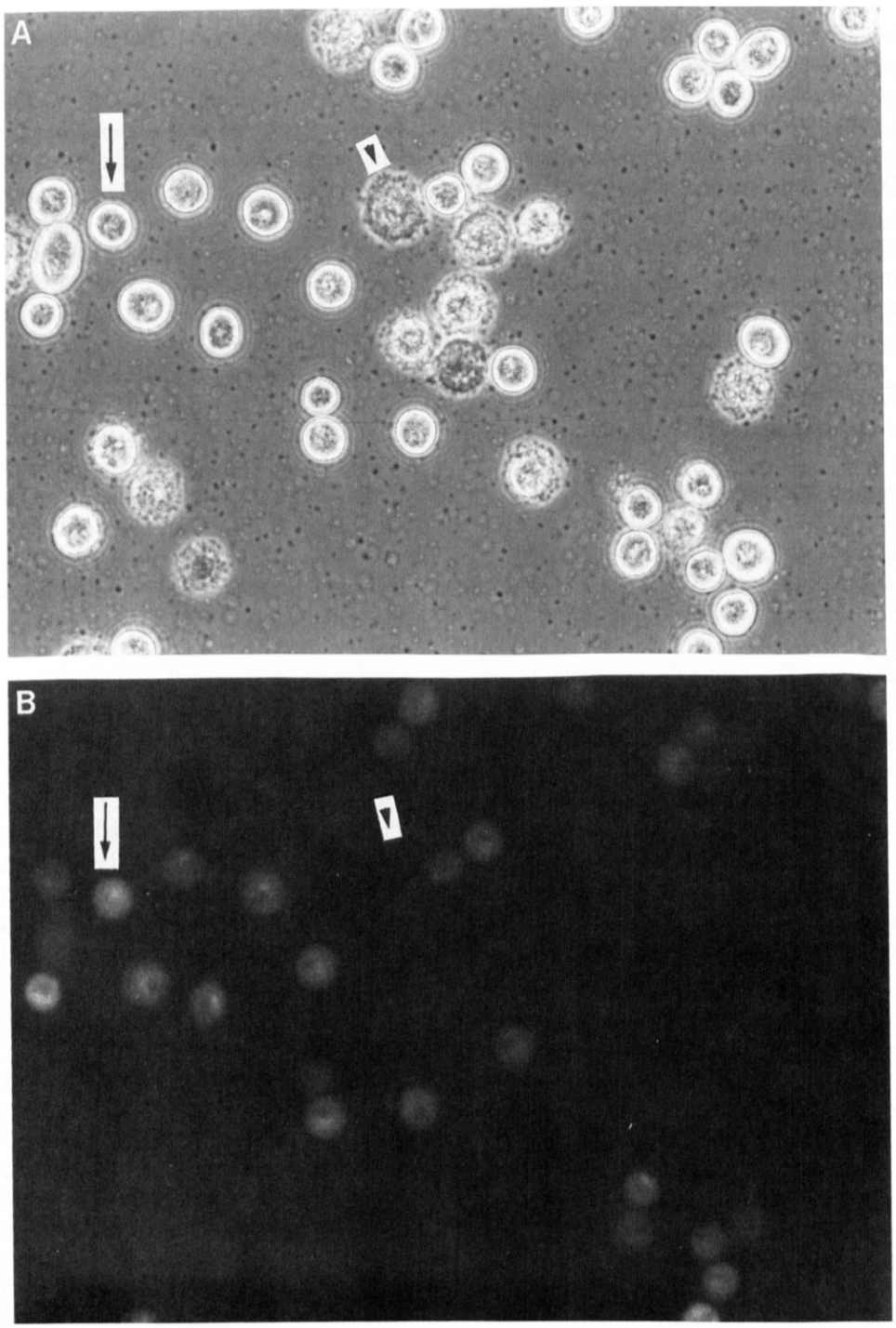

Fig. 2. Bisoxonol labelled rat peritoneal mast cells under bright field (A) and fluorescence illumination (B). The cells were preincubated at 37 °C with 100 nM bisoxonol and then stimulated with 0.2 μg/ml of compound 48/80. The cells were then observed on cover glass slips. Stimulated and unstimulated mast cells can be observed on the same preparation. For instance, a non-stimulated mast cell (arrow) can be seen in (A) and the corresponding labelling in (B). A stimulated mast cell which underwent large exocytosis (arrow head) can easily be observed in (A), but its corresponding image in fluorescence was not detected in B.

leukemia cells (not shown). The stimulation of mast cells, which led to the loss of their regular outline and granule extrusion (Fig. 2A), completely abolished the fluorescence (Fig. 2B), suggesting a dramatical hyperpolarization or simply a dye loss related to the exocytotic process. Another evidence for the dye distribution throughout the cells was obtained by permeabilizing the mast cells with the method using ATP^{4-} [19]. Fig. 3 shows that the fluorescence signals of TMA-DPH and bisoxonol before and after cell permeabilization. We previously demonstrated that, in intact cells, TMA-DPH was only localized in the plasma membrane [17,22] and that this probe underwent a partition equilibrium between the extracellular aqueous phase (where it does

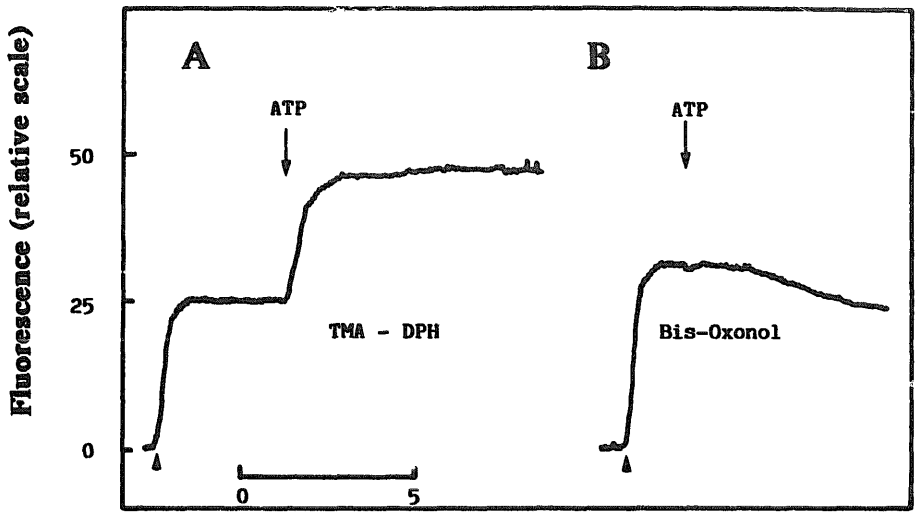

Fig. 3. Effect of ATP on the fluorescence of TMA-DPH and bisoxonol. Purified rat peritoneal mast cells were suspended in a Ca^{2+}- and Mg^{2+}-free medium, containing 20 μM EDTA. Bisoxonol and TMA-DPH were added at the time indicated by the arrow head. (A) Mast cells were labelled with $5 \cdot 10^{-7}$ M of TMA-DPH. Fluorescence intensities were recorded at 340 and 475 nm. (B) Mast cells were labelled with 100 nM of bisoxonol and fluorescence intensities were recorded at 540 and 580 nm. ATP was added at 18 μM (final concentration). Traces were representative of three different experiments. The scale indicates the time in min.

not fluoresce) and the plasma membrane (where it fluoresces). This probe is now also used as a measurable parameter of cell permeation [19]. Fig. 3A shows that the addition of ATP^{4-} to the TMA-DPH-labelled mast cells increased the fluorescence intensity whereas it did not modify that of bisoxonol-labelled cells (Fig. 3B). This result strongly supports that bisoxonol has already labelled the inner membranes of mast cells, such as granule membranes and mitochondria. Thus, changes in the mitochondria membrane potential had to be shown not to affect the fluorescence of bisoxonol.

Fig. 4 shows the effect of rotenone and oligomycin known to affect membrane potential of mitochondria by depleting cells in ATP [15]. In the presence of standard conditions (1 mM of Ca^{2+} and 2.7 mM of KCl), only oligomycin slightly decreased the fluorescence of bisoxonol (Fig. 4A). However, depleting cellular ATP might modify the activity of effectors which participate in maintaining the resting plasma membrane potential. The sodium pump and a Ca^{2+}-dependent process were shown to modulate the fluorescence of bisoxonol in mast cells [18]. Therefore, to short

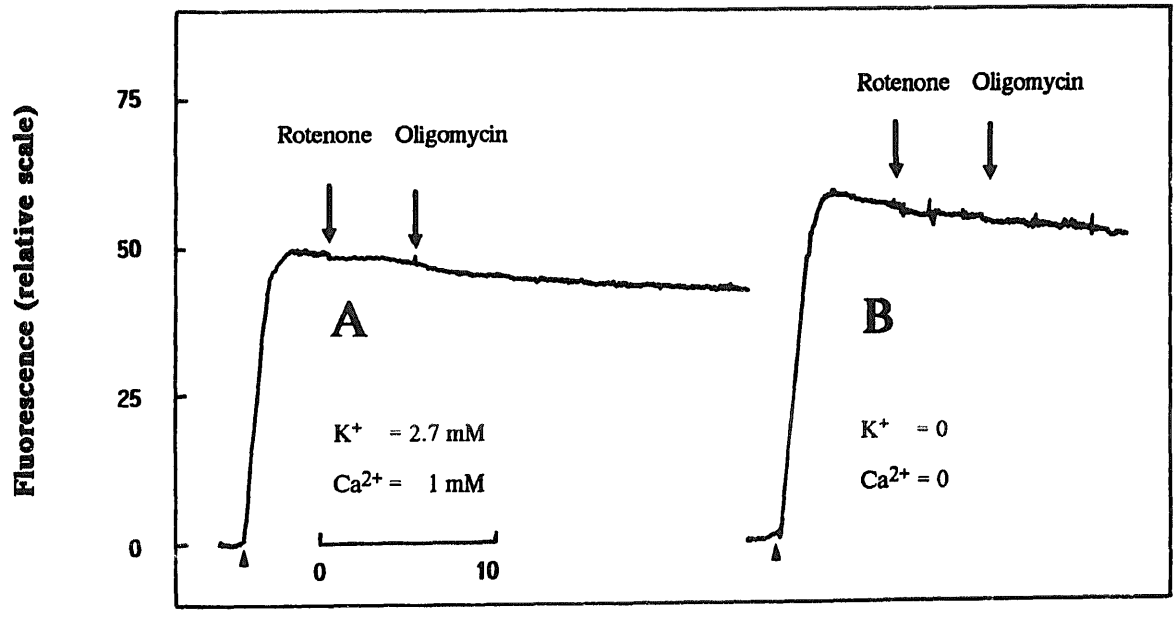

Fig. 4. Effect of rotenone and oligomycin on the fluorescence of bisoxonol under different conditions which were given on traces. Bisoxonol (100 nM) was added at the time indicated by the arrow head. Rotenone and oligomycin were added to obtain final concentrations of 10^{-7} M and 10^{-6} M, respectively. Traces were representative of three different experiments. The scale indicates the time in min.

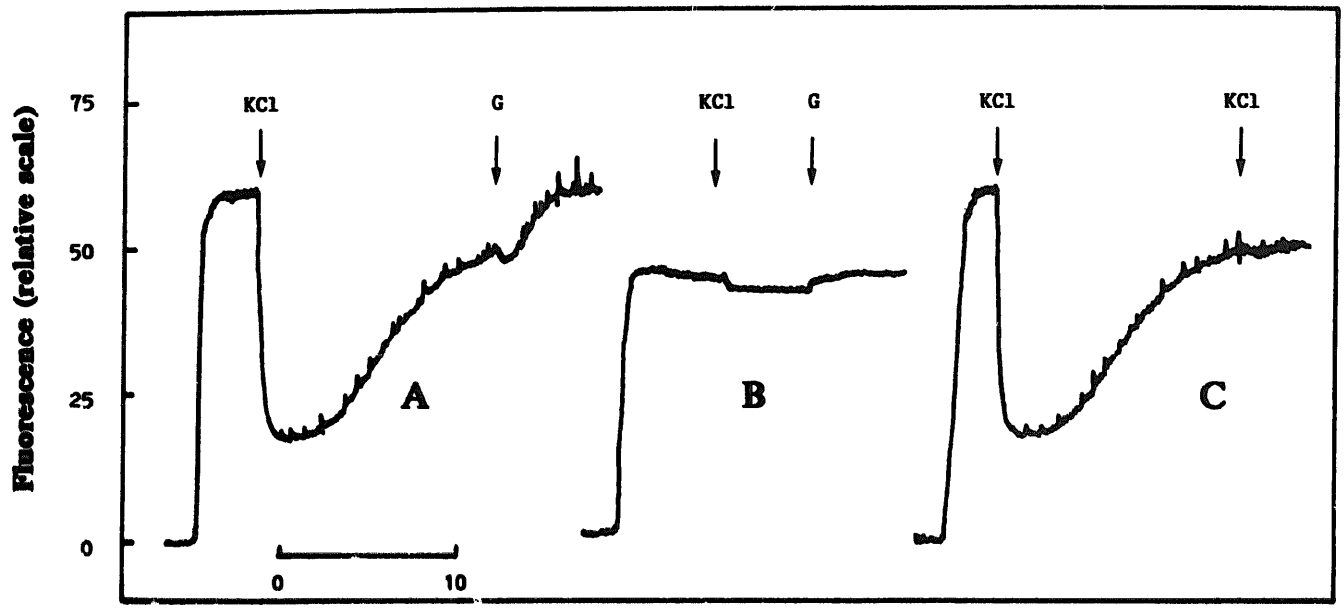

Fig. 5. Effect of KCl and gramicidin on the fluorescence of bisoxonol. (A) Mast cells were preincubated at 37 °C for 45 min in the presence of 5.6 mM glucose before they were placed in the quartz cuvette of the fluorometer. KCl and gramicidin (G) were added at 2.7 mM and 10 μg/ml, respectively. (B) Mast cells were preincubated for 45 min at 37 °C in the presence of 5.6 mM deoxyglycose and 10^{-4} M of dinitrophenol, but in the absence of glucose. Mast cells were washed and resuspended in a glucose free-medium. KCl and gramicidin were added at the concentrations given in (A). (C) Mast cells were treated as described in (A). KCl were added to obtain final concentrations of 2.7 mM (first addition) and 30 mM (second addition). Bisoxonol was added at 100 nM. Traces were representative of four different experiments. The scale indicates the time in min.

circuit these systems we studied the effects of rotenone and oligomycin in the absence of K^+ and Ca^{2+}. Fig. 4B shows that under these conditions neither rotenone nor oligomycin had any effect. Thus, our results show that bisoxonol does not transduce membrane potential changes in mitochondria which is in accordance with data obtained on rat basophilic leukemia cells by other authors [15].

Changes in the fluorescence of bisoxonol in resting mast cells

Fig. 5 shows the effect of potassium on K^+-deprived mast cells. The addition of 2.7 mM induced a fast decrease of fluorescence of bisoxonol followed by a plateau and then by a partial recovering of the fluorescence intensity (Fig. 5A). This decrease was completely abolished in the presence of ouabain [18] showing that

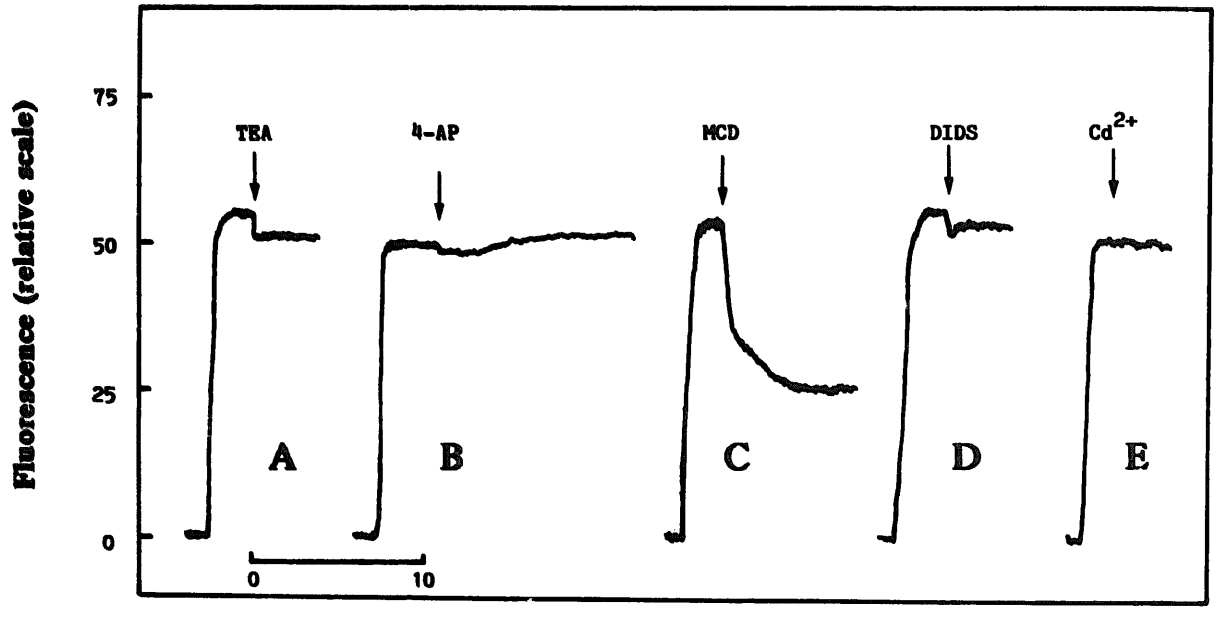

Fig. 6. Effect of ionic channels blockers on the fluorescence of bisoxonol. Mast cells were preincubated 5 min at 37 °C before adding bisoxonol (100 nM). Tetraethylammonium chloride (TEA); 4-aminopyridine (4-AP), mast cell degranulating-peptide (MCD), 4,4'-diisocyanatostilbene-2,2'-disulfonic acid (DIDS) and cadmium (Cd^{2+}) were added at final concentrations of 20 mM, 1 mM, 10^{-6} M, $3 \cdot 10^{-5}$ M, and 10^{-4} M, respectively.

the effect of potassium is entirely dependent on the activity of the sodium pump. Further addition of K^+ at 30 mM did not increase the fluorescence of bisoxonol (Fig. 5C). In contrast, the addition of 10 μg/ml of gramicidin allowed to reach the fluorescence intensity observed in the absence of K^+ (Fig. 5A). When mast cells were pretreated with 5.6 mM deoxyglucose and 10^{-4} M dinitrophenol in order to deplete intracellular stores of ATP, the addition of neither K^+ nor gramicidin significantly changed the fluorescence intensity (Fig. 5B).

Fig. 6 shows the effect of various ionic channels inhibitors. The effect of tetraethylammonium chloride, 4-aminopyridine and mast cell degranulating-peptide which affect Ca^{2+}-activated and voltage-sensitive K^+-channels, were studied. Tetraethylammonium and 4-aminopyridine did not significantly affect the fluorescence of bisoxonol (Figs. 6A and B). A slight decrease was observed for TEA due to the dye dilution. In contrast, mast cell degranulating peptide decreased the fluorescence intensity of bisoxonol (Fig. 6C) suggesting a hyperpolarization of the plasma membrane. However, MCD induced histamine secretion (not shown) under these conditions. Figs. 6D and 6E show the effect of DIDS, an inhibitor of chloride channels in mast cells [3] and Cd^{2+} an inhibitor of Ba^{2+}-sensitive Ca^{2+} currents in mast cells [5,6] on the fluorescence of bisoxonol. DIDS and Cd^{2+} did not significantly modify the fluorescence of bisoxonol. However, a slight decrease was observed for DIDS apparently not due to an inhibitory effect of chloride channels, since a depolarization would be expected, i.e. an increase in the fluorescence of bisoxonol.

Changes in the fluorescence of bisoxonol mast cell activation

The effect of compound 48/80 on the fluorescence of bisoxonol is shown in Fig. 7. When mast cells were triggered by compound 48/80,, a fast decrease of bisoxonol fluorescence was followed by a plateau and then by a slower decrease (Fig. 7A). The addition of gramicidin partially restored the fluorescence which, however, did not reach the initial value (Fig. 7A). In ATP-depleted mast cells, compound 48/80 did not change the intensity of fluorescence (Fig. 7B) clearly demonstrating that changes in the fluorescence of bisoxonol induced by compound 48/80 was an energy-dependent process. Therefore, the decrease of the fluorescence intensity may be due to the secretion process inducing changes in the spectra of bisoxonol and/or a dye extrusion. Both hypotheses were investigated.

Fig. 8 shows the absorption spectra of bisoxonol in stimulated and non stimulated mast cells. The spectrum of bisoxonol was not changed in stimulated mast cells. Only the intensity of bisoxonol was lowered. Similar results were obtained with the emission spectrum (not shown). These results suggest that the decrease of bisoxonol fluorescence observed at 540 nm when compound 48/80 was added to the cells was not due to changes of the physico-chemical properties of bisoxonol.

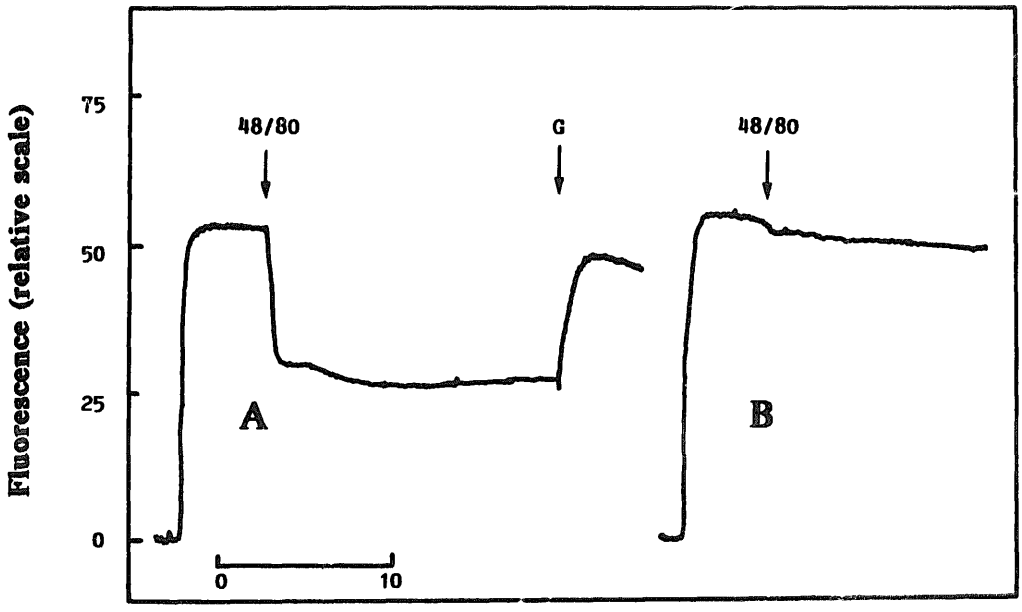

Fig. 7. Effect of compound 48/80 on the fluorescence of bisoxonol. (A) Mast cells were preincubated for 45 min at 37 °C in the presence of 5.6 mM glucose. Compound 48/80 and gramicidin (G) were added at 0.2 μg/ml and 10 gmg/ml, respectively. (B) Mast cells were preincubated for 45 min at 37 °C in the absence of glucose but in the presence of 5.6 mM deoxyglucose and 10^{-4} M dinitrophenol. Compound 48/80 was added at 0.2 μg/ml (final concentration). Traces were representative of three different experiments. The scale indicates the time in min.

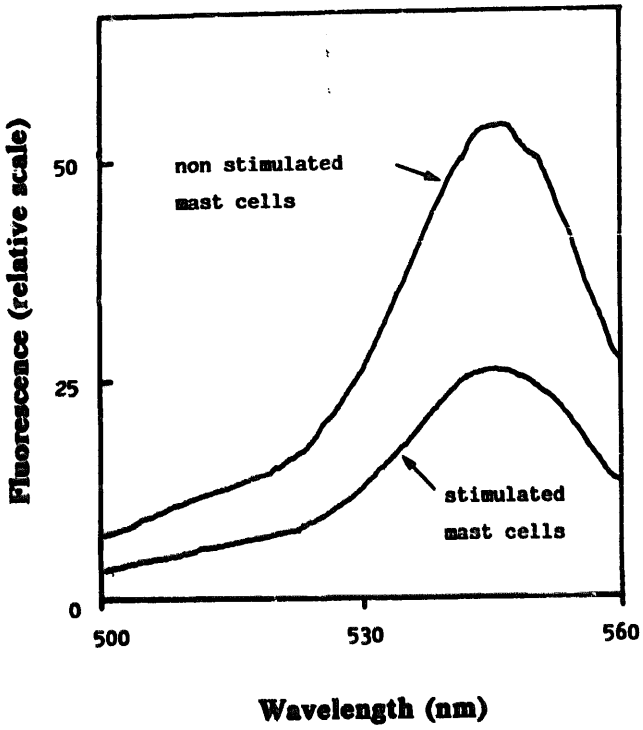

Fig. 8. Fluorescence excitation spectra of bisoxonol before and after the stimulation of mast cells. Fluorescence of bisoxonol was recorded at 580 nM. Mast cells were labeled with 100 nM of bisoxonol and were stimulated with 0.2 μg/ml of compound 48/80. Traces were representative of two separate experiments.

Fig. 9 shows the effect of a pretreatment of mast cells with pertussis toxin in the presence or absence of potassium. Experiments were performed with 0.1 μg/ml of compound 48/80 (Fig. 9A) instead of 0.2 μg/ml. This dose was a more accurate dose to obtain a good separation of the two phases of fluorescence intensity decrease. The first phase was lowered while the delayed one was increased (Fig. 9A). When mast cells were pretreated with pertussis toxin, which inhibits histamine secretion induced by compound 48/80 [23,24] only the delayed phase could be distinguished (Fig. 9B). This was abolished when the sodium pump was inhibited, i.e., mast cells were placed in a potassium free medium (Fig. 9C). Thus, compound 48/80 had no effect when pertussis toxin-pretreated mast cells were placed in a potassium-free medium (Fig. 9D). In summary, compound 48/80 induced two phases of bisoxonol fluorescence decrease, a first one sensitive to pertussis toxin and the second one dependent on the activity of the sodium pump.

Discussion

The plasma membrane potential of rat peritoneal mast cells was assessed by using a fluorescent hydrophobic potential-sensitive probe, bisoxonol. This probe is negatively charged and prefers a hydrophobic environment, according to the membrane polarization state, where the fluorescence quantum yield increases more than 20-fold. Therefore, an increase in the fluorescence of bisoxonol usually corresponds to the depolarization of the plasma membrane and conversely a decrease corresponds to a repolarization, or hyperpolarization of the plasma membrane. Bisoxonol was first used in lymphocytes and was shown to be a better probe than 3,3'-dipropylthiadicarbocyanine because it showed numerous side effects, e.g. it had by itself a depolarizing effect and it inhibited B cell capping [21].

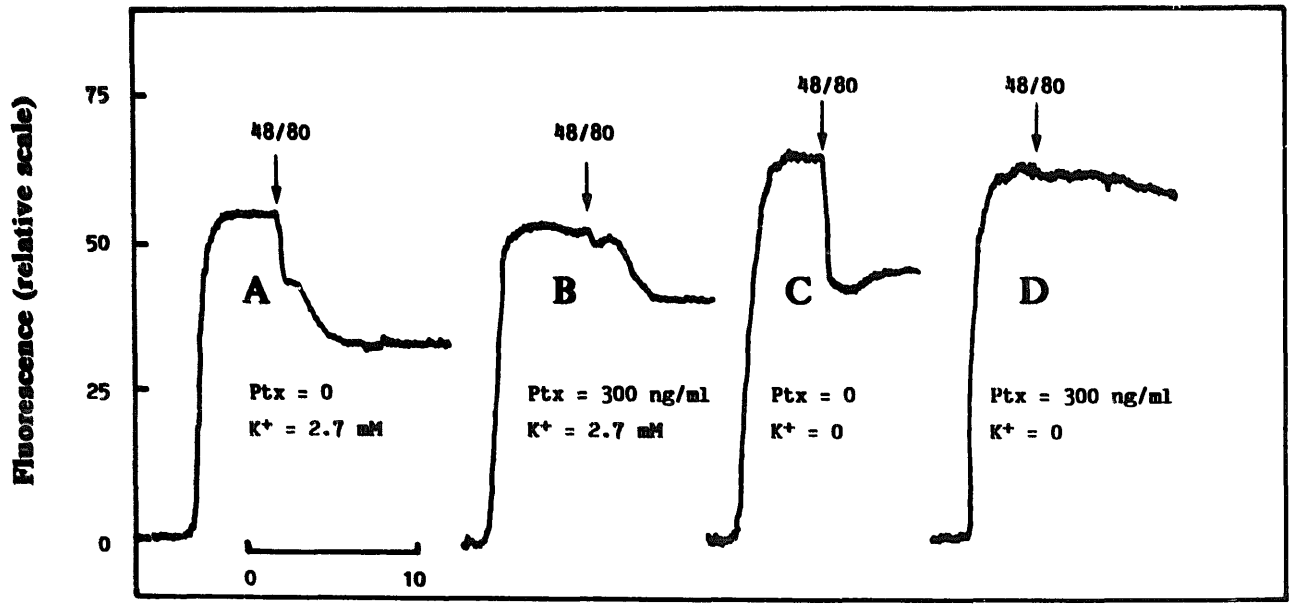

Fig. 9. Effect of compound 48/80 on the fluorescence of bisoxonol under various conditions given on traces. The pretreatment of mast cells with pertussis toxin (Ptx) was carried out for two hours at 37 °C at a concentration of 300 ng/ml. Compound 48/80 was added under each condition at 0.1 μg/ml (final concentration). The scale indicates the time in min. Experiments were performed four times.

However, bisoxonol forms complexes with valinomycin thus ruling out the use of this ionophore which is the current tool to correlate fluorescence intensities of other oxonol dyes and the membrane potential in mV [9,10]. Another probe used as indicator of membrane potential is tetraphenylphosphonium, but its slow equilibrium distribution and its significant accumulation in mitochondria [25] hinders its usefulness. In contrast, it has been reported that bisoxonol is a useful indicator of membrane potential in rat basophilic leukemia cells taking advantage of its rapid equilibration with the cells and that it is not affected by changes in mitochondrial potential [15]. Previously, we reported that rat basophilic leukemia cells behaved differently from mast cells according to data obtained with bisoxonol [18]. We showed that maximal fluorescence intensity of bisoxonol was achieved in mast cells when the sodium pump was inhibited and in rat basophilic leukemia cells when they were placed in high potassium medium. We concluded that the plasma membrane potential of mast cells was controlled mainly by the sodium pump and that of rat basophilic leukemia cells (RBL cells) by a potassium channel permeability which is in accordance with [15]. However, since mast cells and RBL cells are morphologically different cells, e.g. the density and the size of granules, studies had to be undetaken to localize mast cell cellular structures labeled by bisoxonol. Preliminary controls (Fig. 1) showed that 100 nM of bisoxonol and a mast cell density of $4.5 \cdot 10^5$ cells/ml were adequate experimental conditions to monitor changes in the equilibrium of bisoxonol with the cells, since this dye at 100 nM did not saturate binding sites in mast cells and there was still probe available in the aqueous phase. Bisoxonol rapidly equilibrates with mast cells (present results and Ref. 18), i.e. 90% of the fluorescence intensity was obtained within 20 s. Microphotographies showed that the dye was distributed in the whole cell except in the nucleus (Fig. 2). Permeabilization of mast cells did not increase the fluorescence of bisoxonol indicating that this probe was not only localized in the plasma membrane in contrast to TMA-DPH (Fig. 3). A similar behaviour was found for diphenylhexatriene, the cell permeant analog of TMA-DPH, in mast cells [17] and for other cells types (Ref. 26 and references herein).

Bisoxonol belongs to a family of oxonol dyes whose mechanism of transducing changes in the membrane potential has been well studied [27,28]. When a putative potential-sensitive probe is distributed throughout the cytoplasm careful attention has to be paid to the possible interferences of mitochondrial potential. However, as mentioned by Mohr and Fewtrell [15], the negative charges of bisoxonol ensures that the dye does not accumulate in mitrochondria. They showed that membrane depolarization induced by rotenone and oligomycin did not modify the fluorescence of bisoxonol in RBL cells. In this study, we showed that this is also the case for rat peritoneal mast cells (Fig. 4). Therefore, we suggest that in spite of its intracellular localization bisoxonol is a valuable probe for monitoring changes in the plasma membrane potential. We showed that the fluorescence intensity of bisoxonol in mast cells, when the sodium pump was inhibited, was similar to that obtained in the presence of gramicidin when the sodium pump worked (Fig. 5). Considering that gramicidin is currently used to induce maximal depolarization, our results suggest that the inhibition of the sodium pump induced maximal depolarization of mast cells, and rules out a possible involvement of potassium channel permeability in maintaining the resting plasma membrane potential of rat mast cells. This was confirmed by the lack of a significant effect of high concentrations of potassium (Fig. 5), 4-aminopyridine and tetraethylammonium chloride (Fig. 6) which are known to affect most potassium channels (review in Ref. 29). However, mast cell-degranulating peptide (MCD), an inhibitor of some voltage sensitive potassium channels [30], induced a decrease of the fluorescence of bisoxonol similar to that observed with compound 48/80 (Fig. 7). We recently showed that the mechanism of action of MCD in mast cells was similar to that of compound 48/80 [31]. The interpretation of the effect of MCD on bisoxonol must be made in parallel with those for compound 48/80 (see later). The ionic regulation of the sodium pump [32,33] and its role in the mast cell-sensitivity to various secretagogues [34,35] have been sufficiently described elsewhere.

DIDS, a chloride channel inhibitor in mast cells [3], and cadmium, an inhibitor of calcium channels in mast cells [5,6], did not significantly affect the fluorescence of bisoxonol, suggesting that these channels were not involved in the regulation of the plasma membrane potential of resting mast cells. However, these results did not exclude the existence of ionic channels, but do support the idea that they influence the plasma membrane potential in a lower magnitude than the sodium pump does. The fact that the plasma membrane potential is mainly set by the sodium pump is not a unique feature of mast cells since neutrophils and Lettré cells were also concerned [9,10].

The changes of bisoxonol fluorescence during mast cell activation were also studied. We observed that compound 48/80 at 0.2 μg/ml induced a decrease of the fluorescence of bisoxonol in an energy dependent process (Fig. 7). The further addition of gramicidin could not a re-enhance the fluorescence of bisoxonol above the initial value indicating that changes in the fluorescence might not only be related to changes in the membrane potential. Changes in the fluorescence spectra of bisoxonol were not involved (Fig. 8). Dye exocytosis seems to be a reasonable possibility. The

possibility of misinterpretations of results have also been suggested with other intracellular localized dyes (i.e. quin-2 and fura 2), used for monitoring cytosolic calcium changes [13,14]. Significant amounts of these dyes accumulate in granules, which then may be exocytosed and therefore reflects the extracellular calcium concentrations [13,14]. The exocytosis of bisoxonol during mast-cell activation is not demonstrated in the present paper. However, we have observed that activated mast cells completely lost their fluorescence (Fig. 2). Strikingly, the concentration of 0.2 μg/ml compound 48/80 induced the loss of fluorescence in approximately half of the number of mast cells which might therefore be responsible for the 50% decrease of the fluorescence. Thus, this decrease could arise from the complete loss of fluorescence of 50% of the number of mast cells. If this is the case the decrease of fluorescence cannot be due the hyperpolarization of mast cells because this would mean that mast cells undergo drastic hyperpolarization during mast cell activation which has never been reported [2–4,7]. Bisoxonol has been used in RBL-cells stimulated via the immunological pathway. It was shown that the antigenic stimulation of RBL cells induced a depolarization largely occurring before secretion started [15]. Since these authors observed a distinct kinetic of the fluorescence intensity increase and of the secretion process itself, it is clear that these two signals did not interfer. However, compound 48/80-induced exocytosis [17] and the decrease of bisoxonol fluorescence (present results) are fast signals and, thus, hardly distinguishable.

In contrast we obtained interesting results when the exocytotic process was blocked. For this we used pertussis-toxin which is known to interfere with some G proteins and therefore acts at an early stage of stimulus-secretion coupling process. This toxin has been reported to block mast cell exocytosis induced by compound 48/80 [23], mastoparan, substance P [24] and bradykinin [36]. In mast cells treated with pertussis toxin compound 48/80 only induced a decrease of the fluorescence of bisoxonol after only 1.5 min (Fig. 9). This was abolished in the absence of potassium suggesting that it was due to the activation of the sodium pump. Similar results were obtained with zinc gluconate, instead of a pertussis toxin pretreatment [37]. However, the decrease of bisoxonol fluorescence started after 1.5 min ruling out the possibility that it can play a role in the initiation of the secretory process because compound 48/80-induced exocytosis is very rapid and was completely terminated within 1 min [12,17]. However, the activation of the sodium pump and/or the changes in the membrane potential of mast cells might play a role in the termination of the secretory process. We previously suggested that protein kinase C may play a role in the termination of the secretory process induced by compound 48/80 [38]. Furthermore, it was shown, in vitro, that the sodium pump is a substrate for protein kinase C [39]. Such an interaction needs further investigation in mast cells. Whatever the case may be, our results suggest that there are no changes in the plasma membrane potential of rat peritoneal mast cells which might play a possible role in the activation of mast cells. The changes in the membrane potential may rather be a consequence.

In conclusion, we propose that bisoxonol is a useful probe for studying the plasma membrane potential of rat peritoneal mast cells at the resting state or during activation but with blocked-exocytosis.

Acknowledgements

The authors wish to thank Dr. Paul Fontenеau who carried out the fluorescence microphotographies. This work was supported by INSERM (CRE 89-5009 and CJF 91-05) and by the Fondation pour la Recherche Médicale.

References

1 Mousli, M., Bueb, J.L., Bronner, C., Rouot, B. and Landry, Y. (1990) Trends Pharmacol. Sci. 11, 358–362.
2 Tasaka, K., Sugiyama, K., Komoto, S. and Yamasaki, H. (1970) Proc. Japan Acad. 46, 826–830.
3 Penner, R., Matthews, G. and Neher, E. (1988) Nature (London) 334, 499–504.
4 Lindau, M. and Fernandez, J.M. (1986) Nature 319, 150–153.
5 Kuno, M., Kawaguchi, J., Mukai, M. and Nakamura, F. (1990) Am. J. Physiol. 259, C715–C722.
6 Kuno, M., Okada, T. and Shibata, T. (1989) Am. J. Physiol. 256, C560–C568.
7 Lindau, M. and Fernandez, J.M. (1986) J. Gen. Physiol. 88, 349–368.
8 Apell, H.-J. (1989) J. Membr. Biol. 110, 103–114.
9 Bashford, C.L. and Pasternak, C.A. (1984) J. Membr. Biol. 79, 275–284.
10 Bashford, C.L. and Pasternak, C.A. (1986) Trends Biochem. Sci. 11, 113–116.
11 Kuhry, J.G., Bronner, C., Amellal, M. and Landry, Y. (1985) Agent Action 16, 109–112.
12 Bronner, C., Kuhry, J.G. and Landry, Y. (1986) Agent Action 18, 53–56.
13 Bibb, P.C., Cochrane, D.E. and Morel-Laurens, N. (1986) FEBS Lett. 209, 169–174.
14 Almers, W. and Neher, E. (1985) FEBS Lett. 192, 13–18.
15 Mohr, C.F. and Fewtrell, C. (1987) J. Immunol. 138, 1564–1470.
16 Mohr, C.F. and Fewtrell, C. (1987) J. Cell. Biol. 104, 783–792.
17 Bronner, C., Landry, Y., Fonteneau, P. and Kuhry, J.G. (1986) Biochemistry 25, 2149–2154.
18 Bronner, C., Mousli, M., Eleno, N. and Landry, Y. (1989) FEBS Lett. 255, 401–404.
19 Tatham, P.E.R., Cusack, N.J. and Gomperts, B.D. (1988) Eur. J. Pharmacol. 147, 13–21.
20 Tatham, P.E.R. and Lindau, M. (1990) J. Gen. Physiol. 95, 459–476.
21 Rink, T.J., Montecucco, C., Mesketh, T.R. and Tsien, R.V. (1980) Biochim. Biophys. Acta 595, 15–30.

22 Kuhry, J.G., Duportail, G., Bronner, C. and Laustriat, G. (1985) Biochim. Biophys. Acta 845, 60–67.
23 Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584–3593.
24 Mousli, M., Bronner, C., Bueb, J.-L., Tschirhart, E., Gies, J.-P. and Landry, Y. (1989) J. Pharmacol. Exp. Ther. 250, 329–335.
25 Sagi-Eisenberg, R. and Pecht, I. (1983) J. Membr. Biol. 75, 97–104.
26 Kuhry, J.G., Fonteneau, P., Duportail, G., Maechling, C. and Laustriat, G. (1983) Cell Biophys. 5, 129–140.
27 George, E.B., Nyirjezy, P., Basson, M., Ernst, L.A., Pratap, P.R., Freedman, J.C. and Waggoner, A.S. (1988) J. Membr. Biol. 103, 245–253.
28 George, E.B., Nyirjezy, P., Pratap, P.R., Freedman, J.C. and Waggoner, A.S. (1988) J. Memb. Biol. 105, 55–64.
29 Cook, N.S. (1988) Trends Pharmacol. Sci. 9, 21–28.
30 Rehm, H., Bidard, J.-N., Schweitz, M. and Lazdunski, M. (1988) Biochemistry 27, 1827–1832.
31 Mousli, M., Bronner, C., Bueb, J.L. and landry, Y. (1991) Mol. Pharmac. Sci. 207, 249–255.
32 Knudsen, T. and Johansen, T. (1989) Br. J. Pharmacol. 96, 773–778.
33 Knudsen, T., Berthelsen, H. and Johansen, T. (1989) Br. J. Pharmacol. 100, 453–456.
34 Amellal, M., Bronner, C. and Landry, Y. (1985) Br. J. Pharmac. 85, 819–826.
35 Bronner, C., Gies, J.P., Vallé, A. and Landry, Y. (1987) Life Sci. 41, 2555–2562.
36 Bueb, J.L., Mousli, M., Bronner, C., Rouot, B. and Landry, Y. (1990) Mol. Pharmacol. 38, 816–822.
37 Bronner, C., Ratsimbason, M., Pelen, F. and Landry, Y. (1991) Agent Action 33, 88–91.
38 Bronner, C., Vallé, A., Gies, J.P. and Landry, Y. (1986) Ann. Inst. Pasteur/Immunol. 137D, 249–261.
39 Lowndes, J.M., Hokin-Neaverson, M. and Bertics, P.J. (1990) Biochim. Biophys. Acta 1052, 143–151.