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The role of anion channels in the mechanism of acrosome reaction in bull spermatozoa

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The involvement of anion channels in the mechanism of the acrosome reaction (AR) was investigated. The AR was induced by Ca^{2+} or by addition of the Ca^{2+} ionophore A23187. The occurrence of AR was determined by following the release of acrosin from the cells. In order to investigate the role of anion channels in the AR, several anion-channel inhibitors were tested, mainly DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid). Other blockers, like SITS (4-acetamido-4'-isothicyanostilbene-2,2'-disulfonic acid), furosemide, probenecid and pyridoxal 5-phosphate, were also tested. We found that DIDS binds covalently to sperm plasma membrane in a time- and concentration-dependent manner. Maximal binding occurs after 2 h with 0.3 mM DIDS. DIDS and SITS inhibit AR in a concentration-dependent manner. The IC_{50} of DIDS and SITS in the presence of A23187 is 0.15 and 0.22 mM, respectively. Tributyltin chloride (TBTC), an Cl^-/OH^- exchanger, partially overcomes DIDS inhibition of the AR. HCO_3^- is required for a maximal acrosin release and Ca^{2+}-uptake, in the presence or absence of A23187. It is known that HCO_3^- activates adenylate cyclase and therefore, increases the intracellular level of cAMP. The inhibition of the AR by DIDS decreases from 95 to 50% when (dibutyryl cyclic AMP (dbcAMP) was added, i.e., HCO_3^- is no longer required while elevating the level of cAMP in an alternative way. Moreover, we show that the stimulatory effect of HCO_3^- on Ca^{2+}-uptake is completely inhibited by DIDS. We conclude that DIDS inhibits AR by blocking anion channels, including those that transport HCO_3^- into the cell.

Introduction

The acrosome reaction is a prerequisite for successful fertilization in mammals, defined as a Ca^{2+}-dependent exocytotic event in sperm, in which membrane fusion takes place between the outer acrosomal membrane and the overlying plasma membrane, thereby allowing release of the acrosomal contents [1,2].

The involvement of cAMP in acrosome reaction was suggested by Hyne and Garbers [3] and later on they showed that bicarbonate is required for the Ca^{2+}-induced elevations of cAMP in guinea-pig spermatozoa [4]. Stengel and Hamoune [5] observed the activation of ram sperm adenylate cyclase by bicarbonate but not by Ca^{2+}, and Garty and Salomon [6] reported that bicarbonate directly activated partially purified bull sperm adenylate cyclase, results which are consistent with the findings of Okamura et al. [7]. Although the role of bicarbonate and cyclic nucleotides in the sperm acrosome reaction has been widely accepted, the regulation of their metabolism is not clearly understood.

The existence of a bicarbonate transport system in porcine spermatozoa was demonstrated by Okamura et al. [8]. In a more recent report, Visconti et al. [9] have shown that blockers of anion channels inhibit the bicarbonate-dependent phorbol ester enhancing effect on cAMP in hamster spermatozoa, but they did not find any effect on acrosome reaction. In an earlier paper, Hyne [10] demonstrated a requirement of HCO_3^- in monensin (Na^+/H^+ exchanger) induced acrosome reaction in guinea-pig sperm, which was inhibited by low concentrations of the anion-channel blocker diisothiocyanatestilbene-2,2'-disulfonic acid (DIDS). This blocker also prevented the occurrence acrosome reaction in starfish spermatozoa [11]. In porcine epididymal sperm, DIDS enhanced bicarbonate-induced elevation in cAMP content and this effect was not observed in the absence of bicarbonate [12].

In the present report, we were investigating the role of the anion channel in the mechanism of ejaculated

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Abbreviations: DIDS 4,4'diisothiocyanostilbene-2,2'-disulfonic acid; SITS, 4-acetamido-4'-isothicyanostilbene-2,2'-disulfonic acid; TBTC, tributyltin chloride; AR, acrosome reaction.

bull sperm, acrosome reaction and we suggest an explanation for the contradictions found in the literature concerning the effect of anion-channel blockers on the intracellular level of cAMP and acrosome reaction which are described here.

Materials and Methods

Sperm preparation. Frozen bull sperm cells were thawed at 37°C by taking one frozen capsule ($5 \cdot 10^7$ cells) into 0.875 ml of medium comprising of 150 mM NaCl and 10 mM histidine (pH 7.4). The cells were washed by three centrifugations at $780 \times g$, at 25°C for 10 min and were resuspended in TALP medium [13] to reach a final concentration of 10^8 cells/ml. The composition of TALP is: 94.3 mM NaCl, 4.0 mM KCl, 0.7 mM Na_2HPO_4, 26 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 12.8 mM glucose, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate, 3 mg/ml BSA, 10 IU/ml penicilline and 20 mM Hepes (pH 7.4).

DIDS binding to sperm. DIDS binding to the cells was determined by a fluorescent method. Sperm cells (10^8/ml) were incubated in TALP in the presence of DIDS. At the times indicated, the tubes were removed and 4 ml of buffer A (110 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 10.0 mM Mops, pH 7.4) containing 1% BSA and 1 mM NaN_3 were added. The cells were then washed five times by centrifugation at $780 \times g$ for 10 min to remove unbound DIDS, with BSA being omitted from the last two washes. The final cell pellet was resuspended in buffer A to a final concentration of 10^8 cells/ml. Fluorescence measurements of the samples were then made in a Shimadzu RF-5000 recording spectrofluorophotometer equipped with a thermostated stirred cell compartment (39°C). The fluorescence was measured with an excitation of 336 nm and an emission of 435 nm, and corrected for the zero time control.

Determination of acrosome reaction. Cells (10^8/ml) in TALP buffer containing 95% air 5% CO_2 were incubated in sealed tubes and the occurrence of acrosome reaction was determined by following the amount of acrosin released from the cells as described by us earlier [14].

Calcium uptake. ^{45}Ca accumulation in the cells was performed by the filtration technique as described by us elsewhere [15]. Briefly, sperm cells (10^8/ml) were incubated in TALP containing 2 mM $CaCl_2$ and 2 μCi $^{45}CaCl_2$. At apropriate time intervals, 0.1-ml samples were removed and immediately vacuum-filtered on GF/C filters. The cells trapped on the filter were washed three times with ice-cold solution composed of 150 mM NaCl, 10 mM Tris (pH 7.4) and 2 mM EGTA. The dry filters were counted for β-radioactivity. All data are expressed as the experimental value corrected for the zero time control.

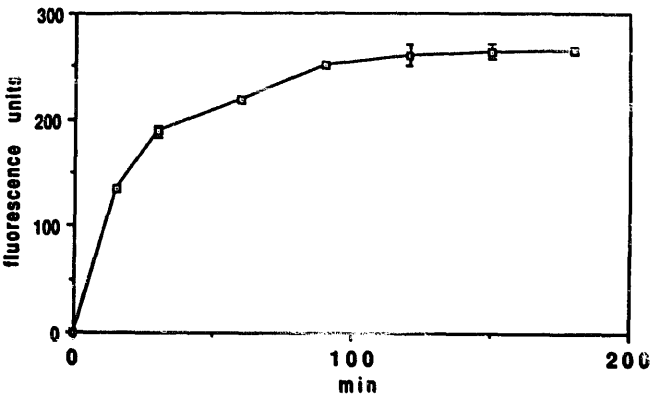

Fig. 1. The kinetics of DIDS binding to sperm cell membranes. Sperm cells (10^8/ml) suspended in TALP were incubated at 39°C in the presence of 0.3 mM DIDS. At appropriate time intervals, the tubes were removed and the cells were washed as described in Materials and Methods, in order to remove the unbound DIDS. Then, the cells were resuspended in 1.0 ml buffer A and the relative fluorescence was measured. Background fluorescence of the buffer and cells, as well as zero time control, were subtracted from the data. The values are means ± S.E. of two different experiments.

Materials. The Ca^{2+} ionophore A23187 (Sigma) and SITS were dissolved in dimethylfuranamide/ethanol (3:1, v/v) and the final concentration of the solvent in the experiments never exceeded 0.1%. DIDS, SITS and dibutyryl cyclic AMP were purchased from Sigma, tributyltin chloride from BDH chemicals and $^{45}CaCl_2$ from New England Nuclear.

Results

DIDS binding to sperm cell plasma membrane

In order to investigate the role of anions and anion channels in the AR, several anion-channel inhibitors were tested, primarily DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), the most potent anion-channel inhibitor so far examined. It binds covalently to the plasma membrane but does not penetrate into the cell [16].

The kinetics of DIDS binding to sperm plasma membrane was studied (Fig. 1). DIDS is a fluorescent compound whose fluorescence is enhanced upon binding. The fluorescence is maximal after 2 h incubation of 10^8 cells/ml with 0.3 mM DIDS, indicating that all binding sites are saturated at this time. The dose response curve for DIDS binding is shown in Fig. 2. Cells were incubated for 120 min with increasing concentrations of DIDS, washed five times to remove unbound DIDS and the fluorescence was measured. The binding was sigmoidal with linear binding between 0.05 and 0.2 mM and reached a plateau at 0.3 mM.

Acrosome reaction

The effect of anion-channel blockers on the acrosome reaction. The effect of anion-channel blockers on AR

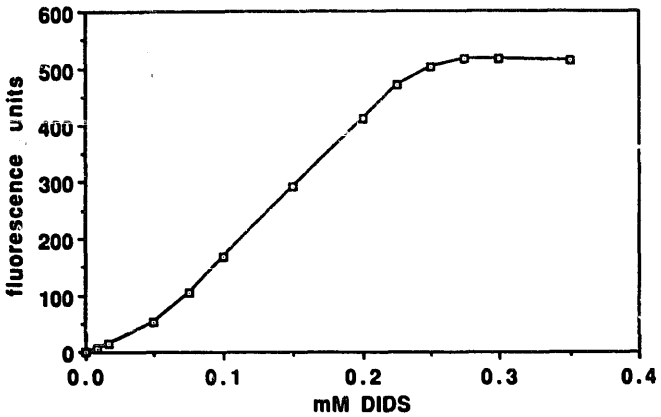

Fig. 2. Dose response for DIDS binding to sperm cell plasma membrane. Sperm cells (10^8/ml) were suspended in TALP and incubated at 39°C for 120 min in the presence of increasing concentrations of DIDS. 2 h later the tubes were removed and cells were washed as described in Materials and Methods. Cells were resuspended in 2.0 ml buffer A and the fluorescence intensity of the cells was measured. The self-fluorescence of the cells and the medium were substracted from the data. The values are means of a representative experiment.

can be seen in Fig. 3. Sperm cells (10^8/ml) were incubated for 3 h in TALP containing 2.0 mM $CaCl_2$, 2.0 μM Ca^{2+} ionophore A23187 and various concentrations of DIDS or SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid). The extent of the acrosome reaction was monitored by measuring acrosin release from the cells. Acrosin activity was measured by following the hydrolysis of benzoyl-arginine ethyl ester (BAEE). The values were corrected for zero time controls. The IC_{50} for DIDS and SITS were 0.15 mM and 0.22 mM, respectively. At 0.3 mM DIDS, we see almost complete inhibition of the in

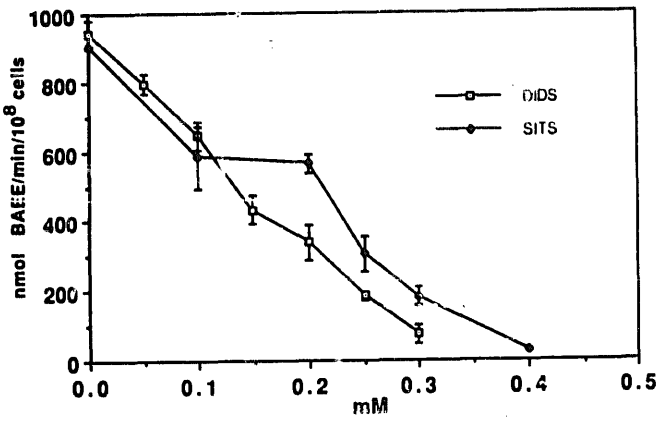

Fig. 3. The effect of DIDS and SITS on acrosin release. Sperm cells (10^8/ml) were suspended in TALP and incubated for 3 h at 39°C in the presence of 2.0 mM $CaCl_2$ and 2.0 μM A23187. Increased concentrations of DIDS and SITS were added from the beginning of the incubation. Acrosin activity was determined by reacting with BAEE as described in Materials and Methods. The zero time or EGTA control was subtracted from the data. Each value represents the mean ± S.E. of duplicates from two different experiments.

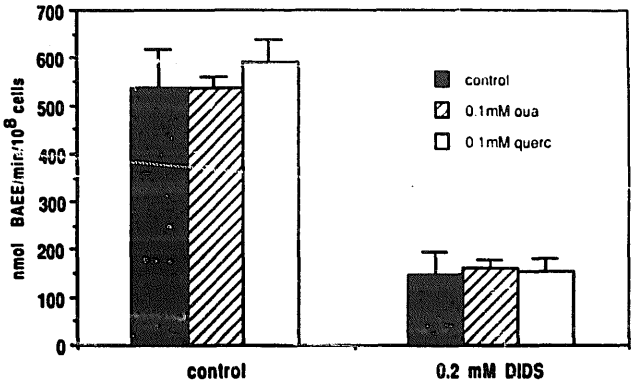

Fig. 4. The effect of ouabain and quercetin on acrosin release. Acrosin preparations were achieved as described in the legend to Fig. 3. Bars represent the means ± S.E. of duplicates from at least two different experiments.

AR, which is in good correlation with the saturation of DIDS binding shown in Fig. 2. The concentrations of the anion-channel blockers used here, although higher than those used with erythrocyte ghosts [17] were similar to those used with other cells, including spermatozoa [8,11].

In order to obtain more information concerning anion channels, we used other blockers, which are: (1), Furosemide, a Na, K–Cl cotransport inhibitor [18]; (2), probencid, an organic anion transport inhibitor [19] and (3), pyridoxal 5-phosphate, an anionic probe for protein amino groups [20], which does not bind covalently in our assay. None of these compounds inhibit the acrosome reaction when 0.4 mM furosemide, 1.0 mM probencid and 0.3 mM pyridoxal 5 phosphate were used (data not shown).

The effect of ATPase inhibitors on the acrosome reaction. It has been shown that DIDS inhibits ATPases in several cell types [21–23]. We thus raised the question whether DIDS inhibits the AR via inhibition of ATPase activity. Therefore, the sensitivity of the AR to other ATPase inhibitors, ouabain and quercetin, was tested. Cells were incubated for 3 h in TALP containing ouabain or quercetin. As is shown in Fig. 4, these ATPase inhibitors did not affect the rate of acrosin release. Quercetin even increased it slightly (10%). Moreover, 0.2 mM DIDS had the same effect (70% inhibition of AR) with or without the ATPase inhibitors, a result which supports the idea that the ATPase inhibitors are not competing for the DIDS binding sites on the cells.

Our next step was to determine which anions are involved in the AR and what is their mechanism of action.

The effect of bicarbonate on the acrosome reaction. Fig. 5 shows the importance of bicarbonate ions for the occurrence of the AR. HCO_3^- increased the rate of acrosin release by 100%. The inhibition of AR by DIDS was greater when HCO_3^- was not added (95%)

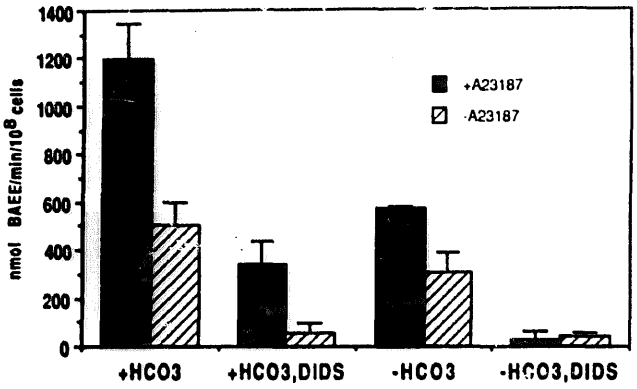

Fig. 5. The effect of DIDS, HCO_3^- and A23187 on acrosin release. Acrosin preparations were achieved as described in the legend to Fig. 3. Sperm cells (10^8/ml) were incubated in the presence or in the absence of 0.2 mM DIDS, 26.0 mM HCO_3^- or 2 μM A23187. Tubes that contained bicarbonate were incubated under 95% air/5% CO_2 and closed with a stopper. Bars represent the means ± S.E. of duplicates from at least three different experiments.

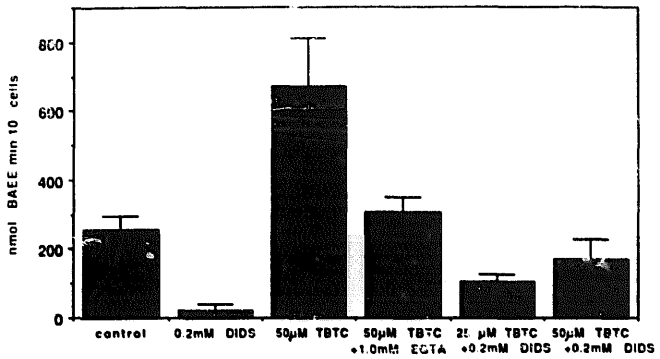

Fig. 6. The effect of TBTC on acrosin release in the absence of A23187. Acrosin preparations were achieved as described in the legend to Fig. 3. All tubes contained 2.0 mM $CaCl_2$ with the exception of the tube which contained 1.0 mM EGTA. Bars represent the means of duplicates from three different experiments.

than in the presence of the anion (73%), indicating that bicarbonate and DIDS might compete for the same binding sites in the cells. In absence of the Ca^{2+} ionophore, the stimulatory effect of HCO_3^- on the rate of acrosin release is smaller (60%), but the inhibition of the AR by DIDS remained basically the same (86 and 89%) whether HCO_3^- was added or not. We can also see that the enhanced effect of HCO_3^- on AR, induced by A23187, is very high (1060%) in the presence of DIDS. In absence of A23187, a very small stimulatory effect of HCO_3^- is seen in the presence of DIDS.

The effect of tributyltin chloride (TBTC) on the acrosome reaction. One of the prerequisites for the occurrence of the AR is the alkalinization of the cytosol [10,24]. HCO_3^- is known to alkalize the cell interior by becoming protonated, and this might be one of the mechanisms by which HCO_3^- stimulates AR. Since bicarbonate transport is blocked by DIDS [8], alkalization of the cytosol artificially would partially bypass the inhibitory effect of DIDS on the AR.

In order to alkalize the cytosol, an artificial Cl^-/OH^- exchanger, tributyltin chloride (TBTC) was used and its effect on AR is shown in Fig. 6.

TBTC caused a 3-fold stimulation in acrosin release, but this effect is evidently unrelated to the Ca^{2+} stimulation of AR, since it can be seen in the presence of EGTA. The stimulatory effect of TBTC was found only in absence of A23187 (Fig. 6), but not in its presence (Fig. 7).

The inhibition of the AR by DIDS was partially recovered in the presence of TBTC. Fig. 6 shows that 50 μM TBTC diminishes the inhibitory effect of DIDS from 90 to 72%. TBTC failed to recover the inhibition by DIDS when A23187 was present (Fig. 7).

The effect of dibutyryl cyclic AMP on acrosome reaction. Garbers and Kopf [25] have documented that cAMP plays an important role in the occurrence of capacitation and/or AR. The permeant cAMP analogue, dibutyryl cyclic AMP (dbcAMP) induces the AR in mammals [26]. HCO_3^- is known to stimulate adenylate cyclase [7]. Therefore, an alternative explanation to the role of HCO_3^- in AR concerns its known stimulatory effect on adenylate cyclase, which catalyzes the production of cAMP in sperm cells [27]. The inhibition of HCO_3^--transport by DIDS would reduce the intracellular level of cAMP, but this should be reversed by introducing exogenous dbcAMP to the cells.

Fig. 8 shows the effects of 1 and 2 mM dbcAMP on the AR in the presence and in the absence of HCO_3^-. When HCO_3^- was added, 1 mM and 2 mM dbcAMP stimulate the AR by a factor 1.25 and 1.65, respectively. The effect of dbcAMP on the AR was much greater when HCO_3^- was not added to the medium. Here, 1.0 mM and 2.0 mM dbcAMP stimulates the AR

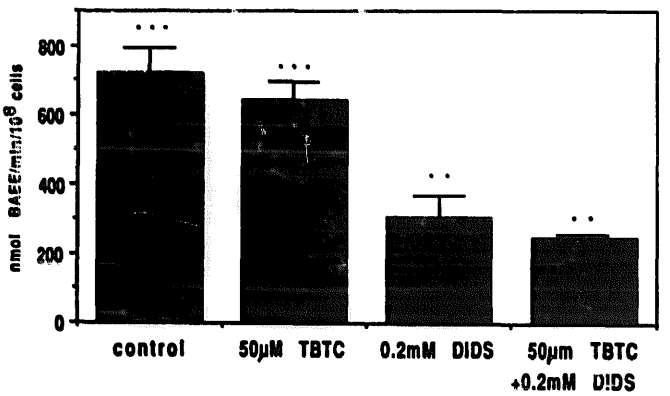

Fig. 7. The effect of TBTC on acrosin release in the presence of A23187. Acrosin preparations were achieved as described in the legend to Fig. 3. All tubes contained 2.0 mM $CaCl_2$ and 2 μM A23187. Bars represent the means ± S.E. of duplicates from two (**) or three (***) experiments.

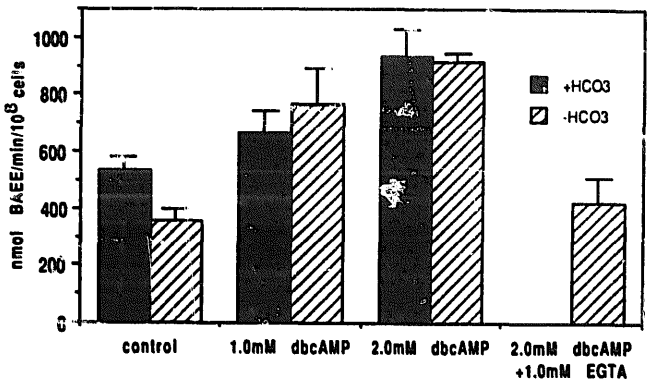

Fig. 8. The effect of dbcAMP on acrosin release. Acrosin preparations were achieved as described in the legend to Fig. 3, except that A23187 was not added. All tubes contained 2.0 mM $CaCl_2$ except that which contained 1.0 mM EGTA. Bars represent the means ± S.E. of duplicates from three different experiments.

by 2.01 and 2.25 times, respectively. It is interesting to note that dbcAMP can induce acrosin exocytosis in absence of extracellular Ca^{2+} (in the presence of 1.0 mM EGTA).

The addition of dbcAMP also partially overcomes the inhibitory effect of DIDS on the AR and this recovery was greater when HCO_3^- was not added to the medium (Fig. 9). In the presence of external HCO_3^-, 0.2 mM DIDS inhibits the rate of acrosin release by 87%, while the addition of dbcAMP decreased this inhibition to 18% only. When HCO_3^- was not added, DIDS inhibited the AR by 83%, while dbcAMP completely eliminated this inhibition and we see even stimulation compared to the control. By comparing the results in Figs. 8 and 9, we can see that dbcAMP cannot completely reverse the inhibition of DIDS. In the presence of 2.0 mM dbcAMP, 0.2 mM DIDS causes 57 and 48% inhibition of the AR, in the presence or absence of HCO_3^-, respectively.

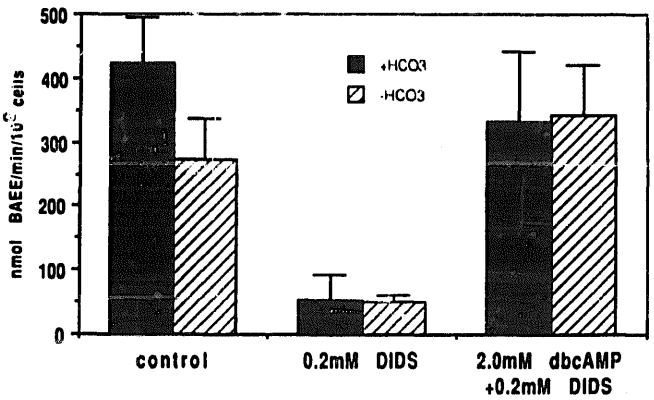

Fig. 9. The effect of dbcAMP on the Inhibition of DIDS on acrosin release. Sperm cells (10^8/ml) were incubated in the presence of 2.0 mM $CaCl_2$ with or without 0.2 mM DIDS and 2.0 mM dbcAMP. Bars represent means ± S.E. of duplicates from at least three different experiments.

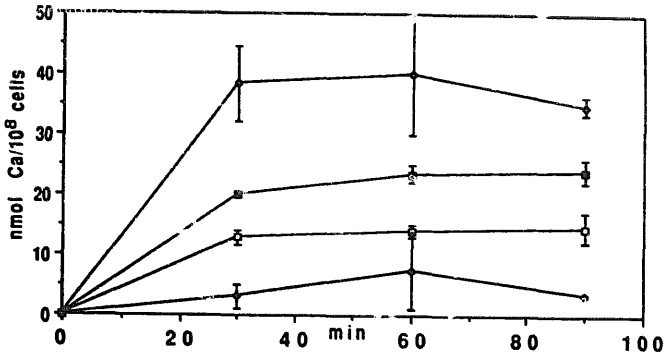

Fig. 10. The effect of HCO_3^- and A23187 on Ca^{2+} uptake into sperm cells. Sperm cells (10^8/ml) were suspended in TALP and incubated at 39°C in the presence or in the absence of 26 mM HCO_3^- and 2.0 μM A23187. The reaction was started by the addition of 2.0 mM $CaCl_2$ containing 2.0 μCi $^{45}CaCl_2$. The uptake of Ca^{2+} into the cells was determined as described under Material and Methods. Each point represents the means ± S.E. of duplicates from at least two experiments. The symbols are: □, control; ◆, with A23187 ■, with HCO_3^-; ◇, with A23187 and HCO_3^-.

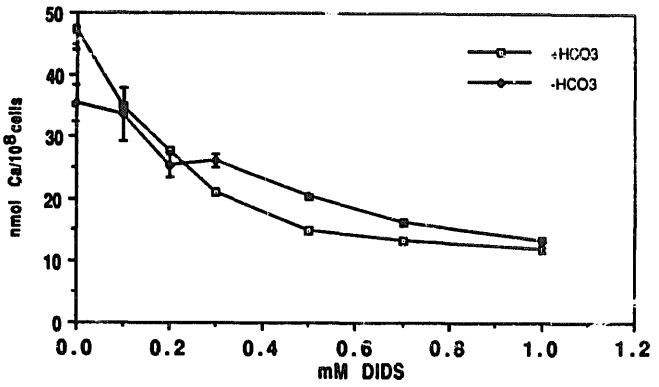

Fig. 11. The effect of DIDS on Ca^{2+}-uptake by the cells. Sperm cells (10^8/ml) were suspended in TALP and incubated for 15 min at 39°C under increased concentrations of DIDS, in the presence or in the absence of 26 mM $NaHCO_3$. Ca^{2+}-uptake was determined as described in the legend to Fig. 10. Each point represents the means ± S.E. of duplicates from at least two different experiments.

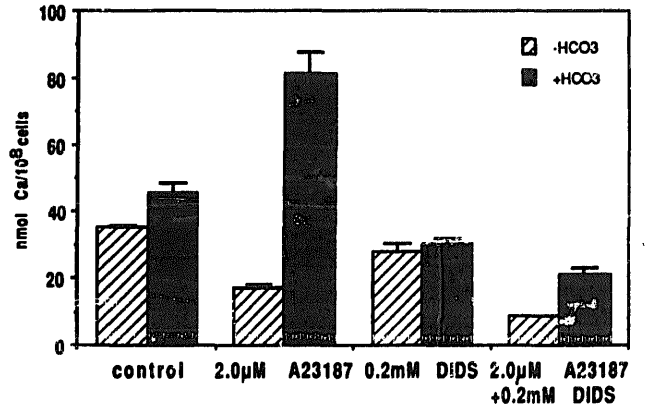

Fig. 12. The effect of DIDS on calcium uptake by the cells. Sperm cells (10^8/ml) were incubated for 30 min in TALP (±26 mM HCO_3^-) at 39°C in the presence or in the absence of 2.0 μM A23187 and 0.2 mM DIDS. Calcium uptake was determined as described in the legend to Fig. 10. Bars represent the means ± S.E. of duplicates from three different experiments.

Calcium uptake study. One of the principal events preceding the occurrence of the AR is a significant accumulation of Ca^{2+} in the cells [28]. If Ca^{2+} is supplied to the incubation medium, a rapid uptake of this cation is observed. HCO_3^- is already known to increase Ca^{2+}-uptake in the presence of external phosphate [29]. Thus, if DIDS blocks HCO_3^--transport into the cells, it would also inhibit Ca^{2+}-uptake. In view of these observations Ca^{2+}-transport into the cells was measured under various conditions.

The kinetics of Ca^{2+}-uptake by the cells are shown in Fig. 10. The Ca^{2+} ionophore, A23187, increased the rate of Ca^{2+}-uptake considerably (170%), but only in the presence of added HCO_3^-. The major influence of A23187 is seen at 30 min, while a plateau is reached. HCO_3^- is shown to increase the rate of Ca^{2+}-uptake by 176% even for long periods of incubation (90 min). When HCO_3^- was not added to the incubation medium, the rate of Ca^{2+}-uptake was 90% inhibited by A23187 (Fig. 12).

Fig. 11 shows the effect of DIDS on Ca^{2+}-uptake in the presence and in the absence of added HCO_3^-. Bicarbonate increases by 22% the rate of Ca^{2+}-uptake. 0.1 mM DIDS causes 25% inhibition of the rate of Ca^{2+}-uptake when HCO_3^- was added and only 5% when HCO_3^- was not added. At higher concentrations of DIDS Ca^{2+}-uptake is inhibited whether HCO_3^- was added or not. The effect of DIDS which is more pronounced in the presence of bicarbonate, indicates again the importance of HCO_3^- for Ca^{2+}-transport.

The effect of DIDS on Ca^{2+}-uptake in the presence of A23187 is shown in Fig. 12. Under these conditions, 0.3 mM DIDS caused 74 and 50% inhibition of Ca^{2+}-uptake in the presence or absence of HCO_3^-, respectively.

Discussion

Anion-channel inhibitors, mainly DIDS, were tested in order to investigate the involvement of anion channels in the mechanism of the AR. The DIDS molecule carries two isothiocyano groups, each of which is capable of reacting with a lysine residue, thereby cross-linking two adjacent segments of the peptide chain [30]. As reported here (Fig. 3), 0.3 mM DIDS almost completely inhibited the AR. SITS, which is also a known anion-channel blocker, inhibited the rate of acrosin release at a similar concentration, suggesting that both DIDS and SITS bind to the same sites. The apparently high concentrations of the inhibitors used here are similar to those used in other studies with sperm cells, although Hyne [10] obtained 100% inhibition of guinea-pig AR by 25.0 μM DIDS, but the final sperm concentration was much lower, $(2–5)\cdot 10^6$ cells/ml, i.e., 100-times fewer cells than we used in this work. On the other hand, Nishyama et al. [11] have shown that AR in starfish sperm is inhibited with concentrations of DIDS and SITS in the range of 0.1–1.0 mM, conditions as we used here.

After a 2-h exposure of the cells to 0.3 mM DIDS, all of the DIDS binding sites are occupied (Fig. 1) and the AR is completely inhibited (Fig. 3). When 10^8 cells/ml were incubated for 2 h with increasing concentrations of DIDS (Fig. 2), the plateau was reached at 0.3 mM, indicating again that at this concentration and after 2 h of incubation all binding sites are saturated.

In order to test whether DIDS inhibits AR via its inhibitory effect on plasma membrane ATPases, we show here that both ouabain and quercetin (ATPase inhibitors) did not affect the rate of the acrosin release and that they also did not interfere with the inhibitory effect of DIDS (Fig. 4). From previous work in our laboratory we know that ouabain and quercetin inhibit ATPases under the conditions used here [15]. Therefore, we conclude that the inhibition of the AR by DIDS is not due to its possible inhibitory effect on plasma membrane ATPases. Consequently, the probable mechanism of action of DIDS is in blocking anion channels that are essential for the occurrence of the AR.

We show here that transport of HCO_3^- into the cells is important for the occurrence of the AR. It is shown that DIDS inhibits the transport of HCO_3^- into sperm cells [8]. The stimulatory effect of HCO_3^- on the AR is significant in the presence or absence of A23187, there is a 2.0- and 1.6-fold increase in the rate of acrosin release in the presence or absence of A23187, respectively. However, we found a very large stimulation of Ca^{2+}-uptake by A23187 only in the presence of HCO_3^- (Fig. 10). A possible explanation for this difference, could be the requirement of HCO_3^- for the action of the ionophore itself.

Tables I and II summarize the data concerning the effects of HCO_3^-, A23187 and DIDS on AR. We calculated from the tables that the effect of HCO_3^- alone on acrosin release (191 nmol BAEE/min per 10^8 cells) plus the effect of A23187 alone (264 nmol BAEE/min per 10^8 cells) is 455 nmol BAEE/min per 10^8 cells, while in the presence of both together the

TABLE I

The effect of HCO_3^- on the acrosome reaction

	nmol BAEE/min per 10^8 cells			
	A23187	A23187 + DIDS	− A23187	− A23187 + DIDS
$+HCO_3^-$	1198	338	500	54
$-HCO_3^-$	573	29	309	44
ΔHCO_3^-	625	309	191	10
% Inhibition by DIDS	–	51		95

TABLE II

The effect of A23187 on the acrosome reaction

	nmol BAEE/min per 10^8 cells			
	$+HCO_3^-$	$+HCO_3^-$ + DIDS	$-HCO_3^-$	$-HCO_3^-$ + DIDS
+ A23187	1198	338	573	29
− A23187	500	54	309	44
Δ A23187	698	284	264	−15
% Inhibition by DIDS	–	59.4	–	100

effect was 661.5 nmol BAEE/min per 10^8 cells (see Fig. 7). Thus, the effect of HCO_3^- and A23187 on AR are not additive but synergistic, indicating that HCO_3^- potentiates the effect of A23187 in AR. This is consistent with the observation that HCO_3^- has a stimulatory effect on the efficiency of the ionophore on Ca^{2+}-uptake (Fig. 12).

In the presence of A23187, 0.2 mM DIDS causes 51% inhibition of the enhanced effect of HCO_3^- on acrosin release and 95% inhibition when A23187 was absent. Thus, A23187 can partially overcome the inhibition of the HCO_3^- effect caused by DIDS. In addition, the 100% inhibition by DIDS of the A23187 effect on acrosin release (Table II) is partially overcome by HCO_3^- (59.4% inhibition only). These data suggest that HCO_3^- is involved in the mechanism by which DIDS inhibits the AR and that DIDS has other mechanisms of inhibition besides blocking HCO_3^--transport. HCO_3^- failed to overcome this inhibition when A23187 was not added. This can be explained by the fact that HCO_3^- can enhance the ionophore activity as an exchanger of Ca^{2+} for two H^+, even in the presence of DIDS. In absence of A23187, the natural $Ca^{2+}/2\ H^+$ exchange through the membrane is very low, therefore, no effect of HCO_3^- can be seen.

These results are in agreement with Lee et al. [31], who showed the necessity of HCO_3^- for successful fertilization of mouse eggs and that HCO_3^- is also required for A23187 induction of the AR. On the other hand, Batacharyya and Yanagimachi [32] reported that guinea-pig eggs are fertilizable in the absence of HCO_3^- at a much lower efficiency than in its presence. Yoshimatsu and Yanagimachi [33] also showed that HCO_3^- is required for the induction of the AR in hamster spermatozoa and that Cl^- appears to be needed for the zona-induced AR. As an anion-channel blocker, DIDS inhibits Cl^--transport across the membrane and this might be one of the mechanisms by which DIDS inhibits the AR.

Tributyltin chloride (TBTC) has been shown to have several effects including mediation of Cl^-/OH^- exchange, inhibition of Na^+/K^--ATPase and Ca^{2+}-translocating ATPase, uncoupling of oxidative phosphorylation and enhanced ion movements [35]. TBTC enhanced AR by 170% (Fig. 6). The acrosin release that is independent of extracellular Ca^{2+} (in the presence of EGTA) was activated by TBTC (Fig. 6) as well.

TBTC acts as a Cl^-/OH^- ionophore, and under the incubation conditions (pH_e 7.6; pH_i 6.8) will alkalinize the interior of the cell, a prerequisite for the induction of the AR. It is possible that TBTC also stimulates AR by inhibiting Ca^{2+}-ATPase which normally drives Ca^{2+} out of the cell, similar to quercetin (the effect of quercetin is much smaller than that of TBTC, probably because it does not alkalize the cytosol) (see Fig. 4).

TBTC was shown to partially reverse the inhibition of AR by DIDS (Fig 6), suggesting that DIDS inhibits the alkalinization of the cytosol, and/or prevents Cl-transport across the membrane. Since TBTC does not affect HCO_3^--transport, it cannot completely recover the inhibition of AR caused by DIDS. However, TBTC and dbcAMP together did completely recover the inhibition of DIDS on the AR (not shown).

When A23187 was present (Fig. 9), TBTC failed to activate the AR or to recover the inhibition of DIDS, possibly because the ionophore itself alkalized the cell interior by exchanging external Ca^{2+} for internal H^+, with no need for TBTC activation.

dbcAMP, an analog of cAMP, induces the AR in mammalian spermatozoa [26]. In the present work, dbcAMP induced AR either in the presence or absence of bicarbonate (Fig. 8). The effect of dbcAMP, in relation to the control, is higher without added HCO_3^-, since HCO_3^- itself activates adenylate cyclase that catalyzes the production of cAMP as was shown in porcine [7] and bull [6] spermatozoa. Indeed, in the presence of dbcAMP, the rate of acrosin release attained the same levels either in the presence or absence of added HCO_3^-.

dbcAMP can induce AR slightly in the absence of added Ca^{2+} (in the presence of EGTA) (Fig. 8), suggesting that one of the possible mechanisms of action of Ca^{2+} is to stimulate adenylate cyclase activity. Although dbcAMP can recover only 40% of the inhibition of AR caused by DIDS, there is a significant enhancement (600%) in the AR under these conditions. In the absence of DIDS, dbcAMP enhanced the AR by 1.6–2.0-fold (Fig. 8) while in its presence there is about 7.0-fold increase in the AR by dbcAMP (Fig. 9), indicating that this recovery is highly significant.

According to Tajima and Okamura [12], DIDS, in the presence of HCO_3^-, increases the cAMP content in porcine sperm cells by blocking the exit of endogenous HCO_3^-, which, in turn, stimulates the production of cAMP by adenylate cyclase. In the present work, we argue that DIDS inhibits AR by inhibiting HCO_3^--transport into the cell. This difference can be explained by the fact that they measured the cAMP content only after a very short time (1 min), but after 3

h of incubation as we did here, HCO_3^- would leave the cell as CO_2 and the inhibition of HCO_3^--entry by DIDS will reduce the intracellular levels of cAMP. Furthermore, they also showed that DIDS inhibited adenylate cyclase activity in membrane fractions or in sperm homogenate, suggesting that DIDS might directly inhibit the membrane-bound adenylate cyclase activity in intact cells and will cause reduction in intracellular level of cAMP after longer period of incubation.

In order to determine whether HCO_3^- is a counteranion for Ca^{2+}-transport, we measured the influence of the anion on Ca^{2+}-uptake into the cells. Maximal uptake was seen while both HCO_3^- and A23187 were present (Fig. 10). But HCO_3^- also activates Ca^{2+}-uptake in the absence of the Ca^{2+} ionophore (32%). In the absence of the ionophore, bicarbonate seems to stimulate Ca^{2+}-uptake through a phosphate-dependent carrier on the plasma membrane [29] or due to a stimulatory effect of HCO_3^- on mitochondrial Ca^{2+}-uptake. Such a stimulatory effect of HCO_3^- on rat-liver mitochondrial calcium uptake is well documented [34].

In the presence of HCO_3^- and A23187, Ca^{2+} accumulates in non-mitochondrial compartments of the cell, since no inhibition of Ca^{2+}-uptake by the uncoupler FCCP was found under these conditions [14]. DIDS inhibited Ca^{2+}-uptake both in the presence or absence of HCO_3^-. Nevertheless, while 0.1 mM DIDS inhibited Ca^{2+}-uptake in the presence of bicarbonate by 25%, this same DIDS concentration inhibited Ca^{2+}-uptake in the absence of the anion only by 5%.

Generally, DIDS inhibits Ca^{2+}-uptake strongly when HCO_3^- is present. In fact, the stimulatory effect of HCO_3^- on Ca^{2+}-uptake is completely inhibited by DIDS (Fig. 12). DIDS causes only 75% inhibition, even at the highest concentrations used (1.0 mM) (Fig. 11) suggesting that there is a Ca^{2+}-transport activity which is not sensitive to DIDS. The fact that DIDS inhibits Ca^{2+}-uptake in the absence of HCO_3^- indicates that the Ca^{2+} transporter, which is HCO_3^- independent, is inhibited by DIDS as well. It is possible that this Ca^{2+}-transport mechanism depends upon other DIDS-sensitive counteranions transport or that the Ca^{2+} transporter itself is inhibited directly by DIDS.

According to Fig. 1, after 15 min 55% of the DIDS binding sites were occupied when 0.3 mM DIDS was used. This is in agreement with the results which show that 0.3 mM DIDS inhibits Ca^{2+}-uptake after 15 min of incubation by 55%.

We conclude that DIDS inhibits the AR by inhibiting HCO_3^- and Ca^{2+}-transport. Although DIDS partially blocks their transport, the sum of these effects and the probable nonspecific effect of the inhibitor would inhibit the AR to a high extent. Bicarbonate induces the AR by stimulating Ca^{2+}-transport into the cells, by alkalizing the cytosol and by stimulating adenylate cyclase activity. TBTC partially recovered the inhibition of the AR by DIDS, due to its ability to alkalize the cytosol and/or by enhancing Cl^--transport. The recovery by TBTC is relatively low, because no stimulation of adenylate cyclase or Ca^{2+}-uptake occurs. The inhibition of AR by DIDS is largely overcome by adding dbcAMP to the incubation medium, indicating that the intracellular level of cAMP is reduced due to the inhibition of HCO_3^--entry caused by DIDS. The recovery by dbcAMP is not complete due to the fact that the inhibition of Ca^{2+}-uptake by DIDS is not recovered by adding dbcAMP to the incubation medium.

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